Chloroplast Biogenesis of Photosystem II Cores Involves a Series of Assembly-Controlled Steps That Regulate Translation

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The biogenesis of photosystem II, one of the major photosynthetic protein complexes, involves a cascade of assembly-controlled regulation of translation of its major chloroplast-encoded subunits. In Chlamydomonas reinhardtii, the presence of the reaction center subunit D2 is required for the expression of the other reaction center subunit D1, while the presence of D1 is required for the expression of the core antenna subunit apoCP47. Using chimeric genes expressed in the chloroplast, we demonstrate that the decreased synthesis of D1 or apoCP47 in the absence of protein assembly is due to a genuine downregulation of translation. This regulation is mediated by the 5’ untranslated region of the corresponding mRNA and originates from negative feedback exerted by the unassembled D1 or apoCP47 polypeptide. However, autoregulation of translation of subunit D1 is not implicated in the recovery from photoinhibition, which involves an increased translation of psbA mRNA in response to the degradation of photodamaged D1. De novo synthesis and repair of photosystem II complexes are independently controlled.

INTRODUCTION

Photosystem II (PSII) is a multimeric protein-pigment complex embedded in the thylakoid membrane, where it initiates linear photosynthetic electron flow. It comprises >20 different subunits, most of which are integral membrane proteins, and binds numerous cofactors. In photosynthetic eukaryotes, PSII subunits, assembled in a 1:1 stoichiometry, are encoded in two different genetic compartments. In the green alga Chlamydomonas reinhardtii, six subunits are nuclear encoded, while 15 subunits are encoded by the chloroplast.

The functional assembly of this highly complex oligomeric protein should require some control mechanisms that set the expression of the constitutive subunits to their proper level. Indeed, PSII assembly is a stepwise process, in which the level of accumulation of PSII subcomplexes increases with the number of their constitutive subunits. The presence of cytochrome b559, which accumulates in the membrane even in the absence of other PSII subunits, is a prerequisite for PSII assembly (Morais et al., 1998). It interacts at a very early step with the reaction center (RC) subunit D2, encoded by the chloroplast gene psbD, to form a precomplex, detected as a high molecular mass species (45 to 200 kD) in etioplasts (Müller and Eichacker, 1999). This precomplex probably serves as a receptor for the assembly of the RC subunit D1, the product of the chloroplast psbA gene. Cytochrome b559 and the heterodimer D1/D2, together with subunits PsbI and PSBW, form the PSII RC that binds all essential redox cofactors of PSII and is the smallest protein complex able to perform light-induced charge separation (Nanba and Satoh, 1987). In subsequent steps, core antenna subunits that bind chlorophyll a, CP47 then CP43, encoded by the psbB and psbC chloroplast genes, respectively, are recruited to form the PSII core complex, allowing the further binding of the oxygen evolving enhancer (OEE) proteins, PSBO, P, and Q, on the luminal side of the membrane.

Strikingly, studies of PSII mutants, mainly performed in C. reinhardtii, revealed that not only the assembly but also the expression of some chloroplast-encoded PSII core subunits is a sequential process. Mutants lacking D1 show a reduced synthesis of CP47 but not of D2 (Bennoun et al., 1986; Jensen et al., 1986; de Vitry et al., 1989). Mutants lacking D2 show a reduced synthesis of both D1 and apoCP47 subunits (Bennoun et al., 1986; Erickson et al., 1986).

This process, whereby the presence of one subunit is required for sustained synthesis of another chloroplast-encoded subunit from the same protein complex, has been termed control by epistasy of synthesis (CES) (Wollman et al., 1999; Choquet and Vallon, 2000; Choquet and Wollman, 2002). The molecular mechanism responsible for CES has been best characterized for cytochrome f from the cytochrome b6f complex. In the absence of its assembly partners, subunit IV or cytochrome b6, the synthesis of cytochrome f decreases 10-fold (Kuras and Wollman, 1994) as a result of an autoregulation of translation initiation (Choquet et al., 1998) mediated by the C-terminal domain of unassembled cytochrome f (Choquet et al., 2003). A similar mechanism
controls the synthesis of PsaA and PsaC, two CES subunits from the photosystem I (PSI) complex (Wostrikoff et al., 2004). PSII is by far the photosynthetic complex whose biogenesis has been studied in greatest detail, in particular because it is the major target for photodestructive processes in the canopy. Under bright illumination, charge separation in PSII generates highly reactive radical species that damage PSII cores, specifically the D1 polypeptide, resulting in a decreased PSII activity described as photoinhibition (reviewed in Ohad et al., 1994). Recovery from photoinhibition involves migration of damaged PSII complexes from stacked to stroma thylakoids, degradation of the damaged subunit D1, and its replacement by neosynthesized D1. During recovery from photoinhibition, the synthesis of D1 is strongly increased (Kettunen et al., 1997).

Although regulation of D1 expression has been extensively studied (reviewed in Baena-Gonzalez and Aro, 2002; Zhang and Aro, 2002), some key aspects of PSII biogenesis remain poorly understood. Whether similar mechanisms control psbA expression during de novo synthesis of PSII complexes or repair of photodamaged D1 remains to be determined. The mechanisms linking assembly of PSII to the rate of production of its individual subunits have not been addressed either. The contribution of the CES process to the high turnover of D1 has not been studied up to now to our knowledge. Here, we used reporter genes translated under the control of the 5’ untranslated regions (UTRs) of the CES genes psbA or psbB to demonstrate that biogenesis of PSII involves a cascade of translational autoregulation mediated by the unassembled D1 and CP47 subunits. We show that recovery from photoinhibition relies on a molecular mechanism distinct from the translational regulation that controls de novo synthesis of PSII cores.

## RESULTS

### Hierarchy in the Synthesis of PSII Core Subunits in *C. reinhardtii*

To investigate the tight coupling in the synthesis of PSII core subunits, we compared various PSII mutants specifically deleted for one of the chloroplast genes encoding major PSII subunits. To this end, we generated the ΔpsbB and ΔpsbD deletion strains, unable to express the core subunit apoCP47 and the RC subunit D2, respectively. We also used the Fud7 chloroplast mutant strain, hereafter referred to as ΔpsbA, lacking expression of the other RC subunit D1 because it carries a deletion of the entire psbA gene (Bennoun et al., 1986), from which we generated a double deletion strain (ΔpsbD, ΔpsbA) Table 1).

The wild-type and deletion strains were pulse labeled with 14C acetate for 5 min in the presence of cycloheximide, an inhibitor of cytoplasmic translation. In the ΔpsbD mutant, the synthesis of D1 and CP47 are strongly reduced, compared with the wild-type strain. The diffuse band (asterisk in Figure 1A) most likely corresponds to a newly synthesized form of D1, since it is absent in psbA deletion strains (ΔpsbD, ΔpsbA) and ΔpsbA. The ΔpsbA mutant, lacking D1, still expressed wild-type levels of D2 but poorly synthesized apoCP47. In the ΔpsbB mutant, lacking apoCP47, the synthesis of D1 or D2 remained unaltered.

