The availability of neither D2 nor CP43 limits the biogenesis of photosystem II in tobacco

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Abstract

The pathway of photosystem II (PSII) assembly is well understood, and multiple auxiliary proteins supporting it have been identified, but little is known about rate-limiting steps controlling PSII biogenesis. In the cyanobacterium Synechocystis PCC6803 and the green alga Chlamydomonas reinhardtii, indications exist that the biosynthesis of the chloroplast-encoded D2 reaction center subunit (PsbD) limits PSII accumulation. To determine the importance of D2 synthesis for PSII accumulation in vascular plants and elucidate the contributions of transcriptional and translational regulation, we modified the 5′-untranslated region of psbD via chloroplast transformation in tobacco (Nicotiana tabacum). A drastic reduction in psbD mRNA abundance resulted in a strong decrease in PSII content, impaired photosynthetic electron transport, and retarded growth under autotrophic conditions. Overexpression of the psbD mRNA also increased transcript abundance of psbC (the CP43 inner antenna protein), which is co-transcribed with psbD. Because translation efficiency remained unaltered, translation output of psbD and psbC increased with mRNA abundance. However, this did not result in increased PSII accumulation. The introduction of point mutations into the Shine–Dalgarno-like sequence or start codon of psbD decreased translation efficiency without causing pronounced effects on PSII accumulation and function. These data show that neither transcription nor translation of psbD and psbC are rate-limiting for PSII biogenesis in vascular plants and that PSII assembly and accumulation in tobacco are controlled by different mechanisms than in cyanobacteria or in C. reinhardtii.

Introduction

Photosystem II (PSII), the water-plastoquinone oxidoreductase protein supercomplex of oxygenic photosynthesis, catalyzes the first step of linear electron flux in the thylakoid membranes of cyanobacteria and photosynthetic eukaryotes (Shen, 2015). Electron transfer within PSII is initiated by a light-induced charge separation at the reaction center (RC) chlorophyll-a dimer P680, which then transfers one electron to the first quinone acceptor Qa. From Qa, the electron is transferred to the plastoquinone-binding site (Qb-site) and reduces plastoquinone to plastosemiquinone. Following a second charge separation, the subsequent reduction of the...
plastoquinone to plastoquinol is coupled to the uptake of two protons from the stroma. Plastoquinol is released into the thylakoid membrane and re-oxidized at the cytochrome b$_{559}$ complex (cyt b$_{559}$). After each charge separation, P$_{680}^+$ is reduced by the oxygen-evolving Mn$_{4O5}Ca$ complex (OEC) near the luminal surface of PSII. After four oxidation steps, two water molecules are oxidized to molecular oxygen, and the Mn$_{4O5}Ca$ cluster is reduced again by the four electrons abstracted from the two water molecules (Dau et al., 2012; Vinyard and Brudvig, 2017).

PSII functions as a dimer, and each monomer is composed of more than 20 core subunits and additional peripheral light-harvesting complex (LHC) antenna proteins. With a molecular mass of up to 1,300 kDa, the PSII–LHC supercomplexes are the largest complexes of the photosynthetic apparatus (Dekker and Boekema, 2005; Kou et al., 2012; Shen, 2015). The PSII RC core is formed by the D1 and D2 heterodimer that binds all redox-active cofactors necessary for rapid electron transfer from water to plastoquinone. D1 and D2 are encoded in the chloroplast genome (plastome) by the psbA and psbD genes, respectively. An additional redox-active cofactor, the heme of cytochrome $b_{599}$ (cyt $b_{559}$), is bound by PsbE and PsbF, which are also plastome-encoded. Cyt $b_{559}$ is an essential structural component and required for PSII assembly (Pakrasi et al., 1989; Morais et al., 1998; Swiatek et al., 2003), but its physiological function is enigmatic. It has been suggested to mediate a cyclic electron flux within PSII when the PSII donor side is inactive (Shinopoulos and Brudvig, 2012; Takagi et al., 2019) and may function as a plastoquinol oxidase (Bondarava et al., 2003; Bondarava et al., 2010).

The PSII RC is surrounded by the inner antenna proteins CP47 (PsbB) and CP43 (PsbC), also encoded in the plastome, and multiple membrane-intrinsic low-molecular mass subunits encoded either in the plastome or the nucleus. Some of these subunits are essential for PSII accumulation or function, while others are not (reviewed by Shi et al., 2012; Plöchinger et al., 2016). Additionally, the three nuclear-encoded extrinsic subunits PsbO, PsbP, and PsbQ are associated with the luminal side of PSII and stabilize the OEC (Bricker et al., 2012).

The process of de novo PSII assembly is largely conserved from cyanobacteria to vascular plants, except that the subcellular localization of some steps varies (Komenda et al., 2012; Nickelsen and Rengstl, 2013). Assembly proceeds in a modular fashion and starts with cyt $b_{559}$, which stably accumulates even in the absence of the other PSII RC subunits (Müller and Eichacker, 1999; Kanervo et al., 2008; Plösch et al., 2009; Schmitz et al., 2012). Subsequently, D2 is co-translationally inserted into the thylakoid membrane (Zoschke and Barkan, 2015) and binds to cyt $b_{559}$ forming the D2-cyt $b_{559}$ subcomplex (Komenda et al., 2004). The addition of a complex consisting of pre-D1, a D1 protein precursor with a short C-terminal extension, and PsbL leads to the formation of the “RC-like complex” (Dobakova et al., 2007), which is stabilized by multiple auxiliary proteins (Li et al., 2019). After maturation of the D1 protein by the luminal C-terminal processing protease CTPA (Che et al., 2013), the “RC47 subcomplex” is formed by binding of CP47 (PsbB; Komenda et al., 2004) and the rapid addition of PsbH, PsbR, and PsbTc (Rokka et al., 2005). Finally, CP43 (PsbC), PsbK, and PsbZ bind to the RC (Rokka et al., 2005; Boehm et al., 2011). This complex is photoactivated by stepwise assembling the Mn$_{4O5}Ca$ cluster, which is further stabilized by binding the three luminal subunits (Mamedov et al., 2000; Mamedov et al., 2008). Ultimately, two PSII monomers form a PSII dimer and bind additional LHCs (Shevela et al., 2016). During the assembly process, more than 20 auxiliary proteins located in the stroma, the thylakoids, and the lumen transiently bind to PSII. They protect and stabilize all assembly intermediates except for the early D2-cyt $b_{559}$ subcomplex, which appears to accumulate without the support of auxiliary proteins (Shi et al., 2012; Nickelsen and Rengstl, 2013; Plöchinger et al., 2016). Some auxiliary proteins mediate chlorophyll and cofactor insertion into the RC and inner antenna proteins of the nascent complex (Bučinská et al., 2018; Hey and Grimm, 2020).

In vascular plants, PSII contents are highly variable (reviewed by Schöttler and Tóth, 2014). In Arabidopsis (Arabidopsis thaliana), PSII contents increase almost fourfold from 2.5 to 9 mmol PSII per mol chlorophyll, at the expense of the peripheral LHClI, when the actinic light intensity increases from 35 to 600 μE m$^{-2}$ s$^{-1}$ (Bailey et al., 2001). Similar changes have been observed in tobacco (Nicotiana tabacum; Petersen et al., 2011; Schöttler et al., 2017) and in Chamerion angustifolium (Murchie and Horton, 1998). The mechanisms, by which these adjustments are achieved, are largely unknown.

