ATP Synthase Repression in Tobacco Restricts Photosynthetic Electron Transport, CO₂ Assimilation, and Plant Growth by Overacidification of the Thylakoid Lumen

Markus Rott,¹ Nádia F. Martins,¹ Wolfgang Thiele,¹ Wolfgang Lein,¹ Ralph Bock,¹ David M. Kramer,² and Mark A. Schöttler¹,²

¹Max-Planck-Institut für Molekulare Pflanzenphysiologie, D-14476 Potsdam-Golm, Germany
²Michigan State University–Department of Energy Plant Research Laboratory and Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824

Tobacco (Nicotiana tabacum) plants strictly adjust the contents of both ATP synthase and cytochrome b₆f complex to the metabolic demand for ATP and NADPH. While the cytochrome b₆f complex catalyzes the rate-limiting step of photosynthetic electron flux and thereby controls assimilation, the functional significance of the ATP synthase adjustment is unknown. Here, we reduced ATP synthase accumulation by an antisense approach directed against the essential nuclear-encoded γ-subunit (AtpC) and by the introduction of point mutations into the translation initiation codon of the plastid-encoded atpB gene (encoding the essential β-subunit) via chloroplast transformation. Both strategies yielded transformants with ATP synthase contents ranging from 100 to <10% of wild-type levels. While the accumulation of the components of the linear electron transport chain was largely unaltered, linear electron flux was strongly inhibited due to decreased rates of plastoquinol reoxidation at the cytochrome b₆f complex (photosynthetic control). Also, nonphotochemical quenching was triggered at very low light intensities, strongly reducing the quantum efficiency of CO₂ fixation. We show evidence that this is due to an increased steady state proton motive force, resulting in strong lumen overacidification, which in turn represses photosynthesis due to photosynthetic control and dissipation of excitation energy in the antenna bed.

INTRODUCTION

The capacity of the photosynthetic light reactions to provide ATP and NADPH must be closely adjusted to their metabolic consumption by the Calvin cycle, the subsequent reactions of dark metabolism such as starch synthesis, and other anabolic pathways within the chloroplast. Upon hyperactivity of the light reactions, the metabolic regeneration of NADP⁺, ADP, and Pᵢ will limit photosynthetic electron transport, resulting in detrimental side reactions. NADP⁺ limitation would result in electron transfer to alternative acceptors, such as O₂, generating reactive oxygen species. These can damage the photosynthetic apparatus itself and also initiate cell death responses (Kim et al., 2008).

Reduced ADP and Pᵢ regeneration results in substrate limitation of the thylakoid ATP synthase, reducing proton efflux from the lumen and resulting in an increase of the proton motive force (pmf) across the thylakoid membrane (Takizawa et al., 2008; Kiirats et al., 2009). Under standard growth conditions, the pmf is partitioned into an electrochemical component (ΔΨ) and a proton gradient (ΔpH) in such a way that the pH value of the thylakoid lumen is usually kept between 7.0 and 6.5 (Takizawa et al., 2007). However, in response to short-term imbalances between proton translocation into the lumen by photosynthetic electron transport and use of the pmf for ATP synthesis, the pH of the thylakoid lumen can drop below 6.5. This initiates photoprotective feedback responses such as nonphotochemical quenching (qN), which is the thermal dissipation of excess excitation energy in the photosystem II (PSII) antenna bed in the form of heat (Szabó et al., 2005). This is achieved through protonation of the PsbS antenna protein and by activation of the violaxanthin deepoxidase, which converts the xanthophyll violaxanthin into zeaxanthin. Also, plastoquinol (PQ) reoxidation at the cytochrome b₆f complex (cyt-bf) is slowed down when the luminal pH falls below 6.5, thereby restricting linear electron flux (reviewed in Kramer et al., 1999). This mechanism is called photosynthetic control.

Although both protective mechanisms become active at pH values below 6.5, they are fully activated only at a luminal pH below 6.0, with the cyt-bf response peaking at slightly lower pH values than qN (Takizawa et al., 2007). In case of a severe imbalance between light reactions and dark metabolism, a more pronounced overacidification of the thylakoid lumen can result in damage to plastocyanin (PC) and disintegration of the oxygen-evolving complex of PSII (Krieger and Weis, 1993; Kramer et al., 1999). However, these types of damage were so far only studied in vitro (Kramer et al., 1999). It is still a matter of debate whether or not these highly acidic pH values in the lumen can be established in vivo or whether they are efficiently avoided by...
downregulation of linear electron flux by qN and decreased PO reoxidation rates. Also, it is still questionable whether plants can control the luminal pH by altering the relative pmf partitioning into ΔΨ and ΔpH. Under nonstressed conditions, the pmf partitioning can be highly variable between different plant species (Takizawa et al., 2007). Therefore, a variable partitioning of pmf could conceivably enable plants to regulate the onset of photoprotective mechanisms and avoid photodamage by over-acidification.

While the short-term responses of plants to an imbalance between light reactions and dark metabolism are well understood, much less is known about the long-term adjustment of photosynthetic electron transport and the proton circuit to a reduced metabolic demand. Long-term changes in assimilation capacity are known to occur during leaf ontogenesis, when assimilation declines with leaf age (Schöttler et al., 2004, 2007a), but also in response to several environmental stresses, such as drought stress (Tezara et al., 1999). Also, light intensities during growth strongly alter leaf assimilation capacity. Under the majority of these conditions, photosystem I (PSI) contents are constant (Anderson et al., 1988; Bailey et al., 2001; Schöttler et al., 2004). Also, PSI contents do not change much, except upon changes in growth light intensity (Bailey et al., 2001). Good indications exist that the capacity of linear electron flux is predominantly controlled by cyt-bf contents, which are highly variable and closely correlated with assimilation capacity (Evans, 1988; Anderson, 1992). This observation is in line with cyt-bf catalyzing the rate-limiting reaction step of linear electron transport, PO reoxidation (Hope, 2000; Kirchhoff et al., 2000). Indeed, a strong contribution of cyt-bf to photosynthetic flux control has been shown by antisense repression of its essential Rieske Fe2-S2– containing subunit (Price et al., 1995, 1998). PC content was also shown to correlate with photosynthetic flux rates (Burkey, 1993, 1994; Schöttler et al., 2004).

Finally, substantial changes in ATP synthase contents in response to changes in light intensity (Anderson et al., 1988; Evans, 1988), leaf age (Schöttler et al., 2007a), and drought stress (Tezara et al., 1999; Kohzuma et al., 2009) have been reported, and these also parallel assimilation capacities. However, the functional significance of the adjustments of ATP synthase content has remained unclear, especially because other components of the photosynthetic apparatus (in particular, cyt-bf) show parallel changes under most of these conditions, possibly suggesting a coregulation.

To determine the contribution of the ATP synthase to photosynthetic flux control, we specifically repressed the ATP synthase content in tobacco (Nicotiana tabacum) using two independent approaches. First, we repressed the AtpC gene encoding the essential γ-subunit with an antisense approach. In Chlamydomonas reinhardtii, it has been shown that AtpC availability controls the biogenesis of the ATP synthase complex (Drapier et al., 2007). Downregulation by an antisense strategy was appropriate because an Arabidopsis thaliana atpc T-DNA insertion line suffered from destabilization of the entire complex and degradation of the other ATP synthase subunits, so that photophosphorylation was entirely abolished (Bosco et al., 2004). In addition, we repressed the translation initiation efficiency of the essential chloroplast-encoded β-subunit, encoded by the atpB gene, which forms part of the catalytic center of the extrinsic CF1 subcomplex of the ATP synthase.

We report here the generation of transgenic and transplasmonic tobacco lines that contain ATP synthase contents from 100% to <10% of wild-type levels. Physiological analysis of lines with greatly reduced ATP synthase revealed a strongly increased pmf across the thylakoid membrane, leading to the activation of photoprotective mechanisms and downregulation of linear electron flux already in low light. This resulted in repressed leaf assimilation and plant growth, supporting a central role of the ATP synthase in regulating photosynthesis. Additionally, the analysis of these mutants enabled the identification of regulated steps of ATP synthase biogenesis in higher plants.