| Table 1. Transformation Experiments |
| Transforms | Recipient Strains | Transforming Plasmid |
| ΔpsbD<sup>a</sup> | WT | pΔpsbD::Kr<sup>c</sup> |
| ΔpsbB<sup>b</sup> | WT | pΔpsbB::Kr<sup>c</sup> |
| ΔpsbD<sup>a</sup> | WT | pΔpsbD::Kr<sup>c</sup> |
| WT:bAK | WT | pB <sup>2H</sup> |
| ΔpsbD:bAK | Δpsb<sup>b</sup> | pB <sup>2H</sup> |
| ΔpsbA:bAK | ΔpsbA (Fud) | pB <sup>2H</sup> |
| bAf | WT | pKaAf<sup>c</sup> |
| (bAf, ΔpsbD) | Δpsb<sup>b</sup> | pKaAf<sup>c</sup> |
| (bAf, ΔpsbA) | ΔpsbA (Fud) | pKaAf<sup>c</sup> |
| (bAf, ΔpsbB) | ΔpsbB<sup>b</sup> | pKaAf<sup>c</sup> |
| bAfbA | WT | pKaAbA<sup>c</sup> |
| bAf | WT | pKaAf<sup>c</sup> |
| D1<sup>a</sup> | WT | pB259StKr<sup>c</sup> |
| (D1<sup>a</sup>, bAf) | D1<sup>b</sup> | pB<sup>2H</sup> |
| bBf | WT | pB<sup>2H</sup> |
| (ΔpsbA, bBf) | ΔpsbA (Fud) | pB<sup>2H</sup> |
| CP47<sup>a</sup> | WT | pKb<sup>2H</sup> |
| (CP47<sup>a</sup>, bBf) | CP47<sup>b</sup> | pKb<sup>2H</sup> |

All recipient strains were mitochondria- and spectinomycin sensitive. Transforms were selected for resistance to spectinomycin (100 μg·mL<sup>−1</sup>). WT, wild type.

<sup>a</sup>Strains, initially selected for spectinomycin resistance due to the presence of the recycling spectinomycin resistance cassette (K<sub>R</sub>), were screened by fluorescence induction kinetics for impaired PSII activity. Once homoplasmic with respect to the PSI mutation, they were grown on TAP medium for several generations to allow the spontaneous loss of the recycling cassette according to Fischer et al. (1998) but not that of the mutated PSI allele.

<sup>b</sup>Strains, which became spectinomycin sensitive again, could then be used as recipient strains in a new round of transformation experiments based on selection for spectinomycin resistance.

The decreased synthesis of the CES subunits, D1 in the absence of D2 and CP47 in the absence of D1 (and therefore in the absence of D2), does not result from a decreased accumulation of psbA or psbB mRNAs that remained unchanged in all strains (Figure 1B), suggesting a translational or early posttranslational regulation.

### The psbB 5’UTR Is Sufficient to Confer a CES Behavior to a Reporter Gene

The mechanism that couples synthesis of apoCP47 to the presence of D1 could be due to an actual regulation of translation, possibly at the step of initiation, as observed for PSI or cytochrome b<sub>f</sub> biogenesis (Choquet et al., 1998; Wostrikoff et al., 2004). Thus, we constructed a chimeric gene made of the promoter and 5’UTR of the psbB gene, fused in frame to the petA coding region that encodes cytochrome f, previously shown to be a convenient reporter gene (Wostrikoff et al., 2004). We associated this psbB-driven chimeric gene with an antibiotic resistance cassette (Goldschmidt-Clermont, 1991) that allows one to select transformed cells on Tris-acetate-phosphate (TAP) medium supplemented with spectinomycin (TAP-Spec) (Figure 2A, Table 1). Using the wild-type strain as a recipient, spectinomycin-resistant
transformants, hereafter referred to as bBf because they express a psbB-driven cytochrome f, also were found to be phototrophic. Expression of the chimeric cytochrome f is high enough to sustain photosynthetic growth. Pulse-labeling experiments showed that it was made at the same rates in the wild-type and bBf transformants (Figure 2B).

To assess the hypothesis of an assembly-dependent regulation of translation initiation of psbB, we examined the susceptibility of translation of the 5’psbB-petA chimeric gene in tba2 progeny resulted from a pleiotropic effect of the tba2 mutation on the expression of both 5’psbA- and 5’psbB-driven genes, we introduced by chloroplast transformation the 5’psbB-petA chimera in the ΔpsbA strain that lacks expression of D1 but has a wild-type nuclear genome (Table 1). Transformed strains, hereafter called (ΔpsbA, bBf), selected for

![Figure 1. Hierarchy of Synthesis among PSII Core Subunits.](image)

(A) Rate of synthesis of the major PSII subunits, determined by 5-min pulse-labeling experiments in wild-type and deletion strains of C. reinhardtii. Analysis on 8% acrylamide-urea gels. An asterisk marks a neosynthesized form of D1.
(B) Accumulation of psbB, psbD, psbA, and petA (loading control) transcripts in the same strains.

![Figure 2. Expression of the 5’psbB-petA Chimeric Gene Decreases in the Absence of D1.](image)

(A) Map of the chloroplast petA gene in wild-type and bBf strains. Relevant restriction sites are indicated: B, BglII; N*, an NcoI site engineered around the petA initiation codon for cloning purposes; H, HincII. The bent arrow indicates transcription start sites. K stands for the spectinomycin resistance cassette, in opposite orientation with respect to petA.
(B) Translation of the chimeric gene in a representative half tetrad from bBf × tba2 crosses and in parental and wild-type strains. Translation of the chimeric gene in the ΔpsbA chloroplast mutant is also shown.
(C) and (D) Protein (C) and transcript (D) accumulation of the chimeric gene in the same strains. Accumulation of OEE2 and of atpB transcript provide loading controls.
resistance to spectinomycin, showed the same reduced expression of the chimera (Figures 2B and 2C, lane \( \Delta psbA, bBf \)) as the tba2 progeny from the cross bBf \( \times \) tba2.

The reduced expression of the reporter cytochrome f did not result from a reduced accumulation of the chimeric mRNA in strains bBf, tba2 or \( \Delta psbA, bBf \) (Figure 2D). Thus, the decreased synthesis of the CES subunit apoCP47 in the absence of its assembly partner D1 is governed by the psbB 5’UTR and, hence, is most likely controlled at the level of translation initiation.

**The Regulation of Translation Initiation of psbB mRNA Is Reflected by Changes in Its Association with Polysomes**

In order to confirm that conclusion, we compared the polysome loading of psbB mRNA by sedimentation of cell extracts on sucrose gradients in the wild type and in strains lacking expression of D1: tba2-F35 and \( \Delta psbA \). The bulk of free mRNA as well as dissociated 50S and 30S ribosome subunits are found in fractions 6 to 10 (as can be seen from the sedimentation pattern of rrnS and psbB RNAs in extracts from wild-type cells treated with EDTA that dissociates ribosomes; Figure 3, WT panel). Therefore, in untreated extracts, transcripts found in fractions 5 to 1 and in the pellet correspond to polysomes of increasing sizes. We compared the distribution of three RNAs: psbD, psbB, and atpB, the latter serving as a control for a chloroplast transcript unrelated to PSII biogenesis. In the wild type, the three mRNAs present roughly a similar distribution: ribosome-free mRNAs peak at fractions 7 and 8, but the maximum in fractions 4 and 5 indicates polysome loading (Figure 3). In the tba2 or \( \Delta psbA \) strains, psbD and atpB mRNAs were still associated with

![Figure 3. psbB mRNA Is Poorly Translated in the Absence of D1.](image)

Left panels: Distribution of psbB, psbD, and atpB mRNAs on sucrose gradients in the wild type and in \( \Delta psbA \) and tba2-F35 mutants lacking expression of D1. The distribution of rrnS and psbB RNAs in extracts from wild-type cells, in conditions preserving polysomes (+CAP) or disrupting polysomes (+EDTA), is shown to substantiate the assignment of polysome and free subunits to specific fractions. Fraction P contains the material found as a pellet at the bottom of the tube. The black circle in panel \( \Delta psbA \) indicates RNA degradation in that specific fraction. Right panels: mRNA distributions were quantified by PhosphorImager scan of \(^{32}\)P labeling bound to the membrane in the three strains and expressed as the percentage of total transcript found in each fraction.
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polyosomes (Figure 3). By contrast, the psbB mRNA was mostly found in fractions 7 and 8, with no peak in fractions P to 5 and <10% of total psbB mRNA in heavy fractions versus almost 40% in the wild type. These observations confirm a defect in the initiation of translation of the psbB mRNA in the absence of D1.

