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Biogenesis of PSI involves a cascade of translational autoregulation in the chloroplast of *Chlamydomonas*

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Photosystem I comprises 13 subunits in *Chlamydomonas reinhardtii*, four of which—the major reaction center I subunits PsaA and PsaB, PsaC and PsaJ—are chloroplast genome-encoded. We demonstrate that PSI biogenesis involves an assembly-governed regulation of synthesis of the major chloroplast-encoded subunits where the presence of PsaB is required to observe significant rates of PsaA synthesis and the presence of PsaA is required to observe significant rates of PsaC synthesis. Using chimeric genes expressed in the chloroplast, we show that these regulatory processes correspond to autoregulation of translation for PsaA and PsaC. The downregulation of translation occurs at some early stage since it arises from the interaction between unassembled PsaA and PsaC polypeptides and 5′ untranslated regions of *psaA* and *psaC* mRNAs, respectively. These assembly-dependent autoregulations of translation represent two new instances of a control by epistasy of synthesis process that turns out to be a general feature of protein expression in the chloroplast of *C. reinhardtii*.

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Introduction

Many enzymatic functions are carried out by hetero-oligomeric proteins. The assembly of their constitutive subunits in an appropriate stoichiometry can be regarded as spontaneous, being a thermodynamically favoured process. Still, biological systems have optimised the rate of protein production in order to avoid wasteful accumulation of unassembled subunits. For instance, in prokaryotes, the operonal organisation of many genes allows a fine-tuning of the coupled transcription/translation rates of subunits from the same protein complex. In many other instances, a proteolytic disposal of unassembled subunits operates as a backup system in biogenesis pathways. The biogenesis of oligomeric protein complexes in the energy transducing membranes of organelles has unique features of particular complexity. Most of these protein complexes comprise more than 10 distinct subunits, some of which are nucleus-encoded, whereas the others are encoded by the organellar genome (Fox, 1996; Wollman *et al*, 1999). Because the gene copy numbers for nucleus- versus organelle-encoded subunits from a same protein complex can differ by as much as four orders of magnitude, organelle-based regulation processes, as well as crosstalks between the nucleo-cytosolic and organelle compartments, should be at work to regulate the level of expression of the various subunits of these oligomeric proteins.

Studies of respiratory mutants from yeast or of photosynthetic mutants from the unicellular green alga *Chlamydomonas reinhardtii* have been instrumental in this major issue in cell biology (Fox, 1996; Wollman *et al*, 1999). These studies showed that the accumulation of the various subunits of these oligomeric proteins is a concerted process: most mutant strains deficient for the expression of a major protein subunit present a pleiotropic loss of the whole set of subunits of the complex. Two main mechanisms are responsible for this concerted accumulation in the chloroplast of *C. reinhardtii* (reviewed in Wollman *et al*, 1999; Choquet and Vallon, 2000). Many unassembled subunits undergo a rapid proteolytic degradation but others show an assembly-dependant regulation of their rates of synthesis. We have defined this assembly-dependent regulatory process as a 'control by epistasy of synthesis' or CES process (Wollman *et al*, 1999; Choquet and Vallon, 2000). In the case of cytochrome *f*, encoded by the chloroplast *petA* gene, we were able to characterise the molecular mechanism underlying the CES process as an autoregulation of *petA* mRNA translation (Choquet *et al*, 1998) that involves negative feedback from the C-terminal domain of the unassembled polypeptide (Choquet *et al*, 2003).

In the present study, we provide evidence that assembly-dependent autoregulation of translation initiation may be a central mechanism in the biogenesis of chloroplast oligomeric proteins in *C. reinhardtii*. Photosystem I (PSI) is a thylakoid-embedded pigment–protein complex that performs light-induced charge separation and drives electron transfer from plastocyanin to ferredoxin. In *C. reinhardtii*, PSI is made of 13 subunits: PsaA, B, C and J are chloroplast-encoded, whereas PsaD, E, F, G, H, K, L, N and O are nucleus-encoded (for a review, see Webber and Bingham, 1998). The core components of PSI are two large transmembrane subunits, PsaA and PsaB, which share strong sequence similarity and may have arisen from gene duplication (reviewed in Baymann *et al*, 2001). Both subunits comprise 11 transmembrane helices. The N-terminus of each subunit faces the chloroplast stroma, whereas their C-termini protrude on the lumenal side of the thylakoid (Sun *et al*, 1997). PsaA and

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PsaB assemble at an early step of PSI biogenesis, forming the chlorophyll *a*–protein complex I (CPI) that binds most of the pigments and redox cofactors of PSI. CPI is the template for assembly of the extrinsic PsaC subunit on the stromal side of the membranes. PsaC, a 9 kDa polypeptide, coordinates the Fe–S clusters F_A and F_B through two cysteine-rich domains. The stromal subunits PsaD and PsaE then assemble coordinately around PsaC (Yu *et al*, 1995).