In principle, PSII accumulation could be limited by the biosynthesis of one of its subunits, by the synthesis and insertion of redox cofactors and chromophores, especially chlorophyll and carotenoids into the nascent complex, or via changes in the abundance of auxiliary proteins. Because PSII assembly starts with cyt $b_{559}$, a limiting role of cyt $b_{559}$ synthesis for the entire assembly process is a reasonable assumption. Supporting this scenario, in the cyanobacterium Synechocystis PCC6803, deletion of the psbEFILJ operon comprising the subunits of cyt $b_{559}$ abolishes accumulation of both D1 and D2 (Pakrasi et al., 1989). Radiolabeling studies reveal that while the absence of D1 in the mutant is mainly attributable to rapid turnover of the unassembled protein, the presence of cyt $b_{559}$ is a prerequisite for translation of D2 (Komenda et al., 2004). However, because a fraction of cyt $b_{559}$ forms part of a higher molecular mass complex of unknown function that does not comprise other PSII subunits, cyt $b_{559}$ cannot be the only factor controlling D2 synthesis. Because D2 accumulates in a ΔpsbA mutant, the downstream assembly partner D1 does not control psbD translation (Komenda et al., 2004). Interestingly, in all of these mutants, the inner antenna proteins CP43 and CP47 accumulate, indicating that their synthesis is independent of
the presence of those RC subcomplexes, into which they later assemble (Pakrasi et al., 1989; Komenda et al., 2004). CP47 forms a pre-assembled pigment–protein complex together with PsbH, PsbL, and PsbT, and CP43 associates with PsbK (Boehm et al., 2011). Clearly, not all subunits of PSII are subject to a tight control of their synthesis by the presence of the essential RC core subunits. However, all observations in Synechocystis PCC6803 point to a limiting role of cyt b₅₉₉ and other, so far unknown factors for the synthesis of D2 and thereby of functional PSII.

Data obtained in photosynthetic eukaryotes suggest major differences in the regulation of PSII biogenesis: In the green alga Chlamydomonas reinhardtii, accumulation of cyt b₅₉₉ is a prerequisite for PSII assembly (Morais et al., 1998). However, different to Synechocystis, cyt b₅₉₉ does not control the synthesis of D2, as in the absence of cyt b₅₉₉ in a ΔpsbE mutant, up to 5% of wild-type (WT) levels of D2 still accumulated, while D1 was not detectable anymore (Morais et al., 1998). Instead, in C. reinhardtii, already relatively small changes in psbD mRNA accumulation and translation have a strong influence on PSII accumulation, supporting a limiting function of D2 synthesis for PSII accumulation. A point mutation in the psbD promoter reduces both psbD mRNA accumulation and D2 protein content to 35% of the WT level (Klinkert et al., 2005). Furthermore, because the AUG start codon is part of an mRNA secondary structure, translation initiation of psbD is controlled by the translational activator RBP40 and the RNA stabilization factor Nac2, which open up the mRNA secondary structure and thereby facilitate translation initiation (Schwarz et al., 2007). Mutations of this repressor element not only result in increased translation of the psbD mRNA, but also lead to a 20% increase in PSII accumulation, clearly supporting a limiting role of PsbD in PSII biogenesis (Klinkert et al., 2006).

In vascular plants, similar to C. reinhardtii, a limiting function of cyt b₅₉₉ can likely be excluded. While knock-out of psbE or psbF abolishes the accumulation of both D1 and D2 (Swiatek et al., 2003), free cyt b₅₉₉ accumulates not only in etioplasts (Müller and Eichacker, 1999; Kanervo et al., 2008), but also in chloroplasts with severely disturbed sugar-phosphate metabolism and redox poise (Schmitz et al., 2012). Assuming that, similar to the situation in C. reinhardtii, D2 plays a limiting role, the underlying molecular mechanisms likely differ, because neither RBP40 nor Nac2 have orthologs in vascular plants. Also, while psbD is encoded by a monocistronic mRNA in C. reinhardtii, in vascular plant plastomes, it forms an operon with psbC (Yao et al., 1989; Christopher et al., 1992; Adachi et al., 2012). In tobacco, the main promoter of this operon is located 905-bp upstream from the ATG codon of psbD. Additionally, a promoter located 194-bp upstream from the start codon of psbC is located within the protein-coding region of psbD (Yao et al., 1989). Because the reading frames of psbD and psbC overlap by 17 nucleotides in tobacco, it has been suggested that their translation is partly coupled. At least some of the ribosomes released after termination of psbD translation may immediately rebind to the psbC 5′-untranslated region (UTR) and initiate CP43 synthesis (Adachi et al., 2012). However, because psbC can be also expressed as a monocistronic transcript (Yao et al., 1989), translational coupling is not a prerequisite for PsbC synthesis.

While in vascular plant chloroplasts, translation regulation is usually considered to be more important than transcript abundance (reviewed by Zoschke and Bock, 2018), a limitation of photosynthetic complex biogenesis by transcript abundance was recently reported for the PetA subunit of the cyt b₆f in tobacco (Schöttler et al., 2017). To determine if D2 or CP43 plays a limiting role for PSII accumulation in vascular plants, here we have generated plastidomic tobacco mutants with altered psbD–psbC transcription abundances. To assess a potential role of psbD translation regulation, we altered psbD translation initiation by the introduction of point mutations into the ATG start codon. Similar approaches have been used by Rott et al. (2011) to reduce atpB translation, and by Moreno et al. (2017) to repress translation of ClpP, the chloroplast-encoded catalytic subunit of the stromal Clp protease (Nishimura and van Wijk, 2015). We also mutated the Shine–Dalgarno (SD)-like sequence in the 5′-UTR of psbD. The SD interacts by complementary base pairing with the anti-SD sequence in the 16S ribosomal RNA to ensure proper positioning of the translation initiation complex close to the start codon. In a transplastomic tobacco line carrying a mutation in the anti-SD sequence, psbD ranked among the genes whose translation was most severely affected (Scharff et al., 2017). Our data suggest that psbD transcript abundance is critical in controlling D2 synthesis, but D2 is not the rate-limiting subunit for the accumulation of PSII in tobacco. We also show that the regulation of PSII biogenesis is markedly different between cyanobacteria, C. reinhardtii and vascular plants.

**Results**

**Generation of transplastomic tobacco lines with altered psbD expression**

To dissect the contributions of transcriptional and translational regulation to the synthesis of the plastid-encoded D2 subunit, we generated chloroplast mutants with altered transcription of the psbD–psbC operon by disrupting the psbD 5′-UTR via insertion of the selectable marker gene aadA (for details, see the “Materials and methods” section). In one set of transplastomic mutants, the selectable marker, whose expression is driven by the strong Prm promoter, was inserted in the plastome in sense orientation relative to psbD. These mutants will subsequently be referred to as “sense” mutants (s-mutants). Read-through transcription from the aadA gene is expected to result in strong over-expression of the downstream psbD–psbC transcription unit (Zhou et al., 2007; Lu et al., 2013; Zhang et al., 2015). In another set of mutants, the selectable marker was inserted in antisense orientation relative to psbD, so that no read-through transcripts can occur. These mutants are referred to as “antisense” (as)-mutants. Because insertion of the
selectable marker in antisense orientation may negatively affect the expression of neighboring genes transcribed in the opposite direction (Loiacono et al., 2019), transcript accumulation of psbD could be strongly reduced in all as-mutants. Additionally, to alter translation initiation efficiency, we introduced point mutations into the translation initiation codon. The standard ATG translation initiation codon was replaced either by a GTG or TTG codon (Figure 1, A), which are both recognized by the chloroplast ribosome as translation initiation sites, but with lower efficiency than the ATG codon (Rott et al., 2011; Moreno et al., 2017). Furthermore, as Scharff et al. (2017) had shown the SD dependency of psbD translation, we mutated the SD of psbD from GGAGGA to GGACGA, reducing translation initiation efficiency than the ATG codon (Rott et al., 2011; Moreno et al., 2017).