**Downregulation of apoCP47 Expression in the Absence of D1 Is Due to an Autoregulation of Translation**

The control on the translation of apoCP47 subunit can be achieved in either of two ways: either D1 per se is required to activate psbB mRNA translation or apoCP47, which remains unassembled because of the absence of D1, exerts a negative feedback on the translation of psbB mRNA. Since the psbB 5'UTR was found sufficient to confer the CES regulation to a reporter gene, we could distinguish between these two possibilities by studying the expression of the 5'psbB-driven cytochrome f in mutant strains lacking both the CES subunit (i.e., apoCP47) and its assembly partner, D1. If the CES process results from a positive control mediated by D1, translational activation should be abolished in the double mutant strain, resulting in low expression of the cytochrome f reporter. By contrast, if the CES protein apoCP47 negatively controls its own translation, the negative feedback will be lost in the double mutant strain, thereby causing a high expression of the cytochrome f reporter.

A possible pitfall in such experiments would be the facilitated recruitment of psbB translational activators by 5'psbB-driven genes in strains lacking the endogenous psbB mRNA, such as ΔpsbB. This may artifactually increase expression of reporter genes. Thus, we generated by transformation another chloroplast mutant, CP47Tr, still able to accumulate and translate the psbB mRNA but unable to accumulate the apoCP47 protein.

This was done by introducing a stop codon in the psbB coding sequence, at position −165 with respect to the initiation codon, in the connecting loop between transmembrane helices III and IV (Figure 4A). The mutated psbB gene was associated with the recycling spectinomycin resistance cassette (Fischer et al., 1996), enabling the selection of transformants on TAP-Spec plates.

Pulse labeling of the CP47Tr strain showed the absence of neosynthesized apoCP47 but the presence of a new translation product with an apparent molecular mass of ~16 kD (Figure 4B, lane CP47Tr). The assignment of this new band to truncated apoCP47 was confirmed by crossing the CP47Tr strain with the nuclear mutant mbb1-222E, deficient for CP47 expression because of the absence of psbB mRNA (Vaijtil et al., 2000). In the resulting tetrad, the two mbb1 members that lacked the psbB mRNA (data not shown; indicated by an asterisk in Figure 4B) also lacked translation of the 16-kD polypeptide. This truncated apoCP47 was found to be very short-lived by pulse-chase experiments (t1/2: 10 min) (Figure 4C). Thus, it is not expected to accumulate to significant levels in the thylakoid membrane, fulfilling our aim to create a strain that accumulates a translatable psbB mRNA, but not its protein product, even in the truncated form.

The CP47Tr strain was then transformed with the 5’psbB-petA reporter gene, associated with the spectinomycin resistance cassette for selection of transformants on TAP-Spec medium (Table 1). The resulting strain {CP47Tr, bBl} expressed the chimeric gene at a rate ~1.7 higher than a strain expressing full-length apoCP47 (Figure 5A, cf. lanes bBl and {CP47Tr, bBl}). We then compared the expression of 5’psbB-driven cytochrome f in the absence of both D1 and apoCP47 among a tetrad progeny from the cross between transformants

![Figure 4](image-url)
CP47Tr, bBf and mutant tba2. Half of the progeny inherited the tba2 nuclear mutation, whereas all inherited both the truncated psbB allele (Figure 5A) and the chimeric petA gene, whose presence can be distinguished from the wild-type petA gene by the higher mobility of its transcript in mRNA gel blots (Figure 5C). As shown in Figures 5A and 5B for a representative half tetrad, the level of translation of the cytochrome f reporter remained high and independent of the presence or absence of D1. Thus, in contrast with what was observed when the full-length psbB gene was expressed, the translation and accumulation of the psbB-driven cytochrome f in the presence of the truncated version of apoCP47 was no longer dependent on the presence of D1. We also note that the rate of synthesis of truncated apoCP47 is insensitive to the absence or presence of D1 (Figure 5A).

Since the expression of both the chimeric 5′psbB-petA gene and the truncated psbB gene product became independent of the presence of D1, we ruled out a positive control of D1 on the translation of apoCP47. Rather, CP47, when unassembled...
because of the absence of D1, exerts a negative feedback on the translation of its own mRNA.

The CES Behavior of D1 Reflects a Genuine Regulation of Translation of 5′ UTR-Driven psbA Transcripts

Evidence for a decreased translation of D1 in the absence of D2 can be found in a comparative analysis of the psbA polysome loading in the wild type and ΔpsbD strains. In the wild type, there is a great contrast between the extent of polysome loading for psaA and psbA mRNAs: while >40% of psaA mRNAs were loaded on polysomes (see also psbB, psbD, and atpB mRNAs in Figure 3, WT panel), <15% of the psbA mRNAs were found in heavy fractions in our culture conditions (10 μE·m⁻²·s⁻¹), most of psbA mRNAs being found in lighter fractions as free mRNA (Figure 6A). This observation correlates well with the poor level of D1 translation observed in pulse-labeling experiments when performed with cells grown in low light (Figure 1A). Still, the limited loading of psbA mRNA on polysomes in the wild type, with a maximum found in fraction 4, was no longer observed in the ΔpsbD strain (Figure 6B).

The significant but limited change in polysome loading of psbA transcripts in the absence of D2 prompted us to seek stronger evidence that translation of psbA is genuinely dependent on the presence of D2. We used a psbA-driven reporter gene approach, similar to the one described above for the regulation of apoCP47 synthesis. We constructed various chimeric genes made of the coding region for cytochrome f, translated under the control of the psbA 5′ UTR, that extended to some degree into the psbA coding region. These chimeric 5′ psbA-petA genes, associated with the spectinomycin resistance cassette to allow selection of the transformed strains (Figure 7A), were introduced into the chloroplast genome of C. reinhardtii. Transformants, selected on TAP-Spec plates, were assayed for phototrophic growth on minimum medium. Only when at least 60 nucleotides of the psbA coding sequence were added in frame, upstream of the reporter gene, did the chimeric mRNA accumulate, enabling the phototrophic growth of the transformants (data not shown). These results point to the existence of cis-acting element stabilizing psbA-driven transcripts within the coding sequence of psbA, as already described for other chloroplast genes in C. reinhardtii (Singh et al., 2001) or for psbA in cyanobacteria (Kulkarni and Golden, 1997). Thus, we used for the rest of our study transformed strains expressing cytochrome f translated under the control of the psbA 5′ UTR, followed by the 60 first nucleotides of the psbA coding sequence, hereafter called bAf because they express a 5′psbA-driven cytochrome f.

We compared the expression of this reporter gene in the presence or absence of D2 in progeny from crosses between bAf...
Figure 8. The psbA 5’UTR Confers a D2-Dependent Expression to the Reporter Gene aadA.

(A) Schematic map of the petA-petD region where the 5’psbA-aadA chimeric gene was inserted in direct orientation with respect to the petA gene, at the neutral EcoRV (V) site. The bent arrows indicate transcription start sites. The small rectangle upstream of aadA coding sequence depicts the 60 nucleotides from the psbA coding sequence.