RESULTS

Generation of AtpC Antisense Lines and Functional Organization of Their Photosynthetic Apparatus

The AtpC antisense plants were obtained from a large collection of antisense lines generated from a tobacco leaf cDNA library (Lein et al., 2008). The tobacco AtpC antisense construct was generated from an EST covering a 601-bp-long fragment of the AtpC gene (spanning positions 760 to 1361 of the coding region). This region is unique to AtpC. Searches of the AtpC EST against all known tobacco ESTs (in the National Center for Biotechnology Information [NCBI]), the complete Arabidopsis genome (in NCBI and The Institute for Genomic Research [TIGR]), and all Solanaeae species (in TIGR) did not reveal any sequence stretches with more than 18 bp homology. Therefore, off-target effects of the chosen antisense sequence are unlikely to occur. However, as the tobacco genome is not yet fully sequenced, they cannot be entirely excluded (see Discussion).

Transformation of tobacco plants with the antisense construct yielded several transgenic lines, two of which were characterized in detail. Whereas one line displayed a strong growth retardation (line atpcC1), the other showed only a mild growth phenotype (line atpcC2; Figure 1A). Flowering of the atpcC2 mutant was delayed on average by 3 to 4 d, while flowering of the atpcC1 mutant was delayed by several weeks in comparison to the wild type. In general, the strength of the growth phenotypes was somewhat variable between individual progeny plants. This is typical of antisense transformants because the underlying epigenetic effect is reset in meiosis (Kooter et al., 1999).

Transcript accumulation was analyzed by RNA gel blot analysis and revealed a much stronger repression of the AtpC transcript abundance in line atpcC1, in agreement with the growth phenotypes (Figure 1B). To analyze adaptive responses of the photosynthetic apparatus to the AtpC antisense repression, we determined several physiological parameters using the youngest fully expanded leaf of plants at the onset of flowering, usually leaf number six or seven from the top. We measured leaf absorbance and chlorophyll content, chlorophyll a/b ratio, assimilation and respiration capacities, the quantum efficiency of CO2 assimilation, maximum quantum efficiency of PSI, and photosynthetic complex contents (Table 1). While chlorophyll content, chlorophyll a/b ratio, and leaf absorbance were comparable in
the wild type and the weak atpC2 antisense line, the strong antisense line suffered from a slight reduction in chlorophyll content, which, however, did not significantly alter leaf absorbance. The most pronounced effect in the strong antisense line was a clear reduction in leaf assimilation capacity to \(~40\%\) of the levels in the wild type and the atpC2 plants. Respiration was unaltered. The quantum efficiency of CO₂ assimilation, as determined from the linear ranges of light response curves between 0 and 40 \(\mu\text{mol photons m}^{-2} \text{s}^{-1}\), was slightly reduced in the strong antisense line.

Interestingly, the chlorophyll \(a/b\) ratio was clearly reduced in the strong atpC1 mutant, suggesting a remodeling of the photosynthetic apparatus. Therefore, photosynthetic complex accumulation was determined spectroscopically. When normalized to chlorophyll content, neither PSII nor cyt-bf contents differ significantly between the wild type and the antisense lines. Also, PSII function does not seem to be impaired in the antisense lines, as indicated by the unaltered \(F_v/F_m\) values of dark-adapted leaves (Table 1). Interestingly, PC accumulation was increased to \(150\%\) of wild-type levels in the strong antisense line, while its PSI contents were slightly reduced, which concurs with its reduced chlorophyll \(a/b\) ratio (Table 1). These spectroscopic data were independently confirmed by protein gel blots (Figure 1C). No changes in the accumulation of PSII ( probed with an antibody directed against the essential reaction center subunit PsbD), cyt-bf ( probed with an antibody against the essential PetA subunit), and PSI ( probed with an antibody against the essential reaction center subunit PsaA) could be detected. The small decrease of redox-active PSI determined spectroscopically is below the resolution of protein gel blots.

The content of ATP synthase, as determined using an antibody against AtpA, the essential \(\alpha\)-subunit of CF1, was clearly diminished in both antisense lines (Figure 1C). While in the weak antisense line atpC2, ATP synthase contents ranging between 100 and \(~65\%)\ of wild-type levels were observed, the ATP synthase content was much more reduced in the strong antisense line atpC1. In this line, ATP synthase contents ranging between 25 and 10\%\ of the wild-type contents were determined. We only probed a single subunit of the ATP synthase, as loss of all subunits had previously been shown for an \(AtpC\) knockout line in \(\text{Arabidopsis}\), indicating that all other subunits are degraded when they cannot assemble together with \(AtpC\) into a stable complex (Bosco et al., 2004).

The effects of \(AtpC\) suppression on antenna complexes was assessed by 77K chlorophyll-\(a\) fluorescence emission spectra (Figure 1D), which, in the mutants, do not differ from the wild type, indicating that the strong antisense line can compensate for the small reduction in PSI reaction center contents by an antenna redistribution between both photosystems. In summary, these data suggest that the pronounced growth phenotype of the strong antisense line is specifically attributable to the reduced ATP synthase abundance.

**Generation and Molecular Characterization of \(atpB\) Translation Initiation Mutants**

To test an independent set of ATP synthase mutants that do not rely on epigenetic suppression of nuclear gene expression, we also wanted to reduce the expression level of an essential plastid genome-encoded ATP synthase subunit by generating transplastomic tobacco plants. To this end, point mutations were introduced into the AUG translation initiation codon of the \(atpB\) mRNA. The translation initiation codon is a major determinant of the rate of plastid translation initiation (Sugiura et al., 1998;
Majeran et al., 2000). In addition to the standard translation initiation codon AUG, two alternative start codons exist in plastids: GUG and UUG. While GUG is used as an initiation codon in both algae and higher plant chloroplasts, UUG has so far been found only in eukaryotic algae (Sugiura et al., 1998; Hirose et al., 1999). In a tobacco in vitro translation system, both alternative start codons can be recognized as translation initiation codons, but their translation initiation efficiency is much lower than that of the standard AUG codon (Hirose et al., 1999; Hirose and Sugiura, 2004). Therefore, transplastomic plants with these point mutations should accumulate reduced amounts of chloroplast ATP synthase.

The TTG and GTG mutations were introduced into a cloned plastid DNA fragment, and a chimeric aadA gene was inserted in the intergenic spacer between the rbcL and aadA plastid DNA fragment, and a chimeric synthase.

To characterize atpB expression in the transplastomic lines, we first compared transcript accumulation for atpB, rbcL, aadA, and the nuclear-encoded AtpC gene by RNA gel blot analysis (Figure 2C). The wild type displayed two similarly abundant bands for the atpB transcript, which may be attributable to the presence of multiple promoters upstream of the atpB gene (Chen et al., 1990). By contrast, all transformants contained only the shorter atpB transcript, which accumulated to similar levels as in the wild type. This indicates that the insertion of the aadA selectable marker gene has disrupted one of the atpB promoters. Accumulation of the rbcL mRNA was unaltered in all transformants, confirming that the insertion of the selectable marker gene between atpB and rbcL did not interfere with rbcL transcript accumulation. As expected, the aadA selectable marker gene was strongly expressed in all mutants and not detectable in the wild type. Expression of the nuclear AtpC gene was unaltered in all transformants when compared with the wild type.