Transplastomic lines were obtained by particle-gun-mediated chloroplast transformation and selection for spectinomycin resistance (conferring the aadA gene). All mutants could be generated and propagated in tissue culture under mixotrophic conditions. However, when plants were transferred to autotrophic growth conditions, neither the as-TTG-psbD-mutant plants nor the as-GGAGGA-psbD-mutant plants survived. Both mutants could only be maintained in tissue culture, and consequently, did not produce seeds. Therefore, these mutants were excluded from further analysis. The homoplasmic state of all other mutants was tested by restriction fragment length polymorphism (RFLP) analysis using a psbD-specific probe and BamHI as restriction enzyme (as indicated in the map in Figure 1, A). Two representative independent lines for each construct were analyzed (Figure 1, B). While the WT showed the expected restriction fragment of 4.5-kb size, all transplastomic mutants showed exclusively a larger fragment of 5.7 kb, which arises from the insertion of the psbD–aadA selectable marker gene and its promoter and terminator. The presence of the point mutations was confirmed by DNA sequencing.

Absence of the smaller WT-like fragment in the mutants strongly suggested the homoplasmic state of all transplastomic lines. To ultimately confirm homoplasmacy, we also performed seed germination tests on spectinomycin-containing medium. This is the most sensitive assay to distinguish between homoplasmic and heteroplasmic transformants (Svab and Maliga, 1993; Bock, 2001; Bock, 2015). As expected, cotyledons of WT seedlings germinated in the presence of spectinomycin were completely white, while inhibition of plastid translation prevents assembly of the photosynthetic apparatus (Figure 1, C). By contrast, all transplastomic lines produced a homogeneous population of green seedlings, thus unequivocally confirming their homoplasmic state. Interestingly, seedlings from the as-mutants were only pale green compared with those from s-mutants, which is in line with the chlorophyll-deficient phenotypes of as-mutants grown in soil (see below).

Transcript accumulation from the psbD operon

To determine general effects of the insertion of the selectable marker gene and the introduction of the point mutations on mRNA accumulation and processing, northern blot analysis was performed for all s-mutants, the as-aadA–psbD control line, and the as-GTG-psbD and as-GGAGGA-psbD mutants, which were viable under autotrophic conditions (Figure 2). Even though the as-GGAGGA-psbD plants had produced some seeds (and, therefore, could be included in the germination assays: Figure 1, C), they only rarely survived autotrophically. Therefore, this mutant was excluded from further analyses. In the WT, using a psbD-specific probe, a complex pattern of transcripts between 4.4- and 2.6-kb size was observed. This pattern arises from mRNA cleavage at a processing site located at position −132 relative to the translation initiation codon (Adachi et al., 2012), and from transcriptional read-through into psbZ. The psbZ gene is encoded downstream of psbC on the same DNA strand (Figures 1, A and 2, B).

The mutants with aadA inserted in s-orientation revealed the presence of two novel bands, which represent read-through transcripts from the strong PrmA promoter in front of the aadA selectable marker gene through the psbD–psbC operon (transcript of approximately 4.0-kb size), or even through psbZ (approximately 4.8-kb size). Furthermore, the abundance of the processed dicistronic mature psbD–psbC transcript of 2.6-kb size was strongly increased, suggesting that the presence of the aadA selectable marker does not interfere with the normal processing of psbD–psbC dicistronic transcripts. In the case of the as-lines, a drastic decrease in psbD transcript abundance was observed, indicating that the aadA insertion indeed massively reduced transcription from the psbD promoter. Only small amounts of the mature dicistronic transcript of 2.6 kb were detectable.

To confirm the identity of the novel large transcripts in the mutants, we used an aadA-specific probe (Figure 2, A). As expected, it did not give any signal in the WT, but in the s-mutants revealed bands at 4.8- and 4.0-kb size, which correspond to the mRNA species already detected with the psbD probe. A stronger band of 1.8-kb size likely represents the processing product cleaved in front of the psbD translation initiation site and still includes the major part of the 5′-UTR of the psbD transcript. The strong 1.0-kb signal only covers the aadA selectable marker gene terminated by the 3′-psbA element downstream of the aadA coding region. This band was also the only prominent signal in the as-mutants.

Next, we used a probe specific for the long 5′-UTR of the primary psbD transcript (Figure 2, C). As expected, in the WT, this probe allowed us to detect major bands of 4.4- and 3.6-kb size (as reported previously; Yao et al., 1989), likely corresponding to the full-length transcripts covering either only the psbD–psbC coding regions or additionally including psbZ. In the s-mutants, by far the most prominent band was a band of 1.8-kb size, which likely corresponds to the processed aadA mRNA transcribed from the psbD operon promoter. Finally, we also used a psbC probe (Figure 2, D), which resulted in the most complex transcript pattern with more than six distinct bands (Figure 2, E). In the
s-mutants, we observed increased accumulation, especially of the full-length transcripts spanning from the selectable marker to psbZ and of the processed mRNAs. In the as-mutants, the long transcripts starting from the promoter in front of psbD were undetectable. However, shorter transcripts of up to 2.6-kb length starting from the psbC-specific promoter within the psbD coding region (Yao et al., 1989) accumulated to almost normal levels.
These either spanned both \( \text{psbC} \) and \( \text{psbZ} \), due to incomplete transcription termination at the end of \( \text{psbC} \), or covered only \( \text{psbC} \).

**Chloroplast translation in the mutants**

To obtain information on changes in \( \text{psbD} \) translation due to the changes in mRNA abundance and the point
mutations introduced into the 5'-UTR or the start codon, we performed polysome loading analyses as a proxy for translation activity (Barkan, 1998). Material was harvested from young leaves with still active thylakoid biogenesis. mRNAs with different ribosome coverage were separated by sucrose density gradient centrifugation into 12 fractions, with fraction 12 being the fraction of highest density. To determine the distribution of free, untrans- 

tlated mRNAs, a puromycin-treated WT sample was also analyzed. Puromycin dissociates ribosomes from the mRNAs, as confirmed by comparison of ethidium bromide-stained agarose gels (upper panels). The major psbD-containing fractions are indicated by horizontal bars below each blot.

Resolution and sensitivity of polysome analyses are rather limited, and in the case of polycistronic transcripts, due to the physical linkage of the reading frames, polysome loading only provides an average value of the total translation of the entire transcript (reviewed by Zoschke and Bock, 2018). Therefore, translation of psbD and psbC cannot be distinguished, and the altered polysome profiles could be due to altered translation of either psbD or psbC, or both. For example, psbD translation might alter translation of psbC due to translational coupling (Adachi et al., 2012).

To characterize transcription and translation in more detail, we selected the s-aadA–psbD control mutant, the two s-translation initiation codon mutants, and the WT for a comprehensive analysis of changes in transcript accumulation and translation of all plastome-encoded genes by transcript and ribosome profiling. To this end, transcript abundances and ribosome footprints were analyzed using high-resolution tiling arrays and then averaged for each chloroplast open reading frame as previously described (Trösch et al. 2018). In each case, ribosomal footprints (“translation output”) and transcript abundances (“RNA”) of the three different mutants were plotted against the signals of WT
To determine the relative translation efficiency of each gene, data were log-transformed and transcript abundance was subtracted from ribosomal footprints (Supplemental Figure S1; Zoschke et al., 2013). The data shown represent averages from three biological replicates (see Supplemental Figure S2). Pronounced changes (more than or equal to two-fold) are highlighted in red color in Figure 4.