(B) Growth of four independent transformants, derived from the recipient strains listed on the left, in the presence of increasing concentrations of spectinomycin plus streptomycin.

(C) Accumulation of the chimeric aadA (and petA as a loading control) transcripts in the wild type and in one representative transformant for each genetic background presented in (B).

Transformants with the nuclear mutant strain mbd1-nac2. This mutant is defective in D2 protein expression because it lacks stable accumulation of psbD mRNA (Kuchka et al., 1989). The chloroplast 5’psbA-petA chimeric gene is easily identified by the faster migration of its transcript (Figure 7D). We observed that the level of translation and accumulation of this chimeric cytochrome f was much lower in the absence of D2 than in its presence (cf. lanes bAf, MBD1 and bAf, mbd1 for a representative half tetrad in Figures 7B and 7C). We could exclude a pleiotropic effect of the mbd1 mutation on the expression of 5’psbA-driven genes since the chimeric gene presented a lower expression when expressed in the ΔpsbD context as well (Figure 7E, lane (∆psbD, bAf)). No reduction in the accumulation of the chimeric mRNA correlated with the absence of the mbd1 allele (Figure 7D). Thus, the absence of D2 causes a decreased translation of psbA as well as of chimeric petA gene translated under the control of the psbA 5’UTR.

As an independent assay for expression changes of a 5’ driven psbA reporter in conditions where D1 can assemble or not with D2, we constructed a 5’psbA-aadA cassette, in which the bacterial aadA reporter gene was translated under the control of the promoter and 5’UTR of the psbA gene, followed by the first 60 nucleotides of the psbA coding sequence. This 5’psbA-aadA chimeric gene was inserted downstream of the petA gene, in plasmid pfbAK, used to transform the chloroplast genome of wild-type and deletion strains ΔpsbD and ΔpsbA (Figure 8A, Table 1). Transformants were recovered on TAP medium supplemented with 100 μg·mL⁻¹ of spectinomycin.

We analyzed the level of expression of the 5’psbA-aadA chimeric gene through the changes in antibiotic resistance (to spectinomycin and streptomycin) in the transformed strains according to Choquet et al. (1998). Four independent transformants, derived from each recipient strain transformed with plasmid pfbAK, were plated on TAP medium supplemented with increasing concentrations of both spectinomycin and streptomycin. As shown in Figure 8B, cells expressing the chimeric gene in a wild-type context demonstrated significant resistance up to 250 μg·mL⁻¹ of both antibiotics. Cells lacking D2, the assembly partner of D1, were barely resisting 62 μg·mL⁻¹ of both antibiotics, whereas clones originating from ΔpsbA were resistant up to 500 μg·mL⁻¹ of both antibiotics. The latter observation demonstrates that the decreased resistance observed in ΔpsbD strains is not a mere consequence of PSII deficiency, as it is not observed in the ΔpsbA strain.

Hence, cells lacking D2, the assembly partner of D1, are unable to express the 5’psbA-driven aadA gene to the same level as the control that expresses D2. Since the 5’psbA-aadA mRNA accumulated to the same level in all transformed strains (Figure 8C), these results point to a specific downregulation of the expression of the chimeric gene operating at the level of translation.

Unassembled D1 Represses Translation of 5’psbA-Driven Genes

To address the mechanism of this translational regulation and determine whether D2 stimulates the expression of D1 or whether unassembled D1 exerts a negative feedback on the translation of 5’psbA-driven mRNAs, we studied the expression of the 5’psbA-driven cytochrome f in the absence of both the assembly partner D1 and the CES subunit D1. Using a strategy similar to that described above for the psbB gene, we generated a strain still able to accumulate and translate the psbA mRNA but lacking accumulation of the D1 protein.

To that end, we substituted by site-directed mutagenesis the codon for residue I259 in the stromal loop connecting helices IV and V by a stop codon, causing synthesis of a truncated D1 protein (Figure 9A). This mutation, associated with a recycling
spectinomycin cassette (Figure 9A), was introduced by trans-
formation in the chloroplast genome of a wild-type strain of C. reinhardtii. Spectinomycin-resistant transformants, hereafter
called $D1_{Tr}$, were screened by fluorescence for PSII deficiency.

The profile of chloroplast translation products in the $D1_{Tr}$ strain
lacked D1 but showed an additional polypeptide with an appar-
ent molecular mass of $\sim 24$ kD that we tentatively assigned to
truncated D1 polypeptide (Figure 9B, lane $D1_{Tr}$). This was con-
firmed by crossing the $D1_{Tr}$ strain with the nuclear mutant strain
tba2-F35, deficient for translation of the $psbA$ mRNA. As ex-
pected from a Mendelian segregation of tba2, only two members
of the resulting tetrads expressed the 24-kD truncated form of D1
(Figure 9B).

Pulse-chase experiments showed that truncated D1 was rap-
idly turning over, with a half-life of $\sim 20$ min (Figure 9C), as already
observed for another, larger truncation of the D1 polypeptide
(Preiss et al., 2001). Thus, this truncated and unstable version of
the D1 protein cannot accumulate in the thylakoid membrane.

The $D1_{Tr}$ strain was then transformed with the 5′$psbA$-petA
chimeric gene associated with the spectinomycin resistance
cassette. Transformants, hereafter referred to as $\{D1_{Tr}, bAf\}$,
were selected on TAP-Spec plates. They expressed the 5′$psbA$-
driven cytochrome $f$ but could not accumulate D1 even in the
truncated form. In those strains, the expression of the chimeric
gene was tremendously increased: it was overexpressed $\sim 12$
times when compared with a wild-type background (Figure 10A).
A closer examination of its electrophoretic properties showed
that instead of migrating as a single sharp band, as in a wild-type
context, cytochrome $f$ migrated as a doublet with the major band
being of slightly faster mobility than regular cytochrome $f$. This
corresponds to the behavior of apocytochrome $f$ (Kuras et al.,
1995) and indicates that such a high level of translation over-
whelms the capability of the cytochrome $c$ synthesis machinery
required for apocytochrome to holocytochrome $f$ conversion (Xie
and Merchant, 1998). Apocytochrome $f$ is highly unstable (Kuras
et al., 1995), and indeed most of the newly synthesized poly-
peptide rapidly turned over in pulse-chase experiments (data not
shown). As a result, the increased accumulation of cytochrome $f$
in the $D1_{Tr}$ context, instead of being $\sim 12$, was only 4 times that
observed in the wild type (Figure 10B).

Double mutants lacking both D2 and full-length D1 subunits
were then generated by crossing the $\{D1_{Tr}, bAf\}$ strains with the
mbd1 mutant. The expression of the 5′$psbA$-petA reporter gene,
uniparentally transmitted to all tetrad members together with the
truncated $psbA$ allele, was compared in representative progeny

![Figure 9. The $D1_{Tr}$ Strain Expresses a Truncated and Short-Lived $psbA$ Gene Product.](image)

(A) Top: Map of the $psbA$ gene. Exons are indicated by gray boxes. The bent arrow indicates transcription start site. Bottom: schematic enlarged view of the insert in p-157BS, were a stop codon (linked to a BglI restriction site) was introduced in the 5th exon of the $psbA$ gene (in the IV to V connecting loop) and associated with the recycling spectinomycin resistance cassette, schematically depicted by the boxed K with chevrons on either side (not to scale). The sequence coding for the fifth transmembrane helix is shown as a white box. Relevant restriction sites are as follows: P, PstI; X, XbaI; A, ApaI; D, DraI; S, SacI; B, BstEII; Bg, BglI; Ba, BamHI. The position of the mutation with respect to the protein sequence is indicated in the schematic structure below.