To assess the effects of the TTG-atpB and GTG-atpB point mutations on translation, we determined the degree of polysome loading of the atpB mRNA as a proxy of translational activity (Barkan, 1998; Figure 2D). Sucrose gradient ultracentrifugation was used to separate mRNAs according to their loading with ribosomes, and six fractions were collected from the gradient after centrifugation. To distinguish between fractions of the sucrose gradient containing free atpB mRNA that is not associated with ribosomes and fractions containing the ribosome-associated, actively translated mRNAs, a sample was treated with puromycin, resulting in ribosome dissociation and release of the mRNA. The puromycin control indicates that free mRNAs are strongly enriched in fraction 2 of the sucrose gradient, while almost no signal is obtained for fractions 4 to 6. Therefore, mRNAs found in fractions 4 to 6 are ribosome associated and actively translated.

For the wild type, the strongest mRNA signals were obtained in fractions 4 and 5 (Figure 2D), while considerably weaker signals were detectable in fractions 3 and 6. Both atpB transcript species showed the same distribution in the gradient, indicating that their translation efficiency is comparable. The aadA-atpB control showed a similar mRNA distribution in the sucrose gradient as the wild type, suggesting that the translation rates are very similar to those in the wild type. The TTG-atpB mutation resulted in a clear shift of the polysome profile toward the upper fractions, indicating reduced translational efficiency. However, the strongest signal was still observed in fraction
Figure 2. Generation and Molecular Characterization of \( atpB \) Translation Mutants.

(A) Physical maps of the wild-type plastome region containing \( atpB \) (top) and the transplastome (bottom). Genes are shown as filled boxes. Genes shown above the line are transcribed from left to right and genes below the line in the opposite direction. Restriction sites used for cloning and restriction fragment length polymorphism analysis are indicated. Sites lost during the cloning process are shown in parentheses.

(B) Restriction fragment length polymorphism analysis of transplastomic plants. DNA was restricted with \( PvuII \). The location of the (\( rbcL \)-derived) hybridization probe is indicated in (A). The wild type (WT) shows the expected restriction fragment of 6.0 kb, while all transformants show only the transplastomic fragment of 7.2 kb (see [A]), confirming their homoplasmic state.

(C) Gene expression analysis in transplastomic plants. Expression of \( atpB, rbcL \) (encoding the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase), the selectable marker gene \( aadA \), and the nuclear-encoded \( AtpC \) gene was tested by RNA gel blot analysis.

(D) Polysome loading analysis to determine \( atpB \) translation rates in the \( atpB \) start codon mutants. Polysome association of the \( atpB \) mRNA was determined by fractionation of the sucrose gradient into six fractions. Polysome dissociation induced by puromycin treatment was used to identify fractions containing free mRNAs. These are only found in fractions 1 to 3, indicating that fractions 4 to 6 contain actively translated mRNAs. Fraction numbers are given above (from top to bottom), and the wedge indicates the increasing sucrose concentration in the gradient. The ethidium bromide–stained agarose gel is shown below each blot.
four, suggesting that the majority of the atpB mRNA is still translated. By contrast, the GTG-atpB mutant showed a severe alteration of the polysome profile. Similar to the puromycin control, signals were only obtained for the upper three fractions (Figure 2D), suggesting that atpB translation was largely abolished in this mutant.

In spite of the translational defects in the TTG-atpB and especially the GTG-atpB mutants, all transformants could grow autotrophically in soil. For Figure 3A, all plants were photographed 12 weeks after germination. While the wild type and the aadA-atpB control plants were phenotypically indistinguishable and started to flower, growth of the TTG-atpB mutant was clearly retarded. On average, onset of flowering was delayed by 1 week. Growth of the GTG-atpB mutant was severely compromised, and the plants flowered only after 6 to 9 months, producing much less flowers and seeds than the wild type and the other mutants. Therefore, the observed growth phenotypes are well in line with the translation rates as estimated from the polysome profiles.

Analysis of the Photosynthetic Apparatus in atpB Translation Mutants

To assess the impact of reduced atpB translation on the photosynthetic apparatus, the same physiological measurements as described for the atpC antisense plants were performed on the youngest fully expanded leaves of plants in the developmental stage shown for the wild type and the aadA-atpB control plants in Figure 3A. Interestingly, neither leaf absorbance, chlorophyll content, chlorophyll a/b ratio, assimilation, respiration, maximum PSII quantum efficiency, nor photosynthetic complex contents showed significant differences between the wild type, the aadA-atpB control transformant, and the TTG-atpB mutant (Table 2). Only the strongly affected GTG-atpB mutant suffered from a >50% reduction of chlorophyll content per leaf area, which also resulted in an ~10% reduction in leaf absorbance (Table 2).

Furthermore, its assimilation capacity per leaf area was repressed to 5% of wild-type levels, and respiration was reduced to 40% of wild-type rates. The quantum efficiency of photosynthesis in the GTG-atpB mutant was strongly reduced. Assimilation of one CO2 required 27 quanta, while in the wild type, the aadA-atpB control, and the TTG-atpB mutant, 11 to 12 quanta per CO2 were sufficient. Furthermore, the chlorophyll a/b ratio was strongly reduced in the GTG-atpB mutant, which, as in the atpC1 line, is attributable to a diminished accumulation of PSI, while PC contents per chlorophyll were strongly increased (Table 2). Accumulation of cyt-bf and PSII were unaltered. Measurements of the maximum quantum efficiency of PSI indicated that the GTG-atpB transformants suffered from mild photoinhibition of PSII.

The spectrosocopically determined contents of the photosynthetic complexes were confirmed by protein gel blots (Figure 3B) using the same antibodies as described for the atpC antisense plants. In spite of their similar growth phenotype, the aadA-atpB control plants showed an ~25% reduction in ATP synthase contents, relative to the wild type. Interestingly, while growth and atpB translation rates were clearly reduced in the TTG-atpB mutant, ATP synthase levels were indistinguishable from the aadA-atpB control (i.e., both had a 25% reduction in ATP synthase contents). ATP synthase accumulation in the strong GTG-atpB mutant was repressed to below 10% of wild-type levels, consistent with the strong reduction of both atpB translation rates and plant growth.

Finally, we also recorded the 77K chlorophyll-a fluorescence emission spectra of wild-type tobacco (dark-blue line), the aadA-atpB control (red line), the TTG-atpB mutant (green line), and the GTG-atpB mutant (light-blue line).
the reduced PSI accumulation and the decreased chlorophyll proteins uncoupled from PSI, which would be in agreement with the shift is indicative of the presence of light-harvesting complex I PSI emission maximum from 733- to 731-nm wavelength. This contrast, the strong GTG- 