When the WT was compared with the s-aadA–psbD control mutant (Figure 4, A), we observed strong increases in transcript abundance of psbD and psbZ, in line with the changes observed by northern blot analysis (Figure 2). psbC transcript abundance also increased but remained slightly below two-fold. Abundance of all other chloroplast transcripts did not change substantially. The translation output of psbD and psbC, but not of psbZ, clearly increased. Unexpectedly, translation of psbK clearly decreased. However, at the level of the relative translation efficiency, not a single pronounced difference between WT and the s-aadA–psbD control mutant was observed (Supplemental Figure S1). The increased transcript abundance of psbD and psbC directly resulted in increased translation output, in agreement with their WT-like polysome distribution profiles (Figure 3).

In the s-GTG-psbD mutant (Figure 4, B), psbD, psbC, and psbZ mRNA accumulation levels increased more than two-fold. This resulted in a pronounced increase in the translation output of both psbD and psbC, suggesting that the GUG initiation codon is almost as efficient as the standard AUG translation initiation codon for psbD. Similar to the other two s-mutants, the s-TTG-psbD mutant displayed clear increases in psbD, psbC, and psbZ transcript abundances (Figure 4, C). However, different from the s-GTG-psbD mutant and despite its increased mRNA abundance, the s-TTG-psbD mutant did not show a pronounced increase in translation output for any of these reading frames. This is due to a strong reduction in the translation efficiency of especially psbD, indicating that the UUG initiation codon is much less efficient than AUG and GUG (Supplemental Figure S1). The strongly diminished translation efficiency of psbD did not hamper translation of psbC. Consequently, in vivo, psbC translation can be considered as uncoupled (or only marginally coupled) to psbD translation initiation, contrasting a conclusion that was previously drawn based on in vitro translation data (Adachi et al., 2012).

Only minor differences in photosynthetic parameters of s-mutants

To determine effects of the altered psbD transcript accumulation and translation initiation on plant growth and photosynthetic performance, the plants expressing the selectable marker gene in s-orientation were grown in soil under long-day conditions and with an actinic light intensity of 350 µE m⁻² s⁻¹. Plant growth and all physiological parameters analyzed (see below) were not significantly different between tobacco analyzed in the same experiment (Figure 4, x-axis).
independent mutant lines harboring the same construct. Therefore, the growth phenotype of one representative plant per construct (Figure 5, A), and average values of all mutant lines per construct (Table 1) are shown. Growth of all mutants was only slightly retarded relative to that of the WT (Figure 5, A).

To determine possible changes in the composition and function of the photosynthetic apparatus in detail, the

Table 1 Average values and standard deviation of chlorophyll content, chlorophyll a/b ratio, leaf absorptance, maximum quantum efficiency of PSII in the dark-adapted state ($F_v/F_m$), and photosynthetic complex contents per leaf area of sense-psbD mutants grown at an actinic light intensity of 350 $\mu$E m$^{-2}$ s$^{-1}$

| Parameter | WT | s-aadA-psbD | s-GTG-psbD | s-TTG-psbD | s-GGACGA-psbD | s-GGAGCA-psbD | s-GGACCA-psbD |
|-----------|----|-------------|-------------|-------------|---------------|---------------|---------------|
| Sample size | 14 | 13 | 12 | 11 | 12 | 12 | 11 |
| Chlorophyll [mg/m$^2$] | 442.3 ± 98.4 | 454.7 ± 102.5 | 436.1 ± 74.6 | 364.7 ± 66.1 | 520.9 ± 91.1 | 519.3 ± 72.1 | 492.1 ± 42.1 |
| Chlorophyll a/b | 4.04 ± 0.16 | **3.84 ± 0.07** | **3.80 ± 0.11** | **3.72 ± 0.13** | **3.85 ± 0.11** | **3.85 ± 0.19** | **3.82 ± 0.10** |
| Leaf absorptance (%) | 88.5 ± 1.8 | 88.2 ± 2.2 | 88.4 ± 2.1 | 86.9 ± 1.9 | 89.8 ± 1.2 | 89.6 ± 1.2 | 89.3 ± 0.6 |
| $F_v/F_m$ | 0.81 ± 0.02 | 0.80 ± 0.02 | 0.80 ± 0.02 | **0.79 ± 0.01** | 0.80 ± 0.02 | 0.81 ± 0.01 | 0.80 ± 0.02 |
| PSII [umol/m$^2$] | 1.11 ± 0.25 | 1.16 ± 0.21 | 1.06 ± 0.17 | **0.81 ± 0.20** | 1.14 ± 0.17 | 1.20 ± 0.19 | 1.05 ± 0.12 |
| Cyt b$_6$f [umol/m$^2$] | 0.49 ± 0.14 | 0.45 ± 0.07 | 0.45 ± 0.12 | **0.37 ± 0.10** | 0.58 ± 0.12 | 0.51 ± 0.10 | 0.52 ± 0.08 |
| Pc [umol/m$^3$] | 2.21 ± 0.65 | 2.55 ± 0.66 | 2.43 ± 0.59 | 1.90 ± 0.73 | **2.93 ± 0.46** | 2.78 ± 0.55 | 2.83 ± 0.41 |
| PSI [umol/m$^3$] | 0.97 ± 0.24 | 0.99 ± 0.22 | 0.95 ± 0.16 | 0.81 ± 0.19 | 1.15 ± 0.17 | 1.13 ± 0.18 | 1.08 ± 0.10 |

Boldface text highlights values, which are significantly different from WT ($P > 0.05$, one-way ANOVA, Holm–Sidak and Dunn’s method).
chlorophyll a/b ratio, the chlorophyll content per leaf area, leaf absorptance, and the maximum quantum efficiency of PSII in the dark-adapted state (Fv/Fm) were determined for the youngest fully expanded leaves of plants at the onset of flowering (Table 1). Then, thylakoids were isolated from these leaves and the contents of both photosystems, the cyt b6f and plastocyanin (Pc), were determined spectroscopically from chemically or light-induced difference absorbance signals of cyt b559 (PSII), cytochromes b6 and f (cyt b6f), and P700 (PSI; see the “Materials and methods” section). Finally, these data were re-normalized to a leaf area basis (Table 1). These quantifications were validated by immunoblots against essential subunits of the different photosynthetic complexes (Figure 6).

Neither chlorophyll content nor leaf absorptance differed significantly between WT and the s-mutants, while the chlorophyll a/b ratio of all mutants was slightly lower than in the WT (Table 1). The maximum quantum efficiency of PSII in the dark-adapted state (Fv/Fm), as well as the PSII and cyt b6f content per leaf area were only slightly lower in the s-TTG-psbD mutant than in the WT. In these parameters, all other mutants did not differ from the WT. For PSI accumulation, no significant differences could be observed for any of the s-mutants. These results were confirmed by immunoblots against the essential PSI RC core subunits PsbA (D1), PsbD (D2), and PsbE, a subunit of cyt b559. LHCB1 accumulation did not reveal any differences between the WT and any of the s-mutants. When PSI, the cyt b6f, and plastocyanin ATP synthase were probed with antibodies against their essential subunits PsaA, PetA (cytochrome f), and AtpB, no obvious differences in protein accumulation between mutants and WT could be observed (Figure 6), in line with the spectroscopic results.