(B) Expression of the truncated D1 polypeptide: $psbA$ translation products were analyzed by pulse-labeling experiments in the wild type, in a transformed strain expressing the truncated $psbA$ allele $D1_{Tr}$, in a representative tetrad progeny from the cross $D1_{Tr} \times tba2$-F35, and in the parental strain tba2-F35. Asterisks mark tetrad members carrying the $tba2$ mutation.

(C) Stability of the truncated protein assessed by pulse-chase experi-
ments. Pulse-labeled wild type is shown for comparison. The positions of D2 and D1 and of the truncated D1 polypeptide are indicated.
that inherited the wild type or the mutated allele of \( \text{MDB1} \) and hence accumulated or not the \( \text{psbD} \) mRNA (Figure 10C). As can be seen in Figure 10A, the level of translation of truncated D1 is identical in a representative half-tetrad, irrespective of D2 synthesis capability. At variance with full-length D1, the synthesis of its truncated version did not depend on the presence of D2. Moreover, the increased translation (Figure 10A) and accumulation (Figure 10B) of the cytochrome \( f \) reporter remained similar regardless of the presence or absence of D2. Thus, when full-length D1 protein fails to accumulate in the thylakoid membrane, the translation of the \( \text{psbA} \)-driven cytochrome \( f \) (as well as that of truncated D1) is no longer repressed in the absence of D2, excluding the D2 positive control hypothesis. Rather, translation of the D1 CES subunit is autoregulated through a negative feedback mechanism mediated by its unassembled state.

Assessment of the Role of CES in the Recovery from Photoinhibition

During recovery from photoinhibition, the synthesis of D1 is enhanced (Kettunen et al., 1997). The underlying mechanism is triggered by the process of D1 degradation itself rather than by the high light conditions (Zer et al., 1994; Krieger-Liszkay et al., 2000). Apparently, a D1-less complex is a prerequisite for productive D1 translation, as would be expected for a CES protein. This is illustrated in Figure 11 by the strong increase in the rate of D1 synthesis in the wild type but not in strains defective for PSII assembly (Figures 11A, \( \Delta \text{psbD} \), or 11B, \( \Delta \text{psbB}, \text{bAf} \)) upon a light intensity switch from 7 to 2000 \( \mu \text{E m}^{-2} \text{s}^{-1} \). We thus investigated whether the CES process could account for the high turnover of D1 in high light conditions. The expression of the

![Figure 10](image_url)

**Figure 10.** The \( 5' \text{psbA}-\text{petA} \) Reporter Gene Is Overtranslated in Strains Expressing Truncated D1.

(A) Chloroplast translation products in representative offspring from the cross \( \text{D1}, \text{bAf} \times \text{mbd1-nac2} \) in wild-type, \( \text{bAf} \), \( \text{D1} \), and parental strains. Lack of D2 synthesis indicates the \( \text{mbd1} \) progeny. Phosphorimager quantification of cytochrome \( f \) synthesis, relative to that observed in the wild type (after normalization to the level of synthesis of the \( \beta \)-subunit from the ATP synthase complex to correct for variations in the efficiency of \( ^{14}\text{C} \) incorporation) is indicated below the figure.

(B) Accumulation of cytochrome \( f \) and OEE2 (loading control) in these strains. Quantification of cytochrome \( f \) accumulation (relative to the wild type) is shown below the panel.

(C) Transcript accumulation for petA, psbA, psbD, and atpB (loading control) in the same strains.

that inherited the wild type or the mutated allele of \( \text{MDB1} \) and hence accumulated or not the \( \text{psbD} \) mRNA (Figure 10C). As can be seen in Figure 10A, the level of translation of truncated D1 is identical in a representative half-tetrad, irrespective of D2 synthesis capability. At variance with full-length D1, the synthesis of its truncated version did not depend on the presence of D2. Moreover, the increased translation (Figure 10A) and accumulation (Figure 10B) of the cytochrome \( f \) reporter remained similar regardless of the presence or absence of D2. Thus, when full-length D1 protein fails to accumulate in the thylakoid membrane, the translation of the \( \text{psbA} \)-driven cytochrome \( f \) (as well as that of truncated D1) is no longer repressed in the absence of D2, excluding the D2 positive control hypothesis. Rather, translation of the D1 CES subunit is autoregulated through a negative feedback mechanism mediated by its unassembled state.

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![Figure 11](image_url)

**Figure 11.** The CES Process Does Not Take Part in the Recovery from Photoinhibition.

Wild type, \( \text{bAf} \), \( \Delta \text{psbA}, \text{bAf} \) and \( \Delta \text{psbD} \) (A) or wild type, \( \Delta \text{psbA}, \text{bAf} \) and \( \Delta \text{psbB}, \text{bAf} \) strains (B) were pulse labeled under normal light (left) or photoinhibition conditions (right). Asterisks indicate the neosynthesized form of D1 described in Figure 1.
5'psbA-petA reporter gene was compared in pulse-labeling experiments using bAf strains grown under dim light (7 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)) or placed in photoinhibition conditions (2000 \( \mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1} \)). Although, in the latter condition, the rate of D1 synthesis was strongly increased, that of chimeric cytochrome \( f \) did not increase significantly (Figure 11A, lane bAf, right part). This lack of stimulation of synthesis is not due to specific limitations in the expression of the chimeric gene since it can be very efficiently overexpressed by more than an order of magnitude in strain \( \Delta \text{psbA, bAf} \) placed either in low or strong light conditions (Figure 11A).

Since the \( \text{psbA} \) 3’UTR may participate in the efficient translation of \( \text{psbA} \) mRNA (Katz and Danon, 2002), we constructed a 5’psbA-petA-3’psbA reporter gene. We also constructed a chimeric gene expressing cytochrome \( f \) translated under the control of the \( \text{psbA} \) 5’UTR, followed by the first 144 codons of \( \text{psbA} \) (i.e., up to the 3rd residue of the third transmembrane helix of D1). Both chimeras, yielding strains \( \text{bAfB} \) and \( \text{bA2Af} \) after chloroplast transformation in \( C. \text{reinhardtii} \), allowed cytochrome \( f \) expression that suggested photoprotective growth. Surprisingly enough, the introduction of >140 residues (and two transmembrane helices) upstream of the lumen targeting peptide of petA did not prevent protein translocation and cleavage of the targeting sequence. However, we failed to observe any significant increase in the expression of the two cytochrome \( f \) reporters when the transformed strains were subjected to photoinhibition (Figure 11B). Two hours after transfer to high light, cytochrome \( f \) expression was even reduced in the \( \text{bA2Af} \) strain, while it was only moderately stimulated (less than twofold, relative to the other chloroplast translation products) in the \( \text{bAfB} \) strain. A weak stimulation of the 5’psbA-driven petA gene was also most often observed in the \( \{ \text{bAf, } \Delta \text{psbB} \} \) strain exposed to strong light. In the latter strain, no photoinhibition-induced stimulation of D1 synthesis occurs since it is defective for PSII assembly. We attribute this marginal increase to a limited light effect on the rate of D1 translation.

Thus, the expression of the very same reporter gene that is under CES control when assembly of PSII cores is prevented is not sensitive to photoinhibition conditions, leading to the conclusion that D1 expression involves widely different mechanisms during PSII biogenesis or repair.

