Knock-out of the Plastid-encoded PetL Subunit Results in Reduced Stability and Accelerated Leaf Age-dependent Loss of the Cytochrome $b_{6}f$ Complex*

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The cytochrome-$b_{6}f$ complex, a key component of the photosynthetic electron transport chain, contains a number of very small protein subunits whose functions are not well defined. Here we have investigated the function of the 31-amino acid PetL subunit encoded in the chloroplast genome in all higher plants. Chloroplast-transformed petL knock-out tobacco plants display no obvious phenotype, suggesting that PetL is not essential for cytochrome $b_{6}f$ complex biogenesis and function (Fiebig, A., Stegemann, S., and Bock, R. (2004) Nucleic Acids Res. 32, 3615–3622). We show here that, whereas young mutant leaves accumulate comparable amounts of cytochrome $b_{6}f$ complex and have an identical assimilation capacity as wild type leaves, both cytochrome $b_{6}f$ complex contents and assimilation capacities of mature and old leaves are strongly reduced in the mutant, indicating that the cytochrome $b_{6}f$ complex is less stable than in the wild type. Reduced complex stability was also confirmed by in vitro treatments of isolated thylakoids with chaotropic reagents. Adaptive responses observed in the knock-out mutants, such as delayed down-regulation of plastocyanin contents, indicate that plants can sense the restricted electron flux to photosystem I yet cannot compensate the reduced stability of the cytochrome $b_{6}f$ complex by adaptive up-regulation of complex synthesis. We propose that efficient cytochrome $b_{6}f$ complex biogenesis occurs only in young leaves and that the capacity for de novo synthesis of the complex is very low in mature and aging leaves. Gene expression analysis indicates that the ontogenetic down-regulation of cytochrome $b_{6}f$ complex biogenesis occurs at the post-transcriptional level.

The Cyt-$bf^{2}$ is the smallest of the three thylakoid membrane-intrinsic multiprotein complexes of photosynthetic electron transport. Cyt-$bf$ functions as a dimer with a molecular mass of 220 kDa and consists of nine subunits per monomer (1). It catalyzes the rate-limiting step of linear electron transport, oxidizing PQ generated by PSII and reducing PC. PC then diffuses through the thylakoid lumen toward PSI and reduces the photo-oxidized chlorophyll $a$ dimer in the PSI reaction center, $P_{700}$. The PQ-PC oxidoreductase activity of the Cyt-$bf$ is coupled to the translocation of at least two protons per transported electron into the thylakoid lumen, thus contributing to the proton motive force required for ATP synthesis (2).

The Cyt-$bf$ is the predominant point of flux control of linear electron transport (3–6); its amount varies strongly in response to growth conditions and the developmental state of the plant, thus adjusting photosynthetic electron flux to the metabolic demand for ATP and NADPH. The mechanisms underlying these adjustments of Cyt-$bf$ concentration are currently not understood. There are some indications that Cyt-$bf$ is a relatively stable complex with lifetimes at least in the range of several days (7), but how Cyt-$bf$ biogenesis is regulated during plant development is completely unknown. Cyt-$bf$ biogenesis is a highly complex process that is regulated not only at the level of subunit gene expression and translation (8–10) but also at the level of complex assembly and requires specific chaperones for cofactor insertion into the complex (11–14).

In addition to its function in linear electron transport and flux control, Cyt-$bf$ has been suggested to oxidize stromal reductants in cyclic electron flux (15–17) and to have proton pumping activity itself (16, 18). It may also function as a redox sensor by activating thylakoid kinases and starting signal transduction chains that ultimately modify the antenna distribution between photosystems (“state transitions”) and alter photosynthetic gene expression (3, 17, 19, 20).