Accordingly, most mutants displayed only subtle changes in the light response curves of the chlorophyll-a fluorescence parameters qL (a measure for the redox state of the PSII acceptor side; Kramer et al., 2004) and qN (a measure for the thermal dissipation of excess excitation energy in the PSII antenna system, Krause and Weis, 1991). Again, average values of all mutant lines per construct are shown (Figure 7). In the s-TTG-psbD mutant, the only s-mutant displaying a significant decrease in PSI accumulation, induction of qN was slightly shifted to lower light intensities, while for the reduction state of the PSII acceptor side, no significant differences were observed (Figure 7, A). Finally, 77K chlorophyll-a fluorescence emission spectra were recorded and normalized to the PSI emission signal at 734-nm wavelength (Figure 7, C). Again, no changes in the maximum emission signal of PSII at 686.5-nm wavelength were observable, strongly suggesting that the LHCII antenna proteins were well coupled to the RC, even in the s-TTG-psbD mutant.

As-mutants suffer from massive growth retardation and impaired photosynthesis

All three autotrophically viable as-mutants showed delayed growth and evidence of reduced photosynthetic performance. They were highly light-sensitive when grown at 350 μE m⁻² s⁻¹, the light intensity used for the characterization of the s-mutants. Therefore, the as-mutants were grown at a reduced light intensity of 100 μE m⁻² s⁻¹. Photographs in
**Figure 7** Chlorophyll-a fluorescence analysis of transplastomic psbD mutants. (A) and (B) Light response curves of qL (redox state of the PSII acceptor sides) and qN (non-photochemical quenching) in the s-psbD (A) and as-psbD (B) transplastomic lines. Error bars indicate the standard deviation of the mean, and the sample size is the same as indicated in Table 1. (C) and (D) 77K chlorophyll-a fluorescence emission spectra in the s-psbD (C) and as-psbD (D) transplastomic lines. The spectra are averaged per construct (n indicated in Table 1) and normalized to the PSI emission maximum at ~735 nm.
On a functional level, the light response curves of qL and qN revealed pronounced differences between the WT and the as-mutants. Both the induction of qN and the reduction of the PSII acceptor side were strongly shifted to lower light intensities, while the full induction of qN in saturating light was impaired in the as-mutants (Figure 7, B). Finally, in the 77K chlorophyll-a fluorescence emission spectra, the maximum emission wavelengths of PSI-LHCl and especially of PSII-LHClII were clearly shifted to lower wavelengths, indicating the presence of free, uncoupled LHC (Figure 7, D). Uncoupled LHCl emit fluorescence at 77K with maxima between 701 and 730 nm (Castelletti et al. 2003; Croce et al., 2004), while the coupled LHCl-PSI in the WT show their typical emission maximum at 734 nm. Likewise, the shift of the PSII-LHClII emission maximum from 686.5 to 682 nm indicates the presence of free, uncoupled LHClII, whose emission maximum is at 680 nm (Krause and Weis, 1991).

**Discussion**

The biogenesis of PSII has been studied for decades, and its assembly sequence is well established. Also, more than 20 auxiliary proteins stabilizing different assembly intermediates and supporting the insertion of pigments and co-factors have been identified (Nickelsen and Rengstl, 2013; Lu, 2016). By contrast, much less is known about the limiting steps of PSII biogenesis, and how PSII accumulation is adjusted to different growth light intensities, which can result in more than four-fold changes in PSII content (reviewed by Schöttler and Tóth, 2014). Therefore, identifying the rate-limiting step(s) of PSII biogenesis is of major importance to...
better understand the acclimation of the photosynthetic apparatus to different environmental conditions, and might pave the way for targeted manipulations of PSII content, without altering its subunit composition and function.

Here, we have addressed a potential limiting role of the two plastome-encoded subunits D2 and CP43, which are expressed from a single operon-type transcription unit in tobacco. Our main focus was on D2, which together with cyt b$_{559}$ initiates the PSII assembly process. We did not consider cyt b$_{559}$ as a candidate for controlling PSII accumulation, because much higher amounts of cyt b$_{559}$ than of functional PSII accumulate in Arabidopsis mutants with massively disturbed primary metabolism (Schmitz et al., 2012). In C. reinhardtii, a reduction in psbD mRNA directly results in a proportional reduction in D2 protein and PSII abundance, pointing to an important regulatory role of psbD transcript accumulation (Klinkert et al., 2005). Mutations in a repressor element of translation initiation increase not only psbD translation initiation efficiency, but also PSII accumulation by up to 20% (Klinkert et al., 2006). A similar overaccumulation of functional PSII by increased synthesis of D2 (either due to increased psbD mRNA abundance or increased rates of translation) would be the ultimate proof that, similar to the situation in C. reinhardtii, D2 is also limiting PSII biogenesis in vascular plants.

Neither D2 nor CP43 limits PSII accumulation in tobacco
To generate transplastomic tobacco with altered psbD transcript abundance, we disrupted the psbD 5′-UTR via insertion of the selectable marker gene aadA. In the s-mutants, read-through transcription from the strong Prm promoter of the aadA massively increased psbD (and psbC) transcript abundance (Figure 2). This increase in psbD mRNA levels was further confirmed by the chloroplast transcriptome profiling data (Figure 4), which additionally showed a strong increase in psbZ mRNA abundance. This again is a consequence of transcriptional read-throughs through the psbD-psbC dicistron into psbZ located further downstream. In the as-mutants, whose strongly decreased psbD transcript abundance is similar to the reduced psbD transcript accumulation in the C. reinhardtii promoter mutant (Klinkert et al., 2005), the massive decrease in transcript abundance resulted in a more than 80% reduction in PSII accumulation (Table 2). This could not be compensated by increased translation of the residual mRNAs (Figure 3). This observation is in line with a limiting role of D2 synthesis for PSII assembly.

However, the strong increase in psbD and psbC transcripts in the s-aadA–psbD mutant did not result in increased PSII accumulation, even though mRNA distribution in the sucrose density gradients used for polysome analysis remained unchanged, indicating that the supernumerary mRNA molecules undergo translation (Figure 3). This conclusion is also supported by ribosome footprint analyses, which show proportional increases of both transcript abundance and translation output for psbD and psbC (Figure 4). Consequently, relative translation efficiency remained unaltered (Supplemental Figure S1). However, neither D2 protein level (Figure 6, A) nor PSII content (Table 1) increased. Also, all other photosynthetic parameters remained unaltered (Table 1). Light response curves of qN and qL did not reveal any major differences to the WT (Figure 7, A). Likely, due to limitation of PSII biogenesis by another so far unknown factor, the additionally synthesized D2 and CP43 are condemned to rapid degradation. Despite the increased transcript abundance and translation of psbD, psbC, and psbZ, the transcript abundance and translation of both psbA and psbB remained unaltered. Also, for the other plastome-encoded subunits of PSII, no obvious changes in transcript abundance or translation were observed in response to the increased transcription and translation of psbD and psbC, except for a slightly reduced translation of psbK (see below). Clearly, no feed-forward stimulation of PSII biosynthesis by the synthesis of supernumerary D2 and CP43 subunits exists in tobacco.