**DISCUSSION**

**The Synthesis of D1 and CP47 Is Regulated at the Level of Translation Initiation**

The biogenesis of PSII involves a CES cascade, where the RC subunit D2 is required for high levels of translation of the RC subunit D1, whose presence is, in turn, necessary for high levels of translation of the core antenna subunit CP47. Whether other chloroplast-encoded PSII subunits participate to this cascade is not known yet. While the synthesis of CP43 appears independent of PSII assembly (de Vitry et al., 1989), the phosphoprotein PsbH is clearly a CES protein since its rate of translation is strongly reduced in the absence of the D1 subunit (K. Wostrikoff and Y. Choquet, unpublished results). It has been proposed that cytochrome \( b_{559} \) could also be part of this cascade, as it would be required for the efficient translation of psbD and psbC mRNAs (Morais et al., 1998). However, we were not able to observe significant alterations in the rate of translation of D2 and CP43 in a \( \text{psbE-} \)null mutant (L. Minai and Y. Choquet, unpublished results). Therefore, the status of cytochrome \( b_{559} \) in the CES cascade is still pending. Further studies will be required to determine the CES behavior of the other PSII subunits.

To assess the importance of translation initiation in the decrease of D1 translation in absence of D2, we used the petA coding sequence, encoding the endogenous cytochrome \( f \) as a reporter gene. Although cytochrome \( f \) is by itself a CES subunit in the course of cytochrome \( b_{559} \) biogenesis, we have previously demonstrated that the regulation of its synthesis requires the petA 5’UTR (Choquet et al., 1998), which is absent in the chimeric constructs. Thus, when expressed from another 5’UTR, cytochrome \( f \) is a faithful reporter protein (Wostrikoff et al., 2004). Here, we showed that the \( \text{psbA} \) 5’UTR (together with the first 60 nucleotides just downstream of the \( \text{psbA} \) initiation codon) was sufficient to confer a D2-dependent rate of synthesis to the petA and \( \text{aadA} \) reporter genes. Their lower expression in the absence of D2 was not accompanied by decreased mRNA levels, excluding significant contribution of a pretranslational step to the D2-dependent regulation of \( \text{psbA} \) expression. The specificity of D2 in this process was confirmed by the preserved expression of the 5’psbA-petA chimera in other strains deficient for PSII assembly, such as \( \{ \Delta \text{psbB, bAf} \} \). The CES control of \( \text{psbA} \) expression occurs at a step earlier than translation elongation or cotranslational degradation of the nascent D1 polypeptide, since both should depend on the \( \text{psbA} \) coding sequence rather than on the \( \text{psbA} \) 5’UTR. Either an activation step prior to initiation or translation initiation itself CES regulates D1 translation. This regulatory mechanism is in contrast with the proposed role of D2 in driving efficient translation of D1, which occurs at the level of translation elongation (Zhang et al., 1999); nascent D1 polypeptides, more specifically the 17- and 25-kD translation intermediates that correspond to the escape of the second and fourth transmembrane helices of D1 from the ribosome tunnel, interact cotranslationally with D2, probably through a transient disulfide bridge. When this interaction is impaired, in an in organello translation system, these intermediates are stabilized, while formation of full-length D1 is decreased (Zhang et al., 2000). Several observations argue for independent participation of translation initiation and translation elongation in the fine-tuning of D1 expression. Besides recovery from photoinhibition, most likely controlled at the level of translation elongation (see below), the elongation, but not the initiation of D1 translation, is altered in \( C. \text{reinhardtii} \) mutant strains \( \text{mf}1 \) and \( \text{mf}2 \), deficient in the synthesis of \( \Delta^{3-}\text{trans} \) hexadecenoic-phosphatidylglycerol (Pineau et al., 2004).

Similarly, the \( \text{psbB} \) 5’UTR is sufficient to confer a D1-dependent regulation to the translation of the cytochrome \( f \) reporter, suggesting a regulation of translation initiation, as confirmed by the marked decrease in polysome loading of \( \text{psbB} \) mRNA in strains deficient for the expression of D1.

**Translation of D1 and CP47 Is Autoregulated**

Two possibilities might explain the decreased translation of D1 and CP47 in the absence of their assembly partners. The latter
could behave as dominant subunits (D2 for D1 or D1 for CP47) that transactivate translation of the CES subunits. We exclude this possibility since the expression of 5’ CES-driven petA genes remains high in the combined absence of the dominant and CES subunits. This behavior fully supports a negative feedback inhibition on translation of their own mRNA by the CES subunits D1 or CP47 when unassembled because of the absence of their assembly partners.

An efficient translational autoregulation requires that unassembled D1 or CP47 accumulate to some extent in the absence of their assembly partners. Indeed, D1 and CP47 remained stable, although synthesized in markedly reduced amounts, in the absence of D2 and D1, respectively (de Vitry et al., 1989). Paradoxically, they are normally translated but rapidly degraded in mutants lacking expression of CP43 (or of CP47 for polypeptide D1) (de Vitry et al., 1989). Most likely, assembly of the CES subunits in their target complex induces a protease-sensitive conformation that allows their proteolysis if PSII assembly cannot proceed further. Similarly, cytochrome f, a stable protein when unassembled (Kuras and Wollman, 1994; Choquet et al., 2003), becomes susceptible to proteolytic degradation upon nitrogen starvation once assembled within cytochrome b6f complexes (Bulte and Wollman, 1992).

As we showed here for PSII and in previous studies of the biogenesis of PSI and cytochrome b6f complexes, the 5’ CES-driven reporter genes were even more translated in the absence of the CES proteins than in a wild-type genetic context, regardless of the presence of their assembly partners. This argues for the presence of a fraction of unassembled CES subunits in wild-type chloroplasts that downregulates their own expression in order to tune their production to the availability of their assembly partners. This feedback inhibition is released when the neo-synthesized CES subunits assemble with their partners (i.e., only when all upstream steps in the sequential assembly pathway of PSII, including an efficient expression of those assembly partners, have been completed). It is thus not a surprise that the CES hierarchy reflects the order of polypeptide assembly (Figure 12), the core antenna subunit CP47 being made only when D1 has successfully assembled with D2.

That a similar process is at work in the biogenesis of PSII in higher plant chloroplasts is supported by the phenotype of the vir15 mutant of barley (Hordeum vulgare) that is primarily affected, in a developmentally regulated manner, in the translation of D1 (Kim et al., 1994) but also in the expression of CP47 (Gamble and Mullet, 1989). The molecular mechanism of this translational coupling between these two PSII subunits in higher plants, however, remains to be studied. By contrast, there is no evidence for a CES process in the expression of PSII subunits in cyanobacteria, where mutations preventing PSII assembly increase the proteolytic disposal of the remaining subunits, with no apparent effect on their rate of transcription or translation (Yu and Vermaas, 1990). The biogenesis of the multimeric photosynthetic proteins obeys widely different rules in photosynthetic prokaryotes and in chloroplasts (Wollman et al., 1999).