Table 2. Photosynthetic Parameters in Wild-Type Tobacco (Petit Havana) and in the atpB Translation Initiation Mutants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-Type PH</th>
<th>aadA-atpB</th>
<th>TTG-atpB</th>
<th>GTG-atpB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorophyll a/b</td>
<td>4.02 ± 0.12</td>
<td>3.96 ± 0.09</td>
<td>3.96 ± 0.09</td>
<td>3.17 ± 0.17</td>
</tr>
<tr>
<td>Chlorophyll (mg m⁻²)</td>
<td>426.5 ± 30.4</td>
<td>438.5 ± 28.4</td>
<td>457.4 ± 61.7</td>
<td>205.7 ± 57.1</td>
</tr>
<tr>
<td>Leaf absorbance (%)</td>
<td>89.4 ± 2.2</td>
<td>87.9 ± 1.8</td>
<td>89.9 ± 1.5</td>
<td>78.1 ± 4.9</td>
</tr>
<tr>
<td>Assimilation (µmol CO₂ m⁻² s⁻¹)</td>
<td>30.4 ± 4.1</td>
<td>27.5 ± 2.8</td>
<td>31.1 ± 5.3</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>Respiration (µmol CO₂ m⁻² s⁻¹)</td>
<td>-1.6 ± 0.4</td>
<td>-1.9 ± 0.3</td>
<td>-1.8 ± 0.7</td>
<td>-0.6 ± 0.2</td>
</tr>
<tr>
<td>Quanta/CO₂</td>
<td>11.7 ± 1.4</td>
<td>12.3 ± 2.3</td>
<td>11.4 ± 0.9</td>
<td>27.0 ± 5.4</td>
</tr>
<tr>
<td>FS/Fm</td>
<td>0.82 ± 0.02</td>
<td>0.83 ± 0.01</td>
<td>0.84 ± 0.01</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>PSII (µmol mol chlorophyll⁻¹)</td>
<td>2.59 ± 0.21</td>
<td>2.57 ± 0.25</td>
<td>2.54 ± 0.24</td>
<td>2.56 ± 0.38</td>
</tr>
<tr>
<td>Cyt-bf (µmol mol chlorophyll⁻¹)</td>
<td>1.09 ± 0.16</td>
<td>1.01 ± 0.13</td>
<td>1.05 ± 0.17</td>
<td>1.11 ± 0.18</td>
</tr>
<tr>
<td>PC (µmol mol chlorophyll⁻¹)</td>
<td>4.44 ± 1.42</td>
<td>5.34 ± 1.12</td>
<td>5.16 ± 1.10</td>
<td>9.37 ± 1.28</td>
</tr>
<tr>
<td>PSI (µmol mol chlorophyll⁻¹)</td>
<td>2.37 ± 0.09</td>
<td>2.39 ± 0.07</td>
<td>2.41 ± 0.07</td>
<td>1.90 ± 0.12</td>
</tr>
<tr>
<td>ECS time constant (ms)</td>
<td>22.7 ± 4.1</td>
<td>22.3 ± 2.3</td>
<td>23.6 ± 1.7</td>
<td>90.2 ± 20.8</td>
</tr>
</tbody>
</table>

All measurements were performed on the youngest fully expanded leaves of plants at the onset of flowering. Six wild-type plants and a minimum of four plants per transformant line were measured. As the two independently generated transplastomic lines for each constructs were indistinguishable, average data and the SD from two lines per construct are shown.

indistinguishable, which is consistent with unaltered photosynthetic complex accumulation and chlorophyll a/b ratios, suggesting that also their antenna organization is unaltered. By contrast, the strong GTG-atpB mutant showed a small shift of the PSI emission maximum from 733- to 731-nm wavelength. This shift is indicative of the presence of light-harvesting complex I proteins uncoupled from PSI, which would be in agreement with the reduced PSI accumulation and the decreased chlorophyll a/b ratio (Table 2).

ATP Synthase Activity Strictly Correlates with Linear Electron Flux and Assimilation Capacity but Not with ATP Synthase Contents

To test if the parallel repression of leaf assimilation and ATP synthase activity is a consequence of a restricted availability of ATP to the Calvin cycle or, alternatively, is due to feedback effects of the reduced thylakoid conductivity on electron transport, we analyzed the dependence of assimilation capacities per leaf area (Figure 4B) and linear electron flux (as determined from the quantum yield of PSII after correction for leaf absorbance; Figure 4C) on ATP synthase activity (gH⁺). In the GTG-atpB mutant and the TTG-atpB translation initiation mutant was a clear correlation between content and activity observed.

Next, we analyzed the dependence of assimilation capacities per leaf area (Figure 4B) and linear electron flux (as determined from the quantum yield of PSII after correction for leaf absorbance; Figure 4C) on ATP synthase activity (gH⁺). In the GTG-atpB mutant and the TTG-atpB translation initiation mutant, we analyzed the dependence of assimilation capacities per leaf area (Figure 4B) and linear electron flux (as determined from the quantum yield of PSII after correction for leaf absorbance;

ATP Synthase Repression Results in Increased Lumen Acidification and Accelerated Induction of Photoprotective Mechanisms

To test if the parallel repression of leaf assimilation and ATP synthase activity is a consequence of a restricted availability of ATP to the Calvin cycle or, alternatively, is due to feedback effects of the reduced thylakoid conductivity on electron transport, we analyzed the responses of linear electron flux and the proton circuit in detail. First, we determined light response curves of the pmf (Figure 5A) and its partitioning into ΔpH and Δψ.
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ΔΨ (Figure 5B). The two components of the pmf can be distinguished by measuring the slow phase of ECS relaxation in the range of seconds, which is attributable to slow counter ion movements across the thylakoid membrane and proportional to the ΔpH component (Cruz et al., 2001; Baker et al., 2007). The ECS$_T$ amplitudes clearly show that, as a consequence of the lower thylakoid conductivity in both the strong antisense line and the GTG-atpB transformant, the maximum pmf across the thylakoid membrane is strongly increased, and its light response curve is shifted to lower light intensities. In both mutants, pmf was fully light saturated at 500 μmol photons m$^{-2}$ s$^{-1}$, and already at 200 μmol photons m$^{-2}$ s$^{-1}$ light intensity, >80% of the maximum pmf was established. In the wild-type plants as well as in theaadA-atpB control and in the TTG-atpB mutant, pmf was only saturated at 2100 μmol photons m$^{-2}$ s$^{-1}$ light intensity. The weak antisense line showed a tendency toward a slightly increased pmf at all light intensities, relative to the wild-type SNN.

While pmf partitioning into ΔpH and ΔΨ was not significantly different at saturating light intensities, the pmf partitioning under low light was clearly shifted in favor of the ΔΨ component both in the strong antisense lines and the GTG-atpB mutant (Figure 5B). However, relative to the wild type, the ΔpH components in the strong antisense line and in the GTG-atpB mutant were still 3 and 2 times larger at 200 μmol photons m$^{-2}$ s$^{-1}$, respectively, resulting in a more acidic thylakoid lumen.

To determine the effects of the increased lumen acidification in atpC1 and GTG-atpB mutants on the efficiency of linear electron flux and PSII antenna function, we measured light response curves of linear electron flux and of the chlorophyll-a fluorescence parameters qN and qL, which represent measures of the thermal dissipation of excess excitation energy and of the redox state of the PSII acceptor side, respectively (Krause and Weis, 1991; Baker et al., 2007). In agreement with the lower assimilation rates determined by gas exchange measurements (Figure 4, Tables 1 and 2), linear electron transport rates were strongly reduced in the atpC1 antisense line and even more so in the GTG-atpB mutant (Figure 6A). The PSII acceptor side was more rapidly reduced already at low light intensities in the atpC1 antisense line and in the GTG-atpB mutant, as revealed by their much lower qL values, suggesting an impaired linear electron flux (Figure 6B). Finally, the onset of qN was accelerated in GTG-atpB and in both antisense lines, though the effect was much weaker in atpC2 than in atpC1 (Figure 6C). From the more acidic thylakoid lumenal pH in the antisense lines, it was expected that the strong induction of qN in low light is largely attributable to its rapidly reversible qE component (i.e., the thermal dissipation of excitation energy in the PSII antenna by the xanthophyll cycle and the PsbS protein). This was confirmed by analyzing the relaxation of qN during the first 15 min after the end of actinic illumination. In both antisense lines and in the wild type, the vast majority of qN relaxed within 10 min after the end of actinic illumination, confirming that quenching is of the qE type (PSII photoinhibitory quenching, qL, relaxes much more slowly; Krause and Weis, 1991). The same holds true for the atpB translation mutants.

The more reduced state of the PSII acceptor side, as deduced from the light response curve of qL (Figure 6B), could either be the consequence of a general reduction of metabolic NADPH consumption due to ATP limitation of the dark metabolism or, alternatively, could be due to impaired linear electron flux from PSII to NADP$^+$ as a result of the more acidic thylakoid lumen. To distinguish between these two possibilities, we next determined the light response behavior of the PSI donor side by quantifying the increase in oxidized inactive PSI with the light intensity (Figure 7A). While the PSII acceptor side and the PQ pool were much more reduced in the strong antisense line and in the GTG-atpB mutant than in the respective wild type (Figure 6B), the PSI donor side showed the opposite redox behavior. It was much more oxidized already at low light intensities, indicating a

Figure 4. ATP Synthase Activity Limits Assimilation Capacity and Linear Electron Flux.