Although Cyt-$bf$ structure has been elucidated both in the photosynthetic prokaryote Mastigocladus laminosus (21) and in a eukaryotic alga, Chlamydomonas reinhardtii (16), the structural basis of its multiple functions is not fully understood. The genetic manipulation of Cyt-$bf$ composition is complicated by six of the nine subunits of the complex being plastid genome-encoded. The only subunits encoded in the nucleus are the Rieske iron-sulfur protein (encoded by the petC gene), the small peripheral PetM subunit, and the ferredoxin-NADP$^{+}$ oxidoreductase encoded by the petH gene. Ferredoxin-NADP$^{+}$ oxidoreductase has been suggested to function as NADPH oxidase in cyclic electron transport (1, 15). It is located at the stromal periphery of the complex and not required for Cyt-$bf$ function in linear electron flow, which instead is mediated by four high molecular mass subunits that bind all redox-active cofactors of the complex: PetA (Cyt-$f$), PetB (Cyt-$b$), PetC, and PetD (subunit IV). Each of these subunits is essential for the assembly and stability of Cyt-$bf$ (8, 22, 23).
In addition to the high molecular mass subunits, the complex contains four small subunits. These are located at the periphery of Cyt-bf, and each spans the thylakoid membrane once in an α-helical conformation. Crystal structure analysis has not revealed unequivocal indications for their function (16, 21). Based on their peripheral location, they have been hypothesized to mediate interactions with other components of the photosynthetic apparatus, such as PSI or the thylakoid kinases, which are redox-regulated by Cyt-bf (16). Genetic studies have revealed that some of the small subunits are essential for stability and assembly of the complex (24). Knock-out of the plastome-encoded PetN subunit in tobacco (25) has resulted in total loss of Cyt-bf. The same was observed after deletion of the PetG subunit in *C. reinhardtii* (26). The function of the nuclear-encoded PetM subunit in photosynthetic eukaryotes is still enigmatic, as no knock-out mutant has been obtained yet. Knock-out of petM in the cyanobacterium *Synechocystis* did not result in any alteration of the redox properties of Cyt-bf, but the accumulation of other complexes of the photosynthetic electron transport chain was found to be altered, suggesting that PetM might be involved in the postulated signal transduction function of the Cyt-bf (27). Currently, the only Cyt-bf subunit directly demonstrated to be nonessential for Cyt-bf function in higher plants is PetL, which is localized in direct proximity to PetM and PetN. In tobacco, a knock-out of the plastome-encoded *petL* gene did not result in any visible growth phenotype (28). These results differ from results obtained in *C. reinhardtii* (29, 30), where deletion of PetL resulted in destabilization of Cyt-bf; the Rieske subunit is bound less tightly to the complex, and the functional dimeric state of the complex is less stable (31). As a consequence, the Cyt-bf content dropped to 50–25% that of the wild type (WT) level during the exponential growth phase, which in turn strongly impaired cell division and growth. Remarkably, a recognizable *petL* gene does not exist in the genome of the cyanobacterium *Synechocystis* (32), possibly suggesting that the Cyt-bf in this prokaryote functions without a PetL-like subunit. In contrast, the Cyt-bf of another cyanobacterium, *M. laminosus*, contains a PetL subunit (21), indicating that the L subunit is not an evolutionarily late acquisition in photosynthetic eukaryotes.

Here, we have studied the function of the PetL subunit in detail in a higher plant, tobacco (*Nicotiana tabacum*). Despite the absence of a visible growth phenotype, the *petL* deletion results in an accelerated loss of the complex in mature and aging leaves. Our data indicate that the accelerated loss of Cyt-bf is due to a reduced stability of the complex in the absence of the PetL subunit. Moreover, our study provides evidence that the capacity for de novo synthesis of Cyt-bf is very low in mature and aging leaves, suggesting that the WT form of Cyt-bf is highly stable.

**EXPERIMENTAL PROCEDURES**

*Plant Material and Growth Conditions—ΔpetL* knock-out tobacco plants (*N. tabacum* L. var. Petit Havana) were generated by disruption of the *petL* gene with the *aadA* selection marker as described earlier (28). WT tobacco and ΔpetL mutants were grown in a controlled environment chamber at 600 μE m⁻² s⁻¹ light intensity at the uppermost leaves. Due to shading by the younger leaves, the light intensity could decrease to less than 150 μE m⁻² s⁻¹ at the level of the lower leaves. The plants were illuminated for 16 h at 22 °C. The night temperature was 18 °C. Humidity was set to 60% throughout the day.

*Gas Exchange Measurements and Chlorophyll a Fluorescence—Leaf assimilation capacity was determined using a closed cuvette system with a Clark-type oxygen electrode (LD2, Hansatech Instruments, Norfolk, England). Measurements were performed on leaf discs in a CO₂-saturated gas mixture (10% CO₂) to completely suppress photospiration. Saturating light was applied by a tungsten halogen lamp (Schott, Mainz, Germany). Chlorophyll a fluorescence was determined using a PAM-101 fluorometer (Walz GmbH, Effeltrich, Germany). The leaves were dark-adapted for 15 min, and respiration rates were determined. After measurement of the dark-adapted F₀/F₅₇ value, the actinic light was switched on, and photosynthetic oxygen evolution and chlorophyll a fluorescence were measured until steady state. The chlorophyll content of the leaf discs was determined after extraction in 80% acetone (33). Assimilation rates were corrected for dark respiration assuming comparable respiration rates in the light and in darkness (34, 35).

*State Transitions—*State Transitions were measured using a Dual-PAM fluorometer (Walz GmbH, Effeltrich, Germany). Leaves were excited using a red light LED (λ = 660 nm, 80 μE m⁻² s⁻¹ light intensity) and either blue light for predominant PSII excitation and reduction of the PQ pool (λ = 440 nm, 25 μE m⁻² s⁻¹) or far-red light (λ = 715 nm) for preferential excitation of PSI and PQ pool oxidation. Measurements were performed and analyzed according to Lunde et al. (36).