PSI mutants lacking either PsaA, PsaB or PsaC display the same severe drop in the accumulation of all PSI subunits, demonstrating that accumulation of these proteins is a concerted process. Strains deleted for *psaC* show wild-type rates of synthesis of PsaB and PsaA, which are then rapidly degraded (Takahashi *et al*, 1991). In contrast, *psaA* mutants show wild-type rates of PsaB synthesis (Girard-Bascou *et al*, 1987; Goldschmidt-Clermont *et al*, 1990) but reduced rates of PsaC synthesis (Takahashi *et al*, 1991). Finally, mutants lacking expression of PsaB show no detectable PsaA synthesis, whether the strains contain mutations in the chloroplast *psaB* gene itself (Girard-Bascou *et al*, 1987), or in the nuclear *TAB1* gene, which is required for *psaB* mRNA translation initiation (Stampacchia *et al*, 1997). A chloroplast mutation in the *psaB* 5′ untranslated region (UTR) suppresses the effect of this nuclear defect and restores translation of both PsaB and PsaA subunits, arguing for a role of PsaB availability in PsaA translation (Stampacchia *et al*, 1997).

In the present work, we used chimeric genes expressing reporter proteins translated under the control of *psaA* or *psaC* 5′ UTRs to provide evidence that the biogenesis of PSI involves a cascade of autoregulation of translation, most likely at the level of initiation, mediated by the unassembled CES subunits PsaA or PsaC.

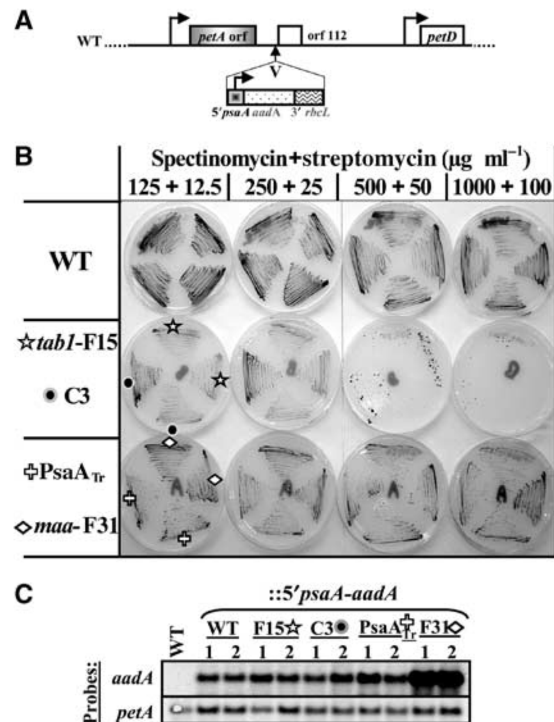

Figure 1 The *psaA* 5′ UTR confers a PsaB-dependent expression to the reporter gene *aadA*. (**A**) Schematic map of the *petA-petD* chloroplast region where the 5′*psaA-aadA* chimeric gene has been inserted, in direct orientation with respect to the *petA* gene, at the neutral *Eco*RV site (V). ↱ indicates transcription start sites. (**B**) Growth of independent transformants, derived from the recipient strains listed in the left, in the presence of increasing concentrations of antibiotics. The $PsaA_{Tr}$ and *maa*-F31 strains are devoid of PsaA expression (see later). (**C**) Accumulation of the chimeric *aadA* (and *petA*, as a loading control) mRNAs in the wild-type strain and in some of the transformants presented in panel B.