Each dot in the graphs represents the results for a single plant. Wild-type (WT) SNN plants are shown as black closed circles, the atpC1 plants as black open circles, and the atpC2 plants as gray filled circles. Color usage for the atpB translation mutants is as in Figure 3.

A) ATP synthase activity (gH$^+$ s$^{-1}$) as a function of ATP synthase content.
B) Assimilation (expressed on a leaf area basis) as a function of ATP synthase activity (gH$^+$ s$^{-1}$).
C) Linear electron flux (on a leaf area basis) as determined from chlorophyll-a fluorescence analysis and corrected for leaf absorptance as a function of ATP synthase activity (gH$^+$ s$^{-1}$).
restricted linear electron flux from PSII toward PSI. No differences between the SNN wild type and the weak antisense line or between the Petit Havana wild type, the aadA-atpB control, and the TTG-atpB mutant was found (Figure 7A). This strongly suggests that in both the GTG-atpB mutant and the strong antisense line, linear electron flux is restricted at the level of cyt-bf or PC.

To determine the exact site of flux limitation, we measured reduction kinetics of cyt-f and P 700 after the end of a saturating light pulse, when the PSII acceptor side and the PQ pool are completely reduced and the high potential chain comprising cyt-f, PC, and the PSI reaction center chlorophyll-a special pair dimer P700 is fully oxidized. After the end of the light pulse, electron flux through the cyt-bf toward PSI was determined. For better comparability, the fully oxidized states of cyt-f (Figure 7B) and P 700 (Figure 7C) were normalized to one, and the completely reduced states were normalized to zero. In agreement with their basically identical light response curves of P 700 oxidation (Figure 7A), the cyt-f and P 700 reduction kinetics of the SNN wild type and the weak antisense line were also indistinguishable (with P 700 reduction half-times being 7 ms). However, the reduction kinetics in the strong antisense line were 2.5-fold slower, with the half-time of P 700 reduction being increased to 17 ms. This finding is well in line with the observed decrease of leaf assimilation and linear electron flux rates (Figures 4A and 4B) and the altered redox poise of the electron transport chain (Figures 6B and 7A).

The same is true for the GTG-atpB mutant, whose P 700 reduction half-time was increased to 22 ms compared with 6 ms in the PH wild type, the aadA-atpB control, and the TTG-atpB mutant. This is again in good agreement with the strong repression of linear electron flux and assimilation.

**DISCUSSION**

**Modification of Chloroplast ATP Synthase Accumulation by AtpC Antisense Repression and Manipulation of atpB Translation**

In the unicellular green alga *C. reinhardtii*, the accumulation of the chloroplast ATP synthase is controlled in part by the synthesis of the nuclear-encoded γ-subunit. In its absence, translation of the plastid-encoded β-subunit (*atpB*) is repressed (Drapier et al., 2007). As accumulation of the β-subunit is required to activate translation of the α-subunit of the ATP synthase, *atpC* and *atpB* function as dominant subunits in a network of translational autoregulation of ATP synthase biogenesis in *C. reinhardtii*. It is currently unknown if the same mechanisms control ATP synthase biogenesis in higher plants. In this work, we targeted both *atpC* (by a nuclear antisense approach) and *atpB* (by reducing its translation initiation in the chloroplast) to repress ATP synthase accumulation in tobacco and study the impact on photosynthesis, assimilation, and plant growth.
Our data show that both AtpC antisense repression and manipulation of atpB translation initiation are feasible strategies to modify chloroplast ATP synthase accumulation. The successful repression of ATP synthase accumulation in our transplastomic approach demonstrates that photosynthetic complex contents in higher plants can be altered by reducing translation of an essential subunit.

In vitro translation assays suggested that both UUG and GUG can serve as alternative start codons in chloroplast translation initiation but are recognized with much lower efficiency than the standard AUG initiator codon (Hirose et al., 1999; Hirose and Sugiura, 2004). However, an exact prediction of in vivo translation initiation rates for these alternative start codons is not possible because their efficiency depends strongly on the sequence context around the initiation codon (Boeck and Kolakofsky, 1994). In our experiments, only the GTG-atpB transplastomic lines suffered from a strong repression of ATP synthase accumulation, assimilation, and linear electron flux (Figure 4) and displayed strongly retarded growth (Figure 3A). Mature leaves of both the TTG-atpB mutant and the aadA-atpB control showed on average a 25% reduction of ATP synthase contents, relative to the wild type (Figure 4A). However, this did not result in a significantly reduced ATP synthase activity. Also, no alterations in electron transport and assimilation capacities were observed (Figures 4B and 4C), and all other photosynthetic parameters were indistinguishable from the control plants (Table 2).

The reduced ATP synthase accumulation in both the aadA-atpB control and the TTG-atpB mutant is most likely attributable to the 50% reduction in atpB mRNA accumulation observed in all atpB translation initiation mutants. The reduced mRNA accumulation is due to the disruption of a second, so far unknown atpB promoter in the tobacco plastome by the insertion of the aadA

Figure 6. Restricted Linear Electron Transport, Overreduction of the PSII Acceptor Side, and Accelerated Induction of qN upon ATP Synthase Repression.

Symbols are used as in Figure 4. Data for the SNN wild type (WT) and the antisense plants are shown in the left panel, and data for the PH wild type and the atpB translation mutants are shown in the right panel. A minimum of 10 individual plants were measured per line, and standard deviations are shown as error bars.

(A) Light saturation curve of linear electron flux as calculated from PSII yield measurements and corrected for leaf absorptance. Linear electron flux is strongly repressed in response to reduced ATP synthase contents.

(B) Redox state of the PSII acceptor side, determined as qL. With increasing light intensity, the PSII acceptor side becomes progressively reduced. Note that this effect is shifted to lower light intensities in the strong antisense line and in GTG-atpB.

(C) Measurement of nonphotochemical quenching (qN). The onset of qN is shifted toward lower light intensities in both antisense lines and in the GTG-atpB mutant, resulting in increased thermal dissipation of excitation energy, reduced quantum efficiency of photosynthesis, and, thus, lower light use efficiency.
selectable marker gene (Figure 2C). It is not surprising that the actual ATP synthase accumulation in the aadA-atpB control and the TTG-atpB mutant is much less affected than atpB mRNA levels, as it is well established that translation initiation rather than transcription is limiting photosynthetic complex accumulation in photosynthetic eukaryotes (Eberhard et al., 2002; Drapier et al., 2007).

Interestingly, polysome analyses revealed that also in the TTG-atpB mutant, atpB translation was somewhat reduced, relative to the wild type and the aadA-atpB control, even though the effects were much less severe than those in the GTG-atpB mutant, in which atpB translation was almost completely abolished. This slight reduction of atpB translation in the TTG-atpB mutant might explain the weak growth retardation relative to the aadA-atpB control, which displayed a similar (~25%) reduction in ATP synthase contents in mature leaves as the TTG-atpB mutant. The reduced atpB translation could slightly delay ATP synthase biogenesis during the early stages of leaf development, when translation rates are highest. This would limit photosynthesis during early phases of plant development, when growth is most sensitive to delayed establishment of a fully functional photosynthetic apparatus and reduced photoassimilate availability. In mature leaves, when the photosynthetic apparatus is fully established and low translation rates are sufficient for its maintenance, ATP synthase contents then reach wild-type levels. Due to the limited availability of young, strongly expanding leaf material, we have not yet been able to test this hypothesis.

As a second independent approach and to obtain mutants covering a wider range of ATP synthase contents, we also used antisense RNA directed against the nuclear-encoded essential

**Figure 7.** Limited Linear Electron Flux through cyt-bf in ATP Synthase Mutants.

The symbols are used as in Figure 4. Data for the SNN wild type (WT) and the antisense plants are shown in the left panel, and data for the PH wild type and the atpB translation mutants are shown in the right panel. A minimum of 10 individual plants were measured for all lines, and standard deviations are shown as error bars in (A).