**Ternary Effectors Involved in the CES Process?**

The true nature of the interaction between unassembled CES proteins and the 5’ UTR of their mRNAs is not known. However, considering the lack of CES process in cyanobacteria but the high conservation of D1 and CP47 sequences from cyanobacteria to higher plants, it seems unlikely that chloroplast CES subunits, D1 and apoCP47, have evolved a specific repressor domain capable of sequence-specific interactions with the 5’ UTR of their mRNAs. We note that an active RNA binding domain has recently been proposed for another conserved CES protein of the photosynthetic apparatus, the large subunit of

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**Figure 12.** Model of the CES Cascade Involved in PSII Biogenesis.
The PSII repair mechanism following photoinhibition involves an increase in the rate of D1 translation, apparently triggered by the degradation of photodamaged D1 and by the formation of D1-less complexes (Adir et al., 1990; Bailey et al., 2002). Such a mechanism is consistent with an autoregulation of D1 synthesis. This prompted us to investigate the contribution of the CES process to the recovery from photoinhibition. The use of reporter constructs carrying variable parts of the psbA gene fused in frame with the coding region of cytochrome f did not allow us to observe any significant stimulation in the expression of the reporter protein upon photoinhibition, although this construct can be highly overexpressed in other conditions. This seems conflicting with the 100-fold increase in the expression of a 5's psbA-uidA reporter gene, when tobacco (Nicotiana tabacum) etiolated cells are transferred to light (Staub and Maliga, 1994). However, biogenesis of PSII and translation of psbA mRNA are arrested in less PSII complexes (Kettunen et al., 1997). By contrast, PSII reporter gene, when tobacco (Nicotiana tabacum) etiolated cells are transferred to light (Staub and Maliga, 1994). However, biogenesis of PSII and translation of psbA mRNA are arrested in less PSII complexes (Kettunen et al., 1997).

The CES Process in PSII Biogenesis

The CES Process Plays No Role in the Recovery from Photoinhibition

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Thus, translation initiation, although being a main regulatory step in psbA gene expression during PSII biogenesis, does not play a significant role in the rapid D1 synthesis during recovery from photoinhibition. Rather, translation elongation represents the key control point of psbA gene expression during recovery from photoinhibition: under high light stalled psbA polytopes are associated with the thylakoid membrane. The presence of D1-less PSII complexes would release this translational arrest (Kettunen et al., 1997).

It is tempting to attribute the two widely different mechanisms for the control of psbA gene expression to different spatial localizations. During the recovery from photoinhibition, the synthesis of D1 takes place in stromal thylakoids, as required for cotranslational interactions with D2 and assembly within D1-less PSII complexes (Kettunen et al., 1997). By contrast, PSII...
biogenesis may develop in a specialized membrane compartment, the low density membranes, biochemically related to the chloroplast inner envelope (Zerges and Rochaix, 1998). Indeed many nuclear encoded factors, required for posttranscriptional steps of chloroplast gene expression, including RB47 involved in psbA translation initiation, are associated with this low density membrane fraction but not with the bulk of thylakoid membrane (Zerges and Rochaix, 1998). Similarly, in cyanobacteria, de novo biogenesis of PSIi complexes is likely to occur in plasma membranes (Zak et al., 2001), while repair of photodamaged complexes takes place in the thylakoid membrane. Most interestingly, an RNA binding protein with a high affinity for the psbA 5′UTR (RB63) has been identified by a biochemical approach in C. reinhardtii (Ossenbuhl et al., 2002). This protein is associated with thylakoids but not with the low density membrane fraction and could be specifically required for psbA expression during the high turnover of D1.

Our study demonstrates that the molecular mechanism for the CES process in PSIi shares similar features to those operating in the biogenesis of cytochrome b$_{5}$f and PSI complexes (Choquet et al., 1998; Wostrikoff et al., 2004). This assembly-dependent autoregulation of translation is then central to the biogenesis of photosynthetic proteins in the chloroplast of *Chlamydomonas*. Given the similar features of the biogenesis of cytochrome oxidase in yeast mitochondria, it is likely that the CES process is a key signature of protein assembly in situations in which interactions between two genetic intracellular compartments prevail (i.e., in the biogenesis of organellar protein complexes).

**METHODS**

**Strains and Growth Conditions**

Wild-type, mutant, and transformed strains of *Chlamydomonas reinhardtii* were grown in TAP medium, pH 7.2 (Harris, 1989), under continuous low light (5 to 10 μE·m$^{-2}$·s$^{-1}$). mt− parental strains mbd1-nac2 (Kuchka et al., 1989), tba2-F35 (Girard-Bascou et al., 1992), and mbdf-222E (Monod et al., 1992; Vaistij et al., 2000) were used for crosses performed as described by Harris (1989). The Fus7 strain (mt+) (Bennoun et al., 1986) is referred to as ΔpsbA.

**Constructs and Nucleic Acid Manipulations**

Standard nucleic acids manipulations were performed according to Sambrook et al. (1989).

**DNA Constructs**

**Construction of psbB and psbD Deletions**

A plasmid carrying a partial deletion of the psbB gene was created by ligation of vector p38.Ncol (Vaistij et al., 2000), digested with Ncol and PvuI, with the 2.95-kb SacI-KpnI fragment from plasmid pKS-aadA-483 (Fischer et al., 1996), both fragments being treated with T4 DNA polymerase to generate blunt ends. In the resulting plasmid, p3psbB::K, the first 614 bp of the psbB coding region were replaced by the recycling aadA cassette, in reverse orientation with respect to psbB.

To create a vector carrying a deletion of the psbD gene, a 5827-bp HindIII-PstI fragment from the chloroplast genome, containing the whole psbD gene (Erickson et al., 1986), was cloned into the pUC9 vector to yield plasmid pHSP. This vector, cut with Bst1107 and PmlI to remove most psbD coding sequence, was ligated to the 2.95-kb recycling aadA cassette, in reverse orientation with respect to psbD to form the ΔpsbD::K, plasmid.

**Construction of Chimeric Genes**

Plasmid P-157, obtained from the Chlamydomonas Genetic Center (www.biology.duke.edu/chlamy), contains a 10-kb BamHI-BglII fragment of the chloroplast genome from strain DCMU4 (Erickson et al., 1984) subcloned into pUC18 vector. It was used as a template with oligonucleotide primers bA$_{prom}$ and bA$_{5′}$ (Table 2) in PCR reaction. The 324-bp amplicon contained the promoter and 5′UTRs of the psbA gene, followed by the first 20 codons of psbA, as well as two restriction sites, Clal, upstream of the psbA promoter, and Ncol, downstream of psbA coding sequences (Table 2, 3rd column).

Similarly, plasmid pBA158 bearing an intronless psbA gene (Sugiura et al., 1998), kindly provided by A.R. Crofts, was used as template with primers bA$_{prom}$ and bA$_{3′}$ in PCR reactions. The 716-bp amplicon contained the psbA promoter and 5′UTRs, followed by the first 144 codons of psbA (up to the 3rd residue from the 3rd transmembrane helix of D1).

A 356-bp DNA fragment carrying the promoter and 5′UTRs of the psbB gene, but no psbB coding sequence, was amplified by PCR with primers bB$_{prom}$ and bB$_{3′}$ from the template vector p38.Ncol.

The three fragments, digested with Clal and Ncol, were ligated into vector pAFFF (Choquet et al., 1998), digested with the same enzymes, to yield plasmids pbAf, pbA$_{259StR}$, or pbBf, in which the EcoRV-Smal 1.9-kb aadA cassette (Goldschmidt-Clermont, 1991) was inserted, in reverse orientation with respect to the petA gene, at the unique restriction site HindIII to form plasmid pbKfAf, pbKbA$_{259StR}$, or pbKbBf.

The 324-bp amplicon (5′psbA), digested by Clal and Ncol, was also cloned into the corresponding sites of vector PUC-ATPX-AAD (Goldschmidt-Clermont, 1991) to yield plasmid pbAK, from which the 5′psbA-aadA chimeric gene was removed by digestion with Smal and EcoRV. It was then inserted downstream of the petA gene, at the EcoRV site from plasmid pWF (Kuras and Wollman, 1994), in direct orientation with respect to the petA gene to create plasmid pbAK.