*Thylakoid Membrane Isolation—*Thylakoid membranes were isolated as described (6). Treatments with chaotropic reagents were conducted by incubating thylakoids equivalent to 200 μg of chlorophyll ml⁻¹ in a low salt buffer (100 μM EDTA, 10 mM KCl, 30 mM HEPES, pH 7.6) with either 2 mM NaBr, 2 mM NaI, or 0.1 M Na₂CO₃ in a total volume of 1000 μl. After incubation for 10 min at room temperature, thylakoids were pelleted by centrifugation (10,000 × g for 1 min) and resuspended in low salt buffer for Cyt-bf quantification.

*Cyt-bf and PSII Quantification—*The cytochromes of the thylakoid membrane were determined in isolated thylakoids equivalent to 50 μg of chlorophyll ml⁻¹ after detaching in a low salt medium to improve the optical properties of the sample (37). The cytochromes were oxidized by the addition of 1 mM ferricyanide and subsequently reduced by the addition of 10 mM ascorbate and dithionite, resulting in reduction of Cyt-f and the high potential form of Cyt-b₅₅₉ (high potential, ascorbate-ferricyanide difference absorption spectrum) and reduction of cytochrome b₅₉ and the low potential form of Cyt-b₅₉ (low potential, dithionite-ascorbate), respectively. At each redox potential absorption spectra were recorded between 575- and 540-nm wavelengths with a spatial resolution of 0.2 nm using a Jasco V550 spectrophotometer (Jasco, Groß-Umstadt, Germany) equipped with a head-on photomultiplier. The spectral bandwidth of the monochromator was set to 1 nm, and the scanning speed was 100 nm min⁻¹. A minimum of 10 spectra were averaged at each redox potential to reduce the noise contribution to the total absorption signals to less than 10⁻⁶. Before curve fitting, a base line was subtracted from the differ-
ence absorption spectra, defined by the wavelengths 540 and 575 nm. Difference absorption spectra were deconvoluted using reference spectra according to Kirchhoff et al. (37). Base line-corrected difference extinction coefficients of 28.0 for Cyt-f and 24.6 for Cyt-b₆ were calculated based on the difference extinction coefficients determined in Metzger et al. (38) and Nelson and Neumann (39). PSII contents were calculated from the sum of the Cyt-b₅₅₀ high potential and low potential difference absorption signals according to Lamkemeyer et al. (40).

PC and P₇₀₀ Redox Kinetics and Quantification—Difference absorption signals of PC and P₇₀₀ were measured in the far-red range of the spectrum, essentially as described (41). The contributions of PC and P₇₀₀ were deconvoluted by measuring difference absorption changes at 830–870 nm (predominantly arising from P₇₀₀) and 870–950 nm (predominantly arising from PC). Measurements were done using a novel instrument developed in cooperation with Christof Klughammer and Ulrich Schreiber (Walz GmbH, Effeltrich, Germany), which will be described elsewhere.³ This instrument allows the simultaneous measurement of both difference absorption signals. Measurements were done on pre-illuminated intact leaves with fully activated Calvin cycle, so that a limitation of PSII photooxidation by metabolic NADP⁺ regeneration could be excluded. P₇₀₀ and PC were photooxidized using far-red light (715 nm wavelength), which selectively excites PSI. After a 10-s exposure to far-red light, a saturating pulse of red light (6000 μE m⁻² s⁻¹, 200 ms duration) was applied, and the far-red light was switched off, so that PC and P₇₀₀ could become fully reduced after the actinic light pulse. The reduction kinetics were fitted with an exponential function to determine the halftimes of PC and P₇₀₀ reduction.

For PSI quantification in isolated thylakoids, membranes equivalent to 50 μg of chlorophyll ml⁻¹ were solubilized in a medium containing 0.2% (w/v) β-dodecyl-maltoside, 100 μM methylviologen as the artificial electron acceptor, and 10 mM ascorbate as donor. P₇₀₀ determined as described for measurements on intact leaves, was photooxidized by application of a saturating pulse of red light (6000 μE m⁻² s⁻¹ light intensity, 200-ms duration).

Protein Gel Electrophoresis and Immunoblotting—Thylakoid proteins were separated by SDS-polyacrylamide gel electrophoresis using the Perfect Blue twin gel system (PeqLab GmbH, Erlangen, Germany). Proteins were transferred to a polyvinylidene difluoride membrane (Hybond P, Amersham Biosciences) using a semidry blotting system (SEDEC-M, PeqLab) and a standard transfer buffer (25 mM Tris, 192 mM glycine, pH 8.3). Immunobiochemical detection was carried out with the ECL system (Amersham Biosciences) according to the instructions of the manufacturer.