(A) Light intensity–dependent oxidation of the donor side of PSI and the high potential chain.

(B) and (C) Restricted linear electron flux due to photosynthetic control, as evidenced by retarded reduction kinetics of cyt-f (B) and P<sub>700</sub> (C).
AtpC subunit. We analyzed two lines suffering from different degrees of ATP synthase repression in detail. One of the lines (atpC1) showed a strong growth phenotype and a clear reduction in ATP synthase accumulation, providing ATP synthase activities between those of the GTG-atpB translation mutant and the wild type. The other line (atpC2) showed only a mild reduction in ATP synthase accumulation, and linear electron flux and assimilation were largely unaltered relative to the wild type. Taking the translation initiation mutants and the antisense plants together, a wide range of ATP synthase contents and activities is covered.

One concern arising from employing an antisense strategy is the possibility that the observed phenotypes are not exclusively related to the repression of the target gene. In theory, other genes could be affected by so-called off-target effects. These could be brought about by posttranscriptional silencing of genes sharing short identical sequence stretches with the gene of interest. As the tobacco genome is not fully sequenced, it is not possible to fully exclude such off-target effects. However, two lines of evidence argue against the presence of off-target effects in our nuclear-transgenic antisense plants. First, no EST from tobacco or any other Solanaceous species was found that would share homologous sequences of >18 bp length with AtpC. More importantly, comparison of phenotypes and photosynthetic parameters in nuclear antisense and the plastid translation mutants provides strong evidence against off-target effects. The GTG-atpB and atpC1 mutants behave in a very similar manner, as far as the effects of reduced ATP synthase activity on linear electron transport and leaf assimilation capacity are concerned (Figures 4B and 4C), with all parameters being slightly more affected in the GTG-atpB mutant due to its more severe ATP synthase repression. Both mutants also suffer from an accelerated induction of qN already at low light intensities (Figure 6C) due to increased lumen acidification (Figures 5A and 5B), resulting in a decreased quantum efficiency of CO2 fixation (Tables 1 and 2). They also suffer from a chronic overreduction of the PSI acceptor side (Figure 6B) and an accelerated oxidation of the PSI donor side (Figure 7A) due to decreased rates of PQ reoxidation at the cyt-bf (Figures 7B and 7C). Finally, both mutants also show a reduced PSI accumulation and an upregulation of PC (see below). Therefore, the observed effects are independent of the chosen strategy for ATP synthase repression.

Decreasing ATP Synthase Contents Reduces CO2 Assimilation, Electron Transfer, and Plant Growth

ATP synthase repression to <50% of wild-type levels resulted in a marked reduction of ATP synthase activity. Strikingly, we observed a strict correlation between the activity of the ATP synthase, measured by the decay of the ECS signal, and assimilation capacity (Figure 4B) as well as linear electron flow (Figure 4C), establishing ATP synthase activity as a potentially limiting or governing factor of photosynthesis. This observation is in agreement with the growth phenotypes reported by Price et al. (1995) for δ-subunit antisense lines, although at that time, no analysis of the molecular mechanisms underlying the growth retardation was undertaken. It has also been observed that mutations affected in the reductive activation of the ATP synthase by thioredoxin (due to amino acid substitutions in the γ-subunit) have an adverse effect on photosynthesis and plant growth in low light (Wu et al., 2007), although also in this case, the mechanistic basis has not been explored.

Steady State pmf Is Increased in ATP Synthase Mutants

In vivo probing of both electron and proton transfer allowed us to follow the effects of reduced ATP synthase expression on the photosynthetic energy budget. We found that reduced ATP synthase activity leads to an increased steady state pmf (Figure 5A). Even though the partitioning of pmf into Δψ and ΔpH components was shifted in favor of Δψ in both atpC1 and GTG-atpB, especially at low light intensities (Figure 5B), the strongly increased pmf led to a higher ΔpH and thus a more acidic luminal pH.

Thus, it appears likely that the growth phenotype of the strong antisense plant represents an indirect inhibition of linear electron flux by the increased acidification of the lumen due to restricted PQ reoxidation, a phenomenon referred to as photosynthetic control (Figures 7B and 7C). PQ oxidation represents a rate-limiting step in linear electron flux, even in wild-type plants under nonstressed conditions (Anderson, 1992; Price et al., 1998), but it is further slowed down when the pH value in the thylakoid lumen drops below 6.5. This is because PQ oxidation now works against a much higher preestablished pmf (Kramer et al., 1999; Hope, 2000). Supporting this view, ATP synthase repression led to a more reduced PQ pool (Figure 6B) and a more oxidized high potential chain (Figure 7A), clearly indicating a restriction in electron flow at the level of cyt-bf. The colimitation (or coregulation) of proton flow through the ATP synthase and electron flow through the photosynthetic chain should result in restrictions in both ATP and NADPH synthesis, which in turn result in down-regulation of the Calvin cycle.

Excess ATP Synthase Capacity in Wild-Type Plants

Interestingly, neither antisense line atpC2 nor the aadA-atpB control or the TTG-atpB mutant, which suffer from an up to 40% reduction in ATP synthase contents, displayed a significant decrease in ATP synthase activity (Figure 4A) and assimilation capacity (Figure 4B). Only in the GTG-atpB mutant and the strong antisense line atpC1, which contain ATP synthase contents between 20 and <5% of wild-type levels, were clear correlations between ATP synthase content and activity observed. We conclude that ATP synthase activity is not exclusively controlled by absolute enzyme contents but instead is highly regulated at the level of enzyme activity. This was previously demonstrated by Kanazawa and Kramer (2002), showing strong effects of CO2 levels on ATP synthase activity. When CO2 was increased from 0 to 2000 ppm, ATP synthase activity increased by approximately sixfold, leading to large changes in the regulation of photosynthesis. It is noteworthy that the maximum ATP synthase activity was not yet reached at 350 ppm CO2, implying that ATP synthase is partly downregulated at ambient CO2. Maximum ATP synthase activity was established at 500 ppm CO2, followed by a minor decrease when CO2 contents were further increased up to 2000 ppm (Kiirats et al., 2009). All these data suggest that the ATP
Luminal pH values below 5.5 have been observed to dramatically accelerate photodamage in vitro, particularly at the donor side of PSII (Krieger and Weis, 1993; Kramer et al., 1999). Also, PC is highly sensitive to pH values below 5.5 (Gross et al., 1994; Kramer et al., 1999). However, in both the strong antisense line and the GTG-atpB mutant, the content of redox-active PC actually increased (Tables 1 and 2). The fact that we observed neither increased PSII photodamage nor inactivation of PC suggests that the luminal pH did not decrease below this threshold value. Alternatively, the tobacco photosynthetic apparatus might be unusually acid resistant. At present, we favor the former scenario, noting that the pH sensitivity of cyt-b/f should act as a feedback regulator preventing overacidification by slowing down electron transfer as pH decreases. Likewise, increased luminal acidification also activates photoprotective qE quenching (Figure 6C), thereby at least partly ameliorating the photo-inhibitory effects of a more reduced PQ pool (Figure 6B).

The increase in redox-active PC can most likely be attributed to the strongly repressed assimilation capacity of the mutants. PC expression is known to be repressed via sugar sensing when high amounts of photoassimilate accumulate in source leaves (Oswald et al., 2001; Schöttler et al., 2004). Thus, the strongly restricted assimilation in both mutants could result in a derepression of PC. Interestingly, while PC accumulation was increased and neither accumulation nor functional integrity of PSII was strongly affected, both the atpC1 and the GTG-atpB mutants suffered from a 20% reduction of redox-active PSI per chlorophyll (Tables 1 and 2). This could indicate that PSI represents the component of the photosynthetic apparatus that is most sensitive to luminal acidification.