**Generation of Truncated Alleles of psbA and psbB Genes**

psbA. Plasmid p-157, cut with BstElI and SpII, was treated with T4 DNA polymerase and religated on itself to yield plasmid p-157BS. Codon l$_{259}$, in the fifth exon of psbA, was substituted by a stop codon associated with the restriction site BglII for RFLP analysis by a two-step PCR procedure (Higuchi, 1990). Two pairs of oligonucleotides (bA$^{5′}$ and bA$_{3′}$ on one hand, and bA$_{259StB}$ and bA$_{3′}$ on the other hand) were used to amplify two partially overlapping fragments from template plasmid p-157BS. The purified fragments were mixed and used as templates in another PCR reaction with the external primers bA$^{5′}$ and bA$^{3′}$. The final amplicon, carrying the mutation, was digested with PstI and XbaI, two restriction sites, upstream and downstream of the introduced mutation, and cloned into plasmid p-157BS digested with the same enzymes to create plasmid pbA$_{259St}$. St.

Using bA$p_{Dir}$/bA$p_{Rev}$ and bA$S_{Ac}l/l_{A'}/bA$3′ oligonucleotide pairs, the same two-step strategy allowed amplification from the template vector P-157BS of a 1013-bp DNA fragment corresponding to regions downstream of the psbA coding sequence where two restriction sites, SacII and Apal, were introduced after the psbA 3′UTR. This fragment, digested with XbaI and DraII, was cloned into the corresponding sites of plasmid pbA$_{259St}$ to yield plasmid pbA$_{259StR}$. The recycling cassette excised with Apal and SacII from plasmid pKS-aadA-483 was then inserted into these sites within plasmid pbA$_{259StR}$ to create plasmid pbA$_{259StR}$.

psbB. Similarly, a stop codon, associated with a HindIII restriction site for RFLP analysis, was introduced in the psbB coding sequence, 495
nucleotides after the initiation codon, using the two oligonucleotide pairs bBS ’/bBSinv and bBST1/bBST3’ and the template plasmid p38.NcoI. The resulting 742-bp amplicon was digested with ApaLI and PvuII, two unique restriction sites within the coding sequence of psbB and ligated with the 5266-bp fragment obtained from vector p38.NcoI by PvuII digestion and ApaLI partial digestion to yield plasmid pBb164-St. The recycling aadA cassette was then introduced in the unique Stul site of this plasmid, in the same orientation as psbB, to create plasmid pKbBb164-St.

Creation of a 5 psbA-petA-3 psbA Chimeric Gene

Partially overlapping fragments were obtained using the oligonucleotide pairs cACod/CASinv with vector pWF as template and bATG/bATGinv with the template vector P-157. The resulting 269- and 349-bp amplicons were mixed together with primers cACod and bATGinv to yield a 579-bp PCR product that contains the C-terminal part of the petA gene, fused to the psbA 3’ UTR. This amplicon, digested with Accl and EcoRV, was cloned into the pkBaf vector digested with the same enzymes to yield plasmid pkBafA.

All constructs were sequenced to assess the presence of appropriate mutations before transformation of C. reinhardtii. RNA gel blot analyses were performed as described by Wostrikoff et al. (2004).

Analysis of Polysomes

The polysome purification protocol was adapted from Rott et al. (1998).

Preparation of Polysome Fractions

One hundred milliliters of cell culture (2 × 10^6 cell mL^–1), preincubated for 10 min with chloramphenicol (CAP; 100 μg mL^–1), were harvested by gentle centrifugation. Cells were resuspended in 2.5 mL of resuspension buffer (0.2 M Tris-HCl, pH 8.0, 0.2 M KCl, 25 mM MgCl2, 25 mM EGTA, 0.2 M sucrose, 1% Triton X-100, 2% polyoxyethylene-13-tridecylether, 50 mM β-mercaptoethanol, 0.5 mg mL^–1 heparin, and 100 μg mL^–1 CAP), broken with a French press (13 kg cm^–2), and centrifuged at 1000g for 5 min to pellet unbroken cells and large cell debris. Supernatant, after addition of deoxycholate (0.5% final), was kept on ice for 5 min to pellet unbroken cells and large cell debris. Supernatant, after addition of deoxycholate (0.5% final), was kept on ice for 5 min and centrifuged at 10,000g for 15 min. Two milliliters of this new supernatant were loaded onto a 55% sucrose (w/v) solution. Gradients were centrifuged at 38,000 rpm in a Beckmann SW41 rotor for 2 h and 30 min.

Ten fractions of 1.1 mL were collected, out of which a 100-μL aliquot was removed to determine the sucrose percentage by refractometry. To assess the presence of pelleted material, the bottom of the tube was assessed the presence of pelleted material, the bottom of the tube was removed to determine the sucrose percentage by refractometry. To assess the presence of pelleted material, the bottom of the tube was removed to determine the sucrose percentage by refractometry.

RNA Preparation and Analysis

From these fractions, nucleic acids were precipitated overnight at –20°C by addition of 400 μL of 2 M KCl and 3 mL absolute ethanol and centrifuged for 15 min at 16,200g. Pellets, resuspended in 400 μL of 5 mM EDTA, 0.1% SDS, were extracted once with 400 μL of phenol/chloroform/IAA and ethanol precipitated. After centrifugation, pellets were washed with 70% ethanol, dried under vacuum for 5 min, resuspended in 25 μL of 5 mM EDTA, 0.1% SDS, and stored at –80°C. Five microliters of these RNA solutions were analyzed by RNA gel blot experiments.

Identification of the ribosome populations present in the sucrose gradient fractions resulted from the analysis of the sedimentation properties of cell extracts treated with EDTA that dissociates ribosome subunits (Figure 3, WT panel).

Transformation Experiments

Cells were transformed by tungsten particle bombardment (Boynton and Gillham, 1993) as described by Kuras and Wollman (1994). Transformants were selected on Tap-Spec (100 μg mL^–1) under low light (5 to 6 μE m^–2 s^–1) and subcloned on selective medium to reach homoplasmy. Proper insertion of transforming DNA and homoplasmy were checked by RFLP analysis of specific PCR amplification products. At least three independent transformants were analyzed for each transformation.

Protein Analysis

Pulse and pulse-chase experiments, protein electrophoresis, and immunoblotting were performed according to Kuras and Wollman (1994). For pulse labeling, we used illumination of 50 μE m^–2 s^–1, except for experiments in Figures 2 and 9, where an illumination of 200 μE m^–2 s^–1 increased 14C incorporation for a better visualization of the decreased level of cytochrome f synthesis. Cells were photoinhibited by transfer for 2 h under 2000 μE m^–2 s^–1 illumination before pulse labeling in the same conditions (Figure 11). Cell extracts were analyzed on 8% (Figure 1) or 12% 18% acrylamide SDS-PAGE in the presence of 8 M urea after loading on equal chlorophyll basis. All pulse-labeling experiments were repeated twice and performed on three independent transformed strains. Quantification of rates of translation was done on PhosphorImager scans of pulse-labeled proteins from dried gels using the program ImageQuant (Molecular Dynamics). Values for the level of synthesis of cytochrome f (either endogenous or chimeric) were corrected for background by measuring in each lane an empty window of the same area below cytochrome f.

Amino-acid synthesis of other polypeptides (including the unrelated C homologue) were corrected for background by measuring in each lane an empty window of the same area below cytochrome f

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