Gene Expression Analysis—RNA was extracted from leaves using pepGoldTrifast reagent (PeqLab). To eliminate polysaccharide contaminations from RNA preparations from old leaves, all samples were subjected to an additional purification step using the NucleoSpin RNA Clean-up kit (Macherey & Nagel, Düren, Germany). Samples equivalent to 8 μg of RNA were separated in 1% formaldehyde-containing agarose gels and blotted onto nylon membranes (Hybond-XL, Amersham Biosciences) according to the instructions of the manufacturer. For hybridization, [α-³²P]dCTP-labeled probes were generated by random priming (Amersham Biosciences) following the instructions of the manufacturer. A petB-specific probe was prepared by PCR amplification with primers PetB5 (5′-TGT-ATTTCCGGAGATGATG-3′) and PetB3 (5′-CTCTATAC-AAGCCCAAGAA-3′) using total plant cDNA as a template. A hybridization probe specific for the Rieske iron-sulfur protein was amplified from cDNA with primers PRi5 (5′-CCAGCAGATGATAGTGCTTG-3′) and PRi3 (5′-TGAGCCAAT-GCCAAGGACAAAAG-3′). A plastocyanin-specific probe was generated from PCR-amplified cDNA with primers PCp5 (5′-ACTCGAGTATTGAAGTGTTGCTTGGTAGCGATG-3′) and PCp3 (5′-TGAATTCATCCACACCGACTGGAATTTCATC-3′). Hybridizations were performed at 65 °C in Rapid-Hyb buffer (Amersham Biosciences) following the manufacturer’s protocol.

Hybridization signals were quantified using a Typhoon Trio+ variable mode imager (Amersham Biosciences) and ImageQuant 5.2 software. Signal intensities were corrected for equal loading of the agarose gels by normalizing to the ribosomal RNA signals determined after ethidium bromide staining using the Infinity 1000 gel documentation system (PeqLab).

RESULTS

Growth Phenotype and Leaf Assimilation Capacities—Tobacco petL knock-out mutants generated by chloroplast transformation do not display any visible growth phenotype (28). Both flowering time and number of leaf generations per plant are identical with WT plants. Also, the chlorophyll contents per leaf area are about identical in WT and mutant plants, with the highest chlorophyll contents measured in youngest leaves (600 mg of Chl m⁻²) and the lowest contents found in the oldest leaves (350 mg of Chl m⁻²). Therefore, the corresponding leaf generations of transformants and WT plants can be directly compared. For most experiments, tobacco plants at the onset of flowering were used. Leaf assimilation capacities were determined from photosynthetic O₂ evolution in saturating light and 10% CO₂. Under these conditions, photorespiration is repressed, so that photosynthetic O₂ production directly reflects the maximum leaf assimilation capacity (6). The youngest leaves that were sufficiently large to be used in gas exchange measurements were leaves no. 2 (from the top of the plant). In both WT and ΔpetL plants, these leaves had comparable average assimilation capacities of 500 μmol of electron pairs mg of Chl⁻¹ h⁻¹ (Fig. 1A). For WT plants, an about 50% reduction of leaf assimilation capacity was observed from leaf no. 2 down to the oldest leaf measured (leaf no. 12). The slow decrease in assimilation capacity started from leaf number 4, which was the first fully expanded and mature leaf, downward. In the ΔpetL mutant, the decrease of assimilation capacity was strongly accelerated, with the assimilation capacity of the oldest leaves being only about 100 μmol of electron pairs mg of Chl⁻¹ h⁻¹.

In parallel to this strong reduction of assimilation, the capacity for non-photochemical quenching (NPQ) of oversaturating light intensities was also found to be significantly reduced in the

³ M. A. Schöttler, C. Klughammer, R. Bock, and U. Schreiber, manuscript in preparation.
mutant plants (Fig. 1B). In WT leaves, the NPQ capacity was about constant (0.8), independent of leaf age. In contrast, the NPQ capacity of older mutant leaves was strongly decreased. Whereas young mutant leaves had only slightly lower NPQ capacities than the WT, NPQ declined strongly from leaf no. 6 downward to levels of only about 0.5. These changes in NPQ indicate that, in addition to assimilation, also thylakoid membrane energizing is increasingly impaired with leaf age in ΔpetL plants. Both effects can most probably be explained with a leaf age dependent limitation of electron flux at the level of the Cyt-bf.

Linear Electron Flux Is Strongly Reduced in Mature and Old Mutant Leaves—To directly demonstrate the strong limitation of photosynthetic electron flux in mature and old mutant leaves at the level of Cyt-bf, chlorophyll a fluorescence relaxation and redox kinetics of PC and P_{700} were determined in intact leaves. A saturating light pulse (6000 μE m^{-2} s^{-1}, 200-ms duration) was applied to completely oxidize the high potential chain and reduce the PQ pool. Afterward, the dark reduction kinetics of PC and P_{700} and the reoxidation of the PQ pool were determined. Normally, these reactions are rate limited by PQ reoxidation at the Cyt-bf (5). In Fig. 2, kinetics of P_{700} reduction are rate limited by PQ reoxidation at the Cyt-bf (5). In Fig. 2, kinetics of P_{700} reduction after a saturating light pulse in young (leaf no. 4; A) and old (leaf no. 11; B) wild type and mutant leaves. Leaves were pre-illuminated to fully activate the Calvin cycle. Then, P_{700} was photo-oxidized by 8 s of exposure to weak far-red light, which selectively excites PSI but not PSII. Afterward, a saturating pulse of red light (5000 μE m^{-2} s^{-1}, 200-ms duration) was applied to fully oxidize P_{700} and reduce the PSII acceptor side of the electron transport chain. The reduction kinetics of P_{700} in the dark phase after the end of the saturating light pulse (t = 0 ms) were then traced. The fully oxidized state of P_{700} was normalized to 1, and the completely reduced state was normalized to 0. The symbols are as stated in the legend to Fig. 1. Young leaves of both WT and mutant show fast reduction kinetics with halftimes of about 5 ms (A). With increasing leaf age, the efficiency of linear electron flux to P_{700} decreases as the halftime of P_{700} reduction is increased to 10 ms in old WT leaves and to 40 ms in old mutant leaves (B).