In C. reinhardtii, it is still unclear how the increased psbD translation initiation resulted in increased PSII accumulation (Klinkert et al., 2006). A negative regulatory mechanism called "Control by Epistasy of Synthesis" (CES), by which the absence of D2 represses the synthesis of other plastome-encoded subunits of PSII, has been characterized in detail (Minai et al., 2006): D2 (or, more likely, the D2-cyt b$_{559}$ subcomplex) is required for efficient translation of D1, because unassembled D1 inhibits its own translation. Different to the situation in Synechocystis PCC6803, where the inner antenna proteins accumulate independent of the RC subunits (Pakrasi et al., 1989; Komenda et al., 2004; Boehm et al., 2011), CP47 is also part of this regulatory circuit. The presence of the "RC-like complex" as acceptor for newly synthesized CP47 is a prerequisite for the translation of psbB, because again, unassembled CP47 blocks its own synthesis (Minai et al., 2006). Thereby, CES is a highly efficient negative feedback mechanism avoiding the wasteful accumulation of supernumerary RC subunits in the absence of either D2 or D1 as assembly partners. Potentially, via this mechanism, increased abundance of D2 and the D2-cyt b$_{559}$ subcomplex might stimulate the synthesis of D1, and the formation of the RC-like complex, in turn, might stimulate translation of CP47 by allowing its assembly into the nascent PSII complex. However, so far, in Chlamydomonas, such a feed-forward activation of translation has not been demonstrated. While our data reported here clearly establish that a feed-forward activation of PSII biogenesis by a CES-like process does not exist in tobacco, this does neither rule out the existence of such a regulatory loop in C. reinhardtii, nor does it exclude the possibility of a feedback inhibitory CES-like process in vascular plants. More detailed studies on plants with diminished synthesis of the D2 protein, including the as-psbD-mutants introduced here, are needed to clarify these questions.

Another important conclusion from the analysis of the s-aadA–psbD mutant is that, in tobacco, translation of psbD...
and psbC is either independent of mRNA-specific translation initiation factors, or these are present in large excess. If any such factor were limiting, overexpression of the transcript would have resulted in lower translation efficiency. Chloroplast mRNAs with an SD in their 5′-UTR may be less dependent on mRNA-specific translation factors than those that do not contain a SD (Scharff et al., 2017; Zoschke and Bock, 2018). Alternatively, because translation of psbK was slightly decreased in the s-aadA–psbD and the s-GTG-psbD mutant (Figure 4), a specific translation factor might be shared between psbD (and/or psbC) and psbK, possibly explaining its decreased translation.

Translation initiation mutants

In addition to increasing and decreasing the mRNA abundance of the operon genes, we altered psbD translation initiation efficiency via the introduction of point mutations into the initiation codon. This approach had been previously applied to reduce accumulation of chloroplast ATP synthase by the introduction of point mutations into the start codon of the catalytic AtpB subunit (Rott et al., 2011), and to repress the essential plastid-encoded ClpP subunit of the stromal Clp protease (Moreno et al., 2017). While the GTG-atpB mutation had a more severe effect on ATP synthase accumulation than the TTG-atpB mutation (Rott et al., 2011), in the case of ClpP, the TTG-clpP mutation resulted in a more severe defect (Moreno et al., 2017). Here, ribosome footprint analysis (Figure 4) revealed that the GUG initiation codon was almost as efficient as the AUG start codon for the case of ClpP, the TTG-clpP mutation resulted in a more severe reduction of PSII content (Table 1). Furthermore, the as-GTG-psbD mutant was viable under autotrophic conditions, similar to as-GTG-psbD mutants did not survive under autotrophic conditions, similar to the as-TTG-psbD mutant.

What limits PSII biogenesis in vascular plants?

In cyanobacteria and green alga, most data point to a critical role of D2 synthesis in controlling the biogenesis of PSII. In the ΔpsbEFl1 mutant of the cyanobacterium Synechocystis PCC6803, translation of psbD is abolished, suggesting a direct role of cyt b559 in regulating D2 synthesis (Komenda et al., 2004). However, in addition to the fraction of cyt b559 bound to PSII, cyt b559 is also part of a complex containing no other subunits of PSII. Therefore, cyt b559 is unlikely to be the only limiting factor for psbD translation and PSII accumulation. D1 is translated in the absence of both cyt b559 and D2 but is rapidly degraded again unless it is stabilized by association with the D2-cyt b559 subcomplex (Komenda et al., 2004), suggesting that D2 is the major limiting factor for PSII assembly and that cyt b559 is one factor controlling the synthesis of D2, while the downstream assembly partner D1 does not exert a similar control over psbD translation (Komenda et al., 2004). Different to the situation in Synechocystis PCC6803, in C. reinhardtii, cyt b559 does not control translation of psbD (Morais et al., 1998). Because a clear increase in psbD translation due to the mutation of a repressor element of translation initiation results in increased accumulation of PSII, D2 synthesis seems to control PSII accumulation (Klinkert et al., 2006).

Because of the previous observation that free cyt b559 can accumulate in vascular plants, and therefore is likely not the limiting factor for PSII biogenesis (Schmitz et al., 2012), and because our data eliminate both D2 and CP43 as limiting factors for PSII accumulation, the rate-limiting step in PSII biogenesis seems to have changed several times during the evolution of oxygenic photosynthesis. For vascular plants, it still remains to be identified. A limiting function of one of the other plastome-encoded subunits of PSII appears unlikely, given that a rate-limiting step should occur early in the pathway. The next subunits to assemble into the D2-cyt b559 subcomplex are D1 and the small PsbL subunit. Psbl is not essential for PSII accumulation (Schwenkert et al., 2006). In vascular plants, for multiple reasons, D1 is an unlikely candidate to control PSII biogenesis. First, an autoregulatory loop may adjust the synthesis of D1 to the presence of assembly partners (Chotewutmontri and Barkan, 2020): Accumulation of D1 in an assembly complex blocked psbA translation, possibly due to repressive interactions with translational activators of psbA. In the absence of the auxiliary protein High Chlorophyll Fluorescence136 (HCF136), which may associate newly translated D1 with this assembly complex, ribosome occupancy of psbA is always high. This autoregulation of D1 synthesis strongly suggests that another assembly step prior to the insertion of D1 limits PSII accumulation, and that D1 synthesis is adjusted to the capacity of this reaction.

Furthermore, because PSII is prone to photodamage and damaged D1 needs to be rapidly replaced in the PSII repair cycle, this could generate the problem to independently regulate D1 synthesis for the PSII repair cycle and for de novo assembly of PSII (reviewed by Mamedov and Styring, 2003;
Järvi et al., 2015). The situation is different in cyanobacteria, where de novo biogenesis and repair of PSII may be spatially separated: The earliest steps of PSII assembly in Synechocystis PCC6803 occur at “thylakoid centers,” where thylakoids and the plasma membrane are in close contact (Stengel et al., 2012; Nickelsen and Rengstl, 2013), while PSII repair may occur in distinct repair zones in the thylakoid membrane (Sacharz et al., 2015). In the chloroplast of C. reinhardtii, de novo biogenesis of both PSII and PSI occurs in specialized “translation zones,” while PSII repair is distributed over the unstacked stroma lamellae (Uniacke and Zerges, 2007; Sun et al., 2019). In vascular plants, which display strong lateral heterogeneity of stacked grana thylakoids and unstacked stroma lamellae, no evidence exists for distinct thylakoid domains similar to “thylakoid centers” or “translation zones.” Instead, the first steps of both de novo biogenesis and PSII repair take place in the stroma lamellae, with PSII then moving toward the grana core during the later steps of assembly and photoactivation (Mamedov et al., 2000; Mamedov et al., 2008).

Among all subunits incorporated at a later point into nascent PSII, our work rules out a limiting function of CP43, leaving CP47 as the only so far uninvestigated candidate among the major subunits. Besides CP47 and the luminal subunits attached late in the PSII assembly process (prior to dimerization and supercomplex formation; Shevela et al., 2016), only low molecular mass subunits would remain as potential candidates for a limiting role. Among these, only PsbH is essential for PSII accumulation in C. reinhardtii (Summer et al., 1997). All other small subunits are not absolutely essential for PSII accumulation, and knock-out mutants only suffer from reduced PSII accumulation and/or impaired activity, such as transplastomic ΔpsbJ and ΔpsbL mutants in tobacco (Regel et al., 2001; Hager et al., 2002; Ohad et al., 2004), and the ΔpsbK mutant in C. reinhardtii (Takahashi et al., 1994). Other mutants affected in small subunits of PSII suffer from milder functional defects (reviewed by Shi and Schröder, 2004; Shi et al., 2012; Plöchinger et al., 2016). Furthermore, because the translational autoregulation of psbA synthesis suggested that an earlier step in PSII biogenesis is highly regulated, and D1 synthesis is adjusted to it, attempts to stepwise up- and down-regulate the synthesis of the small subunits do not seem to deserve a high priority in identifying the rate-limiting step of PSII biogenesis.