Results

Assessment of regulation of translation initiation in the CES behaviour of PsaA, using the aadA reporter gene

One of the two major reaction centre (RCI) subunits of PSI, PsaA, is a CES subunit since it displays a reduced rate of synthesis in the absence of the other RCI subunit, PsaB. This may result from regulation of translation initiation, controlled by the 5′ UTR of the *psaA* gene. To test that hypothesis, we constructed a chimeric gene bearing the *psaA* 5′ UTR fused immediately upstream of the bacterial *aadA* gene coding sequence, which confers resistance to the antibiotics spectinomycin and streptomycin (Goldschmidt-Clermont, 1991). By biolistic transformation, this chimeric gene was inserted downstream of the chloroplast *petA* gene in the wild type and in mutant strains unable to accumulate PsaB (Figure 1A). We chose the *tab1*-F15 nuclear mutant strain, which is deficient for translation of the *psaB* messenger (Stampacchia *et al*, 1997), and the chloroplast mutant strain C3 (Girard-Bascou *et al*, 1987), which expresses a truncated version of PsaB that is rapidly degraded (see Supplementary Figure I). Transformants were recovered from each recipient strain on spectinomycin ($100\,\mu g\,ml^{-1}$)–Tris-acetate-phosphate (TAP) plates. The level of antibiotic resistance conferred by the chimeric gene was determined by plating the transformants on TAP medium supplemented with increasing concentrations of antibiotics, as illustrated in Figure 1B (see also Supplementary Figure II and Supplementary Table I). While the chimeric gene allowed the growth of strains derived from the wild type on antibiotic concentrations higher than $1000\,\mu g\,ml^{-1}$ of spectinomycin plus $100\,\mu g\,ml^{-1}$ of streptomycin, transformants devoid of PsaB (*tab1*-F15 and C3 in Figure 1B) no longer grew when the concentration of antibiotics reached $500+50\,\mu g\,ml^{-1}$ of spectinomycin + strepto-streptomycin. Since the chimeric 5′*psaA-aadA* mRNAs accumulated to the same level in all transformed strains (Figure 1C), these results point to a specific downregulation of translation of the *psaA* 5′ UTR-driven *aadA* gene when expressed in the absence of PsaB.

We could exclude that the reduced resistance to antibiotics observed in strains lacking PsaB was a mere consequence of PSI deficiency by transforming mutant strains lacking expression of PsaA with the same chimeric gene: the transformed strains that were recovered, although deficient for PSI accumulation, presented the same growth properties on TAP–antibiotics plates as those derived from the wild-type recipient strain ($PsaA_{Tr}$ and *maa*-F31 in Figure 1B and C).

The psaA 5′ UTR is sufficient to confer a PsaB-dependent rate of synthesis to the cytochrome f reporter protein

In the above experiments however, we monitored only indirectly expression of the chimeric *5′psaA-aadA* gene through resistance of the transformed strains to antibiotics. As an alternative, we constructed another chimeric gene, 5′*psaA*-

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petA, allowing cytochrome *f* to be translated under the control of the *psaA* 5′ UTR (Figure 2A). We introduced the chimera in the chloroplast genome of the Δ*petA* strain (Table I). Recovery of phototrophic transformants aA*f* indicated that the *psaA* 5′ UTR allowed high enough expression of cytochrome *f* to sustain photoautotrophic growth.

Strain aA*f* was then crossed to the *tab1*-F15 nuclear mutant strain that lacks PsaB. Each member of the resulting tetrads carried the chloroplast chimeric gene, uniparentally transmitted from the *mt*+ parent aA*f*. Half of them (indicated by * in Figure 2B–D) had fluorescence transients typical of impaired PSI activity (Chua *et al*, 1975) (data not shown) and harboured the nuclear *tab1* mutation, which shows Mendelian segregation; the other half inherited a wild-type nuclear genome. Therefore, analysis of cytochrome *f* expression among tetrad progeny by RNA hybridisation, protein pulse labelling and immunodetection with specific antibodies allowed us to compare the expression of the chimeric gene in the presence or absence of the PsaB subunit. We observed a 2:2 segregation in cytochrome *f* expression, in agreement with the above experiments using *aadA* as a reporter: the *tab1* progeny exhibited a drastic decrease in the accumulation of the reporter protein, reaching only 10–15% of that observed in the parental strain aA*f* or in the progeny with a wild-type nuclear genome (a preliminary report of this experiment has been presented at the 673rd meeting of the Biochemical Society; Choquet *et al*, 2001) (Figure 2C). This resulted from a decrease in translation of the 5′*psaA-petA* reporter gene (Figure 2D). However, the chimeric messenger, which migrates faster than wild-type *petA* mRNA, was less accumulated in the *tab1* offspring from aA*f* × *tab1*-F15 crosses (Figure 2B). In contrast, the *psaA* mRNA was similarly accumulated in all progeny, even those lacking detectable PsaA product (*tab1* progeny).