FIGURE 1. Leaf age-dependent changes in assimilation capacity (A) and thylakoid membrane energizing (B). The youngest leaf was assigned no. 1, and the oldest leaf was no. 12. From leaf no. 4 downward, the leaves were fully expanded (mature). The oldest leaves measured were still fully green and not visibly senescent, although chlorophyll content was already reduced to 60% of the chlorophyll content of young leaves. Filled circles represent the wild type plants, and open circles the ΔpetL mutant. The assimilation capacity in A is expressed as electron pairs (EP)/mg of chlorophyll-h and plotted against the leaf number. Measurements were done under light- and CO_{2}-saturated conditions. The decline in assimilation capacity is significantly accelerated in mature mutant leaves. In B, non-photochemical quenching (qN) was measured as an indicator of thylakoid membrane energizing. The capacity for qN, which is triggered by acidification of the thylakoid lumen, declines in parallel to leaf assimilation in mature mutant leaves, indicating that the formation of the proton motive force may be limiting.
Cyt-bf content or, alternatively, to an altered Cyt-bf function, the contents of all redox-active proteins of the electron transport chain were determined in isolated thylakoids by means of difference absorption spectroscopy and immunoblot analyses. Because thylakoid isolation and the complex quantification in isolated thylakoids require high amounts of chlorophyll, the developmental series of thylakoid isolations started with leaf no. 4, the first fully expanded leaf. In addition, leaves no. 6, 9, and 11 were used for thylakoid isolations. To gain further information on Cyt-bf contents in young expanding leaves of WT and ΔpetL mutants, the youngest leaves from several young, non-flowering plants were pooled to collect sufficient leaf material for these experiments. In WT tobacco leaves, a pronounced reduction in Cyt-bf content with leaf age, which parallels a decline of assimilation capacity, is typically observed (6). This leaf-age dependent Cyt-bf decrease is also seen here (Fig. 3A). The highest Cyt-bf amounts were detected in the youngest leaves and in the fully expanded leaf no. 4 with about 1.30 mmol of Cyt-bf mol Chl⁻¹. From leaf no. 6 downward, the Cyt-bf content decreased continuously to 0.55 mmol mol Chl⁻¹ in leaf no. 12. The changes in Cyt-bf content were closely paralleled by a roughly proportional decrease in leaf assimilation capacity, as measured in vivo before thylakoid isolation (Fig. 3C). In the ΔpetL transformants, the Cyt-bf content in the youngest leaves was almost identical to that of the youngest WT leaves (95% of the WT values). However, in contrast to the WT, Cyt-bf contents in the mutant started to decline already in leaf no. 4. The decrease in Cyt-bf content was clearly accelerated in the knock-out plants with their oldest leaves containing only 0.15 mmol mol Chl⁻¹, equivalent to about 25% of the Cyt-bf content in WT leaves of the same age (Fig. 3B). Again, a close correlation was observed between Cyt-bf contents and leaf assimilation capacities (Fig. 3C). In summary, the Cyt-bf content of ΔpetL plants clearly limits photosynthetic fluxes, thus resulting in the increased $P_{700}$ reduction half-times, the reduced thylakoid membrane energizing, and the reduced leaf assimilation capacity.

Cyt-bf Stability Is Reduced in ΔpetL Plants—To confirm the reduced stability of Cyt-bf in the knock-out mutants biochemically, isolated thylakoids were subjected to mechanical, thermal, and ionic stresses. Experiments were done using thylakoids from leaf no. 4 of mutant and WT plants. After each treatment, the residual Cyt-bf content was determined from difference absorption signals. As a control, PSII contents were calculated from the deconvoluted Cyt-b$_{559}$ signals. Neither mechanical stress resulting from 15 cycles of consecutive freezing in liquid nitrogen and thawing at 35 °C (Fig. 4, Cold) nor heat stress (10-min incubation at 50 °C) resulted in a significant difference between WT and ΔpetL thylakoids (Fig. 4A). Remarkably, both PSII and Cyt-bf were completely stable during repeated freezing and thawing. Likewise, no significant difference between WT and mutant thylakoids was observed in response to heat treatment; although both plant lines lost less than 10% of Cyt-bf, the majority of PSII was destroyed in both WT and ΔpetL transformants (Fig. 4B). Because PSII is well known to be the most heat-labile component of the photosynthetic apparatus, this finding is unsurprising. Interestingly, when thylakoids were treated with chaotropic reagents, the mutant Cyt-bf was found to be significantly less stable than the WT form when treated with the strong chaotropic salt NaI (Fig. 4A). Weaker chaotropic reagents, such as NaBr, had no such effect. No chaotrop had a significant effect on PSII stability (Fig. 4B). In conclusion, the mutant Cyt-bf is significantly less stable in response to NaI treatment, whereas thermal and mechanical stresses do not result in pronounced differences in the stability of mutant and WT complexes.