In summary, a rate-limiting function of the subunits of cyt b559, or of D2 and CP43, can be excluded for vascular plants, and all available data also argue against a rate limitation at the level of D1 synthesis. Therefore, it might be appropriate to consider a limiting function of either pigment biosynthesis and/or auxiliary proteins. For example, the plastid-encoded assembly chaperone Ycf3 can restrict PSI biogenesis (Petersen et al., 2011; Schöttler et al., 2011). However, several auxiliary proteins are also shared by de novo assembly and PSII repair (Järvi et al., 2015), potentially causing a problem similar to that of D1 synthesis in regulating PSII repair versus de novo assembly. An exception may be auxiliary proteins supporting very early steps of assembly, especially in connection with membrane insertion of apoproteins and chlorophyll binding to the nascent polypeptides. In Synechocystis PCC6803, binding of pigments and cofactors during membrane insertion of the nascent polypeptides of photosystem subunits is supported by the formation of a supercomplex consisting of chlorophyll synthase, the PSI assembly factor Ycf3, and the thylakoid insertase Albino3 (Chidgey et al., 2014). Furthermore, in the photosynthesis-deficient mutant 68 (Pam68), a complex of ribosomes, the nascent CP47 protein, and the SecY translocase (required for membrane insertion of CP47) is formed and likely facilitates chlorophyll insertion into the apoprotein (Bucinska et al., 2018). In vascular plants, the one-helix proteins OHP1 and OHP2 together with the scaffold protein High Chlorophyll Fluorescence244 (HCF244) deliver pigments to D1 (Hey and Grimm, 2020). In the absence of either OHP1 or OHP2, synthesis of both D1 and D2 and formation of the PSI RC are blocked (Li et al., 2019). On the other hand, in a chlorophyll-deficient maize (Zea mays) mutant, the translation of plastid mRNAs for chlorophyll-binding apoproteins of both photosystems remained unaffected. However, without stabilization by chlorophyll binding, apoproteins undergo rapid co- or post-translational proteolysis (Zoschke et al., 2017). Together, these data may point to a crucial role of the OHPs in coupling pigment biosynthesis and translation of psbA and psbD.

Therefore, in addition to further exploring possible limiting roles of structural subunits in PSII biosynthesis, the function of chlorophyll channeling into the PSII assembly machinery and its coupling with translation especially of D2 also need to be seriously considered as potential rate-limiting processes. The ultimate proof that a subunit or an auxiliary protein indeed limits PSII biogenesis can only be provided by establishing a strict correlation between its content and PSII accumulation over a wide concentration range, including the demonstration that overexpression of the factor leads to overaccumulation of functional PSII, as reported for C. reinhardtii (Klinkert et al., 2006). Also, such a correlation should be validated under different growth conditions. For example, overexpression of the petA mRNA increases cyt b6f accumulation only in low-light conditions. In high light or under adverse growth conditions such as heat or chilling stress, no difference in cyt b6f content between the WT and the petA mRNA overexpressor is discernable (Schöttler et al., 2017).

The identification of the bottleneck in PSII biogenesis may also pave the way to targeted manipulations of PSII content, without interfering with PSII subunit composition and function. The latter has been observed in many mutants with decreased PSII contents due to the repression of individual subunits. Most of these mutants contain less PSII, but the residual PSII is functionally impaired (reviewed by Shi et al., 2012; Plöchinger et al., 2016). By specifically manipulating the rate-limiting step of the assembly process, only fully functional PSII of WT structure should accumulate. Such mutants would be excellent tools for basic research on
photosynthesis, but also would open up new opportunities in biotechnology and plant breeding.

Material and methods

Plant material and growth conditions

Tobacco (N. tabacum cv Petit Havana) plants were grown under aseptic conditions on agar-solidified Murashige and Skoog medium containing 30 g/L sucrose (Murashige and Skoog, 1962). Transplastomic lines were rooted and propagated on the same medium. For seed production, transplastomic plants were grown in soil under standard greenhouse conditions. Inheritance and seedling phenotypes were analyzed by germination of surface-sterilized seeds on Murashige and Skoog medium containing 500 mg/L spectinomycin. For photosynthesis measurements and molecular analyses, seeds of tobacco WT and transplastomic plants were germinated under long-day conditions (16-h light) at a light intensity of 100 μE m⁻² s⁻¹. The day temperature was 22°C, the relative humidity 75%. During the night, temperature and relative humidity were decreased to 18°C and 70%, respectively. Four weeks after germination or later (in the case of mutants severely retarded in growth, see the “Results” section), plants were transferred to a Conviron chamber with 350 l. Measurements were performed on the youngest fully expanded leaves at the onset of flowering.

Vector construction for plastid transformation

The region of the tobacco plastid genome containing a part of the psbD gene was isolated as a 1.5-kbp KpnI/SacI fragment (Figure 1, A) with primers PsbD_F and PsbD_R (Supplemental Table S1), and then cloned into the pBC SK+ plasmid vector (Stratagene). The resulting construct was used to produce the translation initiation codon mutations by PCR. The region surrounding the SD and the ATG start codon was cleaved with BsaHI and EcoO109I (Figure 1, A) and replaced by PCR products carrying the point mutations. The mutations of the SD or the start codon were introduced with one of the five primers with mutated sites (PsbD_mut1_F to PsbD_mut5_F) and primer PsbD_mut_R as the reverse primer for all the PCR amplifications (Supplemental Table S1). 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 Ndel site within the 5′-UTR of the psbD gene (Figure 1, A) to enable selection of transplastomic lines. Digestion of the final transformation vectors with restriction enzyme KpnI was used to identify the orientations of aadA insertion. Both the sense and the antisense orientation of the aadA relative to psbD were used for plastid transformation.

Generation of transplastomic plants

Young sterile leaves of tobacco cultivar Petit Havana were bombarded with the respective transformation vectors bound to gold particles (0.6-μm diameter) using a helium-driven biologic gun (PDS-1000 He; Bio-Rad). Primary transformants were selected from 5 × 5-mm leaf pieces exposed to plant regeneration medium containing 500 mg/L spectinomycin. Several independent transplastomic lines were then subjected to a maximum of three additional rounds of regeneration on spectinomycin-containing medium to enrich the transplastome and select against residual copies of the WT plastome. Spontaneous spectinomycin-resistant plants were eliminated by a double resistance test on medium supplemented with 500 mg/L spectinomycin and 500 mg/L streptomycin (Svab and Maliga, 1993; Bock, 2001; Bock, 2015). Homoplasmic transplastomic lines were transferred to the greenhouse for seed production. The homoplasmic state of the progeny was confirmed by inheritance test and RFLP analysis (Figure 1, B and C). The presence of the mutations was confirmed by DNA sequencing. It should be noted that the as-aadA–psbD mutant had been previously used as a control by Krech et al. (2013).