Alternatively, the observed PSI repression could be due to retrograde signals elicited by the repression of ATP synthase. However, the observed repression of PSI cannot be reconciled with any of the known retrograde signals, which should rather result in an upregulation of PSI in the mutants because their PQ pool is chronically overreduced. This should increase PSI contents and repress PSII accumulation because a reduced PQ pool would be a major target of such a pathway because it was unaltered in the atpB translation mutants. Future investigations should reveal if the reduced PSI accumulation is attributable to direct damage by increased luminal acidification or rather to altered photosynthetic gene expression.
The ATP Synthase as a Potent Control Point for Photosynthesis

Our data establish the ATP synthase as an additional control point of the photosynthetic light reactions, as previously suggested by Kramer et al. (2003), in addition to cyt-bf (Anderson, 1992; Price et al., 1995; Kirchhoff et al., 2000) and potentially PC (Schöttler et al., 2004). However, while already very small changes in cyt-bf content and activity linearly affect linear electron flux and assimilation (Price et al., 1998; Kirchhoff et al., 2000), an up to 50% decrease in ATP synthase content can be compensated on a posttranslational level. The relevance of ATP synthase adjustment is obvious from the fact that an up to fivefold downregulation of ATP synthase contents occur during leaf ageing (Schöttler et al., 2007a) and in response to drought stress (Tezara et al., 1999; Kohzuma et al., 2009). Flux control by such strong changes could be even more efficient than by downregulation of cyt-bf. While cyt-bf and PC repression would reduce linear flux directly, ATP synthase regulation would reduce it indirectly due to increased pmf across the thylakoid membrane and photosynthetic control.

However, upon ATP synthase repression, the onset of photoprotective mechanisms is shifted to lower light intensities because lower proton influx rates can sufficiently acidify the lumen to trigger qN. This results in a decrease of the photosynthetic quantum efficiency and thus reduces plant growth. By contrast, selective suppression of cyt-bf without a parallel downregulation of the ATP synthase should have a lower impact on quantum efficiency because it reduces only the capacity for proton influx into the lumen, whereas proton efflux through the ATP synthase would remain high. This should result in a lower steady state pmf and, consequently, in a less efficient induction of the photoprotective mechanisms. Evidence for this scenario comes from our characterization of a ΔpetL mutant suffering from accelerated leaf age-dependent loss of cyt-bf and reduced electron flux capacity due to absence of a small subunit of the cyt-bf. As the ATP synthase is not degraded in parallel with cyt-bf, but instead maintained at wild-type levels, nonphotochemical quenching in these mutants is compromised (Schöttler et al., 2007b).

By a strict coregulation of ATP synthase and cyt-bf contents during leaf ageing in tobacco (Schöttler et al., 2007a), plants can balance the rates of proton influx via electron transport and proton efflux through the ATP synthase in a way that under nonstressed conditions lumen acidification is kept close to but above the threshold value for triggering photoprotective mechanisms, such as qN and photosynthetic control. In this way, optimum photosynthesis with a high quantum efficiency is ensured. However, when ATP and NADPH consumption by the Calvin cycle decreases, the reduced proton efflux through the ATP synthase results in an immediate decrease of the luminal pH below the threshold value for activation of the photoprotective mechanisms, thus enabling a rapid response of the plant to this disturbance. Therefore, we postulate that the ATP synthase as a major control point in the long-term adjustment of the photosynthetic light reactions to changing metabolic demands. Furthermore, we suggest that coregulation of linear electron flux (via cyt-bf and PC contents) with the proton circuit (via ATP synthase accumulation) is required to maintain an optimum steady state luminal pH value under unstressed conditions. This mechanism allows the plant to tightly control the induction of photoprotective mechanisms, thus optimizing photosynthetic quantum efficiency.

METHODS

Plant Material and Growth Conditions

Tobacco (Nicotiana tabacum) AtpC antisense lines were raised from seeds germinated on Petri dishes containing Murashige and Skoog medium supplemented with 2% (w/v) sucrose and 300 μg mL⁻¹ kanamycin. Wild-type seedlings were grown on the medium without kanamycin. Fourteen days after germination, the seedlings were transferred to a soil-vermiculite mixture (2:1; Floragard Vertriebs) and grown in a controlled-environment chamber. Transplastomic plants were grown on soil. All plants were fertilized with Hakaphos Special (Compo Expert) to keep the soil moist. All measurements were performed on the youngest fully expanded leaves at the onset of flowering. The actinic light intensity was 300 μmol photons m⁻² s⁻¹ at the level of these leaves. The plants were illuminated for 16 h at 22°C. The growth temperature was reduced to 18°C during the night. Relative humidity was set to 75%.

Generation of Antisense Constructs and Nuclear Transformation

The antisense construct was expressed under the control of the cauliflower mosaic virus 35S promoter and assembled in a plasmid vector described previously (Lein et al., 2008). Correct insertion of the cassette was verified by PCR with genomic DNA as template and primers 35S (5′-GGGAGTGTAGTGATCTTC-3′) and octopine synthase (5′-GTAAGGACTCTGAAGTACAT-3′) followed by direct sequencing of the PCR products (Lein et al., 2008). Tobacco plants (cv Samsun NN) were transformed with the antisense construct by Agrobacterium tumefaciens–mediated gene transfer using A. tumefaciens strain C58C1:pGV2260 (Rosahl et al., 1987).

Vector Construction for Plastid Transformation and Generation of Transplastomic Plants

The region of the tobacco plastid genome containing part of the accD gene, the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (rbcL), and the 5′ portion of the atpB gene was isolated as a 5132-bp XhoI/Stul restriction fragment corresponding to plastome positions 55360 to 60492 (Figure 2A). The fragment was cloned into a pBS SK vector (Stratagene) cut with XhoI and EcoRI (3616). To remove the Eco0109 site from the remaining multiple cloning site, the resulting plasmid was linearized with Eco0109I, and the recessed ends were filled in using the Klenow enzyme followed by religation. A chimeric aadA gene fused to chloroplast-specific expression signals and conferring resistance to the aminoglycoside antibiotics spectinomycin and streptomycin (Svab and Maliga, 1993) was cloned into a unique ClaI site (position 57175) to enable selection of transplastomic lines. Constructs containing the aadA selectable marker gene in the same orientation as the rbcL gene were chosen for all further cloning steps. This construct was called aadA-atpB, as it contained the selectable marker gene and the atpB gene without any point mutations in its translation initiation codon.

The aadA-atpB construct was then used to produce the translation initiation codon mutations by PCR. The region surrounding the ATG start codon was excised by digestion with Nhel and Eco0109I (restriction sites correspond to nucleotide positions 58844 and 56410, respectively) and replaced by PCR products carrying the point mutations. The mutations were introduced with the primer sequences (see below), yielding the final transformation vectors TTG-atpB and G TG-atpB. Primer oR1l_pR111 Faul
The quantum efficiency of CO₂ fixation was determined in the linear light response range between 0 and 60 μmol photons m⁻² s⁻¹ (wild type and weak mutants) or between 0 and 40 μmol photons m⁻² s⁻¹ (strong mutants). After the end of the gas exchange measurements, leaf absorbance was determined using an integrating sphere (ISV-469; Jasco) attached to the V550 spectrophotometer (Jasco). The spectral bandwidth was set to 1 nm, and the scanning speed was 200 nm min⁻¹. The trans-mission and reflectance spectra were measured between 700- and 400-nm wavelength, and leaf absorbance was calculated as 100% – transmittance (% – reflectance (%)). Finally, the chlorophyll content of the measured leaf discs was determined according to Porra et al. (1989). Assimilation rates were corrected for the dark respiration, assuming comparable respiratory activities in the light and in darkness (Fernie et al., 2004).