Lack of Adaptive Responses of the Electron Transport Chain to Reduced Cyt-bf Stability—To determine whether other components of the electron transport chain respond to the reduced Cyt-bf stability by adaptive changes in their contents, we also
determined the contents of the photosystems and of PC (Fig. 5). For the photosystems, no significant differences between the WT and the ΔpetL transformant could be observed in young leaves. In both the WT and the transformant, PSII contents declined from about 3 mmol of PSII mol Chl\(^{-1}\) in young leaves to about 2 mmol of PSII mol Chl\(^{-1}\) in old leaves (Fig. 5A). PC contents were highest in young WT leaves (about 10 mmol mol Chl\(^{-1}\)) and declined in parallel with assimilation and Cyt-bf contents to about one-third that of the PC content of young leaves, indicating that PC and Cyt-bf might both contribute to flux control (Fig. 5B). Such a close correlation between PC contents and photosynthetic flux capacity was observed previously (6). In the ΔpetL plants, the leaf age-dependent decline in PC content was slowed down, potentially representing an adaptive response of the photosynthetic apparatus to the accelerated loss of Cyt-bf.

These spectroscopic quantifications were confirmed by immunoblot analysis using marker subunits of both photosystems, the Cyt-bf and the ATP synthase. To test the dynamic range of the immunobiochemical detection reactions, a dilution series of the WT samples (100, 50, 20, and 10%) was used (Fig. 6). Proteins from WT and ΔpetLc mutant leaves no. 4, 6, 9, and 11 were semiquantitatively analyzed by direct comparison with the dilution series (Fig. 6). For Cyt-f (PetA), which served as a diagnostic marker subunit for Cyt-bf, an accelerated decrease with increasing leaf age was observed in the transformants (Fig. 6). This decline closely parallels the decrease of functional Cyt-bf contents determined by difference absorption spectroscopy, indicating that no inactive complex accumulates in the knock-out plants and, instead, suggesting that the instable Cyt-bf fraction is condemned to rapid degradation. For
PetL Function in Cytochrome bf Complex Stability

**FIGURE 6.** Immunoblot analysis of marker proteins for the four multisubunit complexes of the photosynthetic light reactions. PetA (Cyt-b) is an essential subunit of the Cyt-bf, AtpA is an essential subunit of the chloroplast ATP synthase, PsaF is part of the PSI reaction center and essential for PSI accumulation, and PsbD (D2-protein) is essential for PSII biogenesis and stability. The first four lanes of each blot show undiluted WT thylakoids from leaves no. 3 and 4 and dilutions to 50, 20, and 10% of the WT sample. The next four lanes display WT thylakoids isolated from leaf no. 4 (lane 5), leaf no. 6 (lane 6), leaf no. 9 (lane 7), and leaf no. 11 (lane 8). Lines 9–12 show the analogous series of samples from the ΔpetL mutant. Whereas the Cyt-bf is almost completely lost in aging mutant leaves, the decreases in the photosystems are far less pronounced in both WT and ΔpetL thylakoids, confirming the results of the spectroscopic quantifications of thylakoid complexes. The ATP synthase displays a clear leaf age-dependent reduction in its content in both WT and mutant thylakoids. Double signals for AtpA are due to cross-reactions with the slightly smaller AtpB subunit, and the D2 antibody also cross-reacts with the homologous but slightly smaller D1 protein.

PSI and PSII, the immunoblots also closely resemble the spectroscopic quantifications. The weak lower molecular weight signal observed in the PsbD (D2) immunoblot most likely arises from cross-reaction of the antibody with the D1 protein, which has high sequence similarity to D2 but migrates at a slightly lower molecular mass. The chloroplast ATP synthase was also quantified using an AtpA antibody. Again, a double band signal was observed. In this case the weakly hybridizing lower band can be attributed to cross-reaction of the antibody with the D1 protein, which has high sequence similarity to D2 but migrates at a slightly lower molecular mass. The chloroplast ATP synthase was also quantified using an AtpA antibody. Again, a double band signal was observed. In this case the weakly hybridizing lower band can be attributed to cross-reaction of the antibody with the D1 protein, which has high sequence similarity to D2 but migrates at a slightly lower molecular mass.

### DISCUSSION

PetL is the only Cyt-bf subunit in photosynthetic eukaryotes, which has been shown to be nonessential for photosynthetic function (28). These results can be reconciled with the crystal
PetL Function in Cytochrome bf Complex Stability

structure of the Cyt-bf, which revealed PetL to be localized at the periphery of the Cyt-bf, far away from the redox-active cofactors (16). Based on these structural data, PetL was predicted to participate in the assembly and/or stability of the Cyt-bf and to possibly be involved in signal transduction processes by mediating molecular interactions with other thylakoid proteins, such as the thylakoid kinases (16, 21, 24). Here, we demonstrate that PetL is important for Cyt-bf stability in higher plants but is not involved in Cyt-bf assembly or in thylakoid kinase activation. Our finding that the youngest leaves of the knock-out plants accumulate Cyt-bf to WT amounts suggests strongly that only complex stability is affected, whereas complex assembly is normal. The absence of a state-transitions phenotype in the ΔpetL mutant is somewhat surprising, because a chimeric PetL-subunit IV fusion in C. reinhardtii resulted in impaired state transitions (30), which was interpreted as circumstantial evidence of a function of PetL in signal transduction.

Although the ΔpetL knock-out plants do not display a visible growth phenotype, the photosynthetic apparatus of mature and old leaves of ΔpetL knock-out plants differs clearly from that of the WT; whereas the Cyt-bf content of young mutant leaves is comparable with that of the WT, the leaf age-dependent decrease of Cyt-bf content is clearly accelerated in the mutant (Fig. 3, A and B). Because the Cyt-bf is rate-limiting for linear electron flow (3, 5, 6), linear electron flux (Fig. 2), thylakoid membrane energizing (Fig. 1B), and leaf assimilation capacities (Fig. 1A) of the mature and old mutant leaves are strongly reduced relative to the WT. The absence of a visible growth phenotype in the ΔpetL mutants can be ascribed to the fact that the predominant contribution to overall assimilation is made by the young leaves, not the least because the older leaves are at least partly shaded by the young leaves. Thus, because the overall contribution of the old leaves to assimilation is marginal, the reduced electron transport capacity of these leaves might be easily compensated by the much higher photosynthesis rates of the younger, fully illuminated leaves. It is, therefore, not all too surprising that the accelerated loss of Cyt-bf in the knock-out plants does not result in a reduced growth rate and altered plant development. Shading by the young leaves might also explain why the older leaves do not suffer from oxidative stress and do not show any symptoms of photobleaching. In full sunlight, the

been shown to be rate-limiting for Cyt-bf biogenesis, transcript abundance of petB (encoding Cyt-bf) was analyzed. Because petB is part of a large operon (43), a complex transcript pattern was observed. The most abundant mRNA species represents the tricistronic psbH-petB-petD RNA (1.9 kb) and the 1.5-kb dicistronic petB-petD RNA. The minor band at 1.2 kb represents dicistronic psbH-petB. As a key nucleus-encoded Cyt-bf subunit, transcript abundance for the petC gene (encoding the Rieske iron-sulfur protein) was determined. A single signal at the expected size of ~1 kb was observed in all lanes. No difference between the WT and the ΔpetL mutant in petB, petD, and petC transcript patterns or RNA abundance and no changes with leaf age were seen. In contrast, for the nuclear-encoded petE gene encoding PC, strong changes in transcript abundance with leaf age and pronounced differences between the WT and the mutant were observed. In the WT, petE transcript abundance strongly decreases in mature and old leaves, paralleling the changes in the content of redox-active PC. In the ΔpetL mutant, petE transcripts accumulate to much higher amounts than in the WT in all developmental stages. Strong expression of petE in the oldest leaves might contribute to the decelerated decrease of PC contents in the mutant. rRNA, ribosomal RNA.

FIGURE 7. Expression of key Cyt-bf subunits and of PC in the WT and the ΔpetL mutant. Northern blots for two genes encoding subunits of Cyt-bf (petB, petC) and for petE encoding PC are shown. Equal loading of RNA samples was controlled by comparing the abundance of the rRNAs (gel photograph shown below each blot). As a chloroplast-encoded subunit, which has
PetL Function in Cytochrome bf Complex Stability

progressive restriction of electron transport in the mature and old leaves of the transformants would most likely result in over-reduction of the PQ pool and oxidative damage to PSII. As a consequence of the shading of the older leaves, PSII excitation rates in these leaves are well below the threshold level required for quantitative reduction of the PQ pool (as revealed by light saturation curves of leaf assimilation; data not shown). The incident light intensity at the level of the old leaves was only 25% that of the light intensity received by the youngest leaves. Therefore, despite restricted linear electron flux and impaired non-photochemical quenching (due to reduced membrane energizing), old mutant leaves do not suffer from pronounced oxidative stress.

The observed reduced Cyt-bf stability could be due to at least two different effects that are not mutually exclusive; the absence of PetL could result in a general weakening of subunit interactions in the complex and/or it could enhance susceptibility of the Cyt-bf to proteolytic degradation. The latter could be due to some domains of the other subunits becoming more accessible to thylakoid proteases. Although some of the proteases involved in the degradation of the Cyt-bf have been identified, the exact mechanism of proteolysis and the specific domains that are attacked by these enzymes have not yet been determined (45, 46).

Currently, a reduced Cyt-bf stability due to weakened molecular interactions seems to be sufficient to explain the accelerated loss of Cyt-bf in the transformants; chaotropic treatments of thylakoid membranes revealed a significant destabilization of the mutant Cyt-bf (Fig. 4), suggesting that in the absence of the PetL subunit the molecular interactions between the subunits of the complex are less tight, and the complex dissociates more readily. The released subunits are likely to be rapidly degraded, as evidenced by the cytochrome f (PetA) subunit contents decreasing in parallel with the spectroscopically active Cyt-bf (Fig. 6). Unassembled subunits of the Cyt-bf are known to be highly unstable in tobacco (8). Whether or not the lack of the PetL subunit also results in increased exposure of specific subunits or domains to the chloroplast proteolytic machinery remains to be determined.

To explain the accelerated loss of the Cyt-bf in mature and old leaves, we have to assume that, in addition to a reduced stability of the complex, also de novo Cyt-bf biogenesis is irreversibly down-regulated in mature and old leaves. Otherwise, mature leaves should compensate the reduced Cyt-bf stability and the restricted capacity for linear electron flux by increased de novo synthesis. Indeed, indications do exist that the Cyt-bf is highly stable (7); the half-life time of Cyt-bf is in the range of several days. This value was obtained by analyzing the decline in Cyt-bf in Chlamydomonas cells after inhibition of de novo protein synthesis with chloramphenicol. In reality the stability of the complex may be even higher because a general inhibition of chloroplast translation is likely to result in an overall destabilization of the thylakoid membrane due to the shorter lifetime of PSII, which in turn leads to increased production of reactive oxygen species and, thus, could have accelerated Cyt-bf degradation in these inhibitor experiments. It, thus, seems conceivable that the stability of the Cyt-bf is in the range of several weeks. Assuming such a high stability, it would make sense to strongly reduce or even switch off de novo Cyt-bf biogenesis in mature leaves. Because these leaves become increasingly shaded, which results in progressive light limitation of electron flux, leaf age-dependent loss of Cyt-bf by slow degradation in WT leaves remains without physiological consequences in that it proceeds in parallel to the gradually reduced demand for electron flux capacity. The same could also be the case for the ATP synthase, for which a parallel leaf age-dependent decrease was observed as for the Cyt-bf (Fig. 6; see also Ref. 42). It seems plausible that the ontogenetic switching-off of Cyt-bf and ATP synthase biogenesis might reflect an economic strategy to minimize the costs for the maintenance of the photosynthetic apparatus.

To address the molecular basis of the leaf age-dependent down-regulation of Cyt-bf biogenesis, we determined the expression of key subunits of the Cyt-bf complex by Northern blot analysis (Fig. 7). Leaf age-dependent changes in transcript abundance could not be observed for either the plastome-encoded Cyt-b (petB) or the nuclear-encoded Rieske iron-sulfur protein (petC). Because subunit IV (petD) is transcribed from the same oligocistronic mRNAs as petB (43), we can assume that also petD transcript abundance is independent of leaf age. This finding argues strongly against a transcriptional control of Cyt-bf biogenesis. Rather, the ontogenetic down-regulation of Cyt-bf concentration seems to occur at a post-transcriptional level of gene expression, most likely at the translational level and/or post-translational level. This observation is unsurprising, because in Chlamydomonas, post-transcriptional control of Cyt-bf synthesis has already been established; greater than 10-fold changes in the contents of chloroplast mRNAs encoding Cyt-bf subunits had no effect on Cyt-bf complex biogenesis (47). Instead, translation initiation is highly regulated (10). Therefore, reduced translation of Cyt-bf mRNAs in mature and old leaves may provide the most probable explanation for the down-regulated Cyt-bf biogenesis in older leaves. As far as the underlying signals and mechanisms are concerned, we currently can only speculate. The ATP synthase, which seems to be subject to a similar leaf-age dependent down-regulation as the Cyt-bf complex (Fig. 6), is strongly regulated by cytokinins at the translational level (48). This suggests that hormonal gradients across the plants can directly determine the rate of photosynthetic complex biogenesis. Whether or not a similar mechanism also operates in the regulation of Cyt-bf biogenesis remains to be determined.

In conclusion, our data reported here may have implications for our understanding of the ontogenetic program of the plant in that ceased synthesis of Cyt-bf and ATP synthase may represent the first dedicated step during leaf aging, which occurs before any visible symptoms of leaf senescence. Experiments focusing on the post-transcriptional processes (translation initiation, complex assembly, and stability), by which the leaf age-dependent down-regulation of photosynthetic complexes could be achieved, are currently under way.

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PetL Function in Cytochrome b Complex Stability

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