Isolation of nucleic acids and hybridization procedures

Total plant DNA was isolated from fresh leaf material by a rapid cetyltrimethylammoniumbromide-based miniprep procedure (Doyle and Doyle, 1990). DNA samples digested with restriction enzymes were separated in 1% w/v agarose gels and blotted onto Hybond-XL nylon membranes (GE Healthcare) according to the manufacturer’s instructions. RNA was extracted using the peqGold TriFast reagent (Peqlab). RNA samples were separated in 1% formaldehyde-containing agarose gels and blotted onto Hybond-XL nylon membranes. Hybridization probes were generated by PCR amplification using specific oligonucleotides (Supplemental Table S1). Prior to labeling, DNA fragments were purified by agarose gel electrophoresis followed by extraction from an excised gel slice using a NucleoSpin Extract II kit (Macherey-Nagel). Hybridization probes were labeled with α[32P]dCTP by random priming (Multiprime DNA labeling kit; GE Healthcare). Hybridizations were performed at 65°C in Rapid-Hyb buffer (GE Healthcare) according to the manufacturer’s instructions. Hybridization signals were quantified using a Typhoon Trio+ variable mode image (Amersham Biosciences) and Image Quant 5.2 software.

Polysome loading assays

Isolation of polysomes and RNA extraction from sucrose gradient fractions were performed as described previously (Barkan, 1998; Rott et al., 2011). Equal aliquots of extracted RNAs from each fraction were separated by denaturing agarose gel electrophoresis as described above. For the puromycin control, polysome samples with 0.5 mg/mL puromycin were incubated at 37°C for 10 min prior to ultracentrifugation.
Ribosome profiling

Ribosome footprints and total RNA were isolated as described in Zoschke et al. (2013), except that after microcentrifugal nucleic acid treatment to degrade nuclease-accessible mRNA and cleave polysomes into monosomes, 4-mL lysate was layered onto a 1-mL sucrose cushion (30% [w/v] sucrose, 0.1-M KCl, 40-mM Tris–acetate, pH 8.0, 15-mM MgCl₂, 5-mM 2-mercaptoethanol, 100 μg/mL chloramphenicol, and 100 μg/mL cycloheximide) and centrifuged for 1.5 h at 50,000 rpm at 4°C in a SW55 Ti rotor (Beckman). RNA labeling was performed according to Zoschke et al. (2013) with the following minor modification: 4-μg purified footprints and 3.5-μg fragmented total RNA derived from mutant and WT plants were differentially labeled with Cy3 and Cy5 (ULS Small RNA Labeling Kit, Kreatech Diagnostics), respectively, following the manufacturer’s instructions.

Ribosome and transcriptome profiling data were analyzed as described in Trösch et al. (2018). Briefly, all local background-subtracted single-channel signals (F635-B635 and F532-B532, respectively) were normalized to the average signal of the datasets of the three mutants and their corresponding WT including all replicates of ribosome footprints and total mRNA to remove biases introduced by technical variation. The average of the probe signals for each reading frame (RF) was then log₂-transformed. The relative abundances of ribosome footprints, total mRNA, and trans- 

Thylakoid membrane isolation and photosynthetic complex quantification

Thylakoid membranes were isolated as described previously (Schöttler et al. 2004). The chlorophyll content and a/b ratio were determined in 80% (v/v) acetone according to Porra et al. (1989). The contents of PSI and the cyt b₅f were determined from difference absorbance signals of cyt b₅₅₉ (PSII) and the cytochromes b₆ and f in destacked thylakoids equivalent to 50-μg chlorophyll ml⁻¹ (Kirchhoff et al., 2002). All cytochromes were fully oxidized by the addition of 1 mM potassium hexacyanoferrate (III). Then, 10 mM sodium ascorbate was added to reduce the high-potential form of cyt b₅₅₉ and cytochrome f. Finally, the addition of 10 mM sodium dithionite reduced the low potential form of cyt b₅₅₉ and the two b-type hemes of cytochrome b₆. Using a V-550 spectrophotometer equipped with a head-on photomultiplier (Jasco GmbH) at each of the three redox potentials, absorbance spectra were measured between 575 and 540 nm. The spectral bandwidth was 1 nm and the scanning speed 100 nm min⁻¹. Ten spectra were averaged per redox condition. Difference spectra were calculated by subtracting the spectrum measured in the presence of hexacyanoferrate from the ascorbate spectrum, and by subtracting the ascorbate spectrum from the spectrum measured in the presence of dithionite, respectively. Finally, a baseline calculated between 540 and 575 nm was subtracted from the signals. Then, the difference spectra were deconvoluted using reference spectra as previously described (Kirchhoff et al., 2002). PSI was quantified from light-induced difference absorbance changes of P700. Thylakoids equivalent to 50 μg chlorophyll ml⁻¹ were solubilized in the presence of 0.2% (w/v) n-dodecyl-β-D-maltoside (DDM). After the addition of 10 mM sodium ascorbate as the electron donor and 100 μM methylviolesin as the electron acceptor, P₇₀₀ photo-oxidation was achieved by applying a light pulse of 250 ms (2,000 μmol photons m⁻² s⁻¹). Measurements were performed with the Pc-P₇₀₀ version of the Dual-PAM instrument (Heinz Walz GmbH). Pc contents, relative to PSI, were determined in intact leaves and then recalculated based on the absolute PSI quantification performed in isolated thylakoids (Schöttler et al., 2007).

Chlorophyll-a fluorescence measurements and leaf absorbance

A F-6500 fluorometer (Jasco Inc., Groß-Umstadt, Germany) was used to measure 77K chlorophyll-a fluorescence emission spectra on freshly isolated thylakoid membranes equivalent to 10-μg chlorophyll ml⁻¹. The sample was excited at 430-nm wavelength with a bandwidth of 10 nm, and the emission spectrum was recorded between 655 and 800 nm in 0.5-nm intervals with a bandwidth of 1 nm. The spectra were normalized to the PSI emission maximum at 734 nm, or to the maximum emission of PSI-LHCl in the as-mutants with shifted emission maximum.

In vivo measurements of chlorophyll-a fluorescence parameters at 22°C were performed using the modular transmittance version of the Dual-PAM (Heinz Walz GmbH). Light-response curves of linear electron flux, non-

photochemical quenching (qN; Krause and Weis, 1991), and the redox state of the PSII acceptor side (qL; Kramer et al., 2004) were measured after 30 min of dark adaptation. The light intensity was increased stepwise from 0 to 2,500 μE m⁻² s⁻¹, with a measuring time of 150 s for each light intensity under light-limited conditions and of 60 s under light-saturated conditions. Linear electron transport was corrected for leaf absorbance, which was calculated from leaf transmittance and reflectance spectra as 100% minus transmittance (%) minus reflectance (%). Spectra were measured between 400- and 700-nm wavelengths using an integrating sphere attached to a photometer (V-550, Jasco Inc.). The spectral bandwidth was set to 1 nm and the scanning speed was 200 nm min⁻¹.
Protein gel electrophoresis and immunoblotting
Thylakoid proteins were separated by SDS-PAGE and then transferred to a polyvinylidene membrane (Hybond P, GE Healthcare) using a tank blot system (Perfect Blue Web M, VWR International GmbH, Darmstadt, Germany). Immunoblotting was performed using an enhanced chemiluminescence detection reagent (ECL Prime, GE Healthcare) according to the manufacturer’s instructions. Chemiluminescence was detected on X-ray ﬁlm. Antibodies against the photosynthetic proteins were purchased from Agrisera AB (Vännäs, Sweden).

Accession numbers
Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NC_001879.2 (tobacco chloroplast genome).

Supplemental data
Supplemental Figure S1. Ratios of relative average translation output and transcript accumulation levels.
Supplemental Figure S2. Reproducibility of transcript abundance and ribosome footprint data between biological replicates.
Supplemental Table S1. Summary of oligonucleotide sequences
Supplemental Data set S1. Data from array-based ribosome profiling experiments.

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