**pmf Measurements and ATP Synthase Activity**

The electrochromic absorption shift (ECS) was used as an in vivo probe of the pmf across the thylakoid membrane (Kramer et al., 2003; Takizawa et al., 2007). The difference transmission signal was measured using a KLAS-100 LED-array spectrophotometer (Heinz Walz), allowing the simultaneous measurement of light-induced difference absorption signals at eight pairs of wavelengths in the visible range of the spectrum between 505 and 570 nm. The first two wavelength pairs at 505 to 520 and 520 to 535 nm are dominated by the electrochromic shift as well as by the slow zeaxanthin to violaxanthin conversion (505 to 520 nm) and the scattering signal most likely arising from PsbS protonation (520 to 535 nm). The other wavelength pairs (540 to 545 nm, 545 to 550 nm, 550 to 554 nm, 554 to 559 nm, 559 to 563 nm, and 563 to 570 nm) are mainly used for the measurements of the C550 pheophytin signal and of cytochrome signals. The ECS was deconvoluted from signals arising from zeaxanthin formation, scattering effects, the C550 signal, and from redox changes of the cytochromes f, b₅, and b₅₅₈ essentially as described by Klughammer et al. (1990). The deconvoluted transmission signals (ΔT/ΔΦ) were then normalized to the chlorophyll content of the measured leaf section.

The maximum amplitude of the ECS (ΔΦₚ) was used as a measure for the light-induced pmf across the thylakoid membrane. The measurements were performed at 20°C under CO₂-saturated conditions (2000 ppm). Leaves were illuminated for 10 min prior to each measurement to allow photosynthesis to reach steady state. ECSₚ was determined after illuminating the leaves with saturating light (2100 μmol photons m⁻² s⁻¹), which then was interrupted by a short interval of darkness (15 s), and the dark-interval relaxation kinetics of the ECS and of cyt-f (cyt-f) reduction were measured. Dark intervals (15 s) were sufficient for complete relaxation of the pmf. To obtain light saturation curves of the pmf, dark-interval relaxation kinetics after illumination at saturating light intensities between 200 and 2100 μmol photons m⁻² s⁻¹ were measured, pmf partitioning into ΔpH and Δν was resolved using the slowly relaxing phase of the ECS between 1 and 15 s of darkness as described by Takizawa et al. (2007).

To determine ATP synthase activity, the fast phase (between 0 and 500 ms after the end of actinic illumination) of the dark-interval relaxation kinetics measured at saturating light (2100 μmol photons m⁻² s⁻¹) was fitted with a single exponential decay function. The reciprocal value of the halftime of the ECS decay, the thylakoid conductivity (gH⁺), was used as a measure of ATP synthase activity because fast ECS decay is exclusively attributable to proton efflux through the ATP synthase (Cruz et al., 2001; Baker et al., 2007).

**Thylakoid Membrane Isolation and Quantititation of Photosynthetic Complexes**

Thylakoid membranes were isolated according to Schöttler et al. (2004). The contents of PSII and cyt-bf were determined from difference
absorption signals of cyt-b_{f}, P_{700}, and cyt-f and b_{569} (cyt-bc). Thyloalkoids equivalent to 50 μg chlorophyll mL⁻¹ were destacked in a low salt medium to improve the optical properties of the probe (Kirchhoff et al., 2002). All cytochromes were oxidized by the addition of 1 mM potassium ferricyanide (+III) and subsequently reduced by addition of 10 mM sodium ascorbate and dithionite, resulting in the reduction of cyt-f and the high-potential form of cyt-b_{559} (ascorbate-ferricyanide difference spectrum) and of cyt-b_{569} and the low-potential form of cyt-b_{559}, respectively. At each redox potential, absorption spectra were measured between 575- and 540-nm wavelength with a V-550 spectrophotometer (Jasco) equipped with a head-on photomultiplier. The spectral bandwidth was 1 nm, and the scanning speed was 100 nm min⁻¹. Difference absorption spectra were deconvoluted using reference spectra and difference extinction coefficients as by Kirchhoff et al. (2002). PSII contents were calculated from the sum of the high- and low-potential difference absorption signals of cyt-b_{559} (Lamkemeyer et al., 2006).

The content of redox-active PSI was determined from light-induced difference absorption changes of P_{700}, the PSI reaction center chlorophyll-a special pair dimer. Isolated thyloalkoids equivalent to 50 μg chlorophyll mL⁻¹ were solubilized with 0.2% (w/v) β-dodecymethylsulfos in the presence of 100 μM pararosanilin as electron acceptor and of 10 mM sodium ascorbate as electron donor. P_{700} was oxidized by the application of a saturating light pulse (2000 μmol photons m⁻² s⁻¹ red light, 200-ms duration). Measurements were done using the Dual-PAM instrument (Heinz Walz) in its PC-P_{700} version (see below).

PC contents, relative to P_{700}, were determined by in vivo difference absorption spectroscopy in the far-red range of the spectrum and then recalculated based on the absolute P_{700} quantification in isolated thyloalkoids (see above). Light-induced absorption changes at 800- to 870-nm wavelength (where the contribution of P_{700} is predominant) and at 870- to 950-nm wavelength (preliminarily arising from PC) were measured on preilluminated leaves with a fully activated Calvin cycle to avoid acceptor side limitation of PSI (Schötterl et al., 2007a). To determine the maximum difference absorption signals of PC and PSI, preilluminated leaves were transferred into darkness for a few seconds to fully reduce both components, and this was followed by 8 s of illumination with far-red light (715-nm wavelength) to selectively excite PSI. Then, a saturating pulse of red light was applied (635-nm wavelength, 5000 μmol photons m⁻² s⁻¹, 200-ms duration), to completely oxidize PC and PSI and reduce the PSI side of the electron transport chain. At the end of the actinic light pulse, all light sources were switched off, and PC and PSI reduction kinetics were determined.

Chlorophyll-a Fluorescence

The 77K chlorophyll-a fluorescence emission was determined on freshly isolated thyloalkoids equivalent to 10 μg chlorophyll mL⁻¹ using an F-6500 fluorometer (Jasco). The sample was excited at 430-nm wavelength (10-nm bandwidth). The emission spectra between 655 and 800 nm were recorded with a bandwidth of 1 nm. Chlorophyll-a fluorescence of intact leaves was measured at room temperature using a Dual-PAM-100 instrument (Heinz Walz). Light-response curves of linear electron flux and of nonphotochemical quenching as well as of the redox state of the PQ pool were recorded on intact leaves after 30 min of dark adaptation. The redox state of the PSII acceptor side was calculated from the QL parameter (Kramer et al., 2004), while qN was determined according to Krause and Weis (1991). Electron transport rates were corrected for leaf absorbance (see above).

Protein Gel Electrophoresis and Protein Gel Blots

Thylakoids proteins separated by SDS-PAGE (Perfect Blue twin gel system; PeqLab) were transferred to a polyvinylidene difluoride membrane (Hybond P) using a tank blotting system (Perfect Blue Web M; Protein Gel Electrophoresis and Protein Gel Blots).

**ACKNOWLEDGMENTS**

We thank Stephanie Ruf and Yvonne Weber for chloroplast transformation, selection, and plant regeneration and Britta Hausmann for plant cultivation. This project was supported by a grant from the Deutsche Forschungsgemeinschaft to M.A.S. and R.B. (SFB 429, Project A12).

Received August 27, 2010; revised December 13, 2010; accepted January 5, 2011; published January 28, 2011.

**REFERENCES**


**ATP Synthase Repression in Tobacco Restricts Photosynthetic Electron Transport, CO$_2$ Assimilation, and Plant Growth by Overacidification of the Thylakoid Lumen**

Markus Rott, Nádia F. Martins, Wolfram Thiele, Wolfgang Lein, Ralph Bock, David M. Kramer and Mark A. Schöttler

*Plant Cell* 2011;23;304-321; originally published online January 28, 2011;
DOI 10.1105/tpc.110.079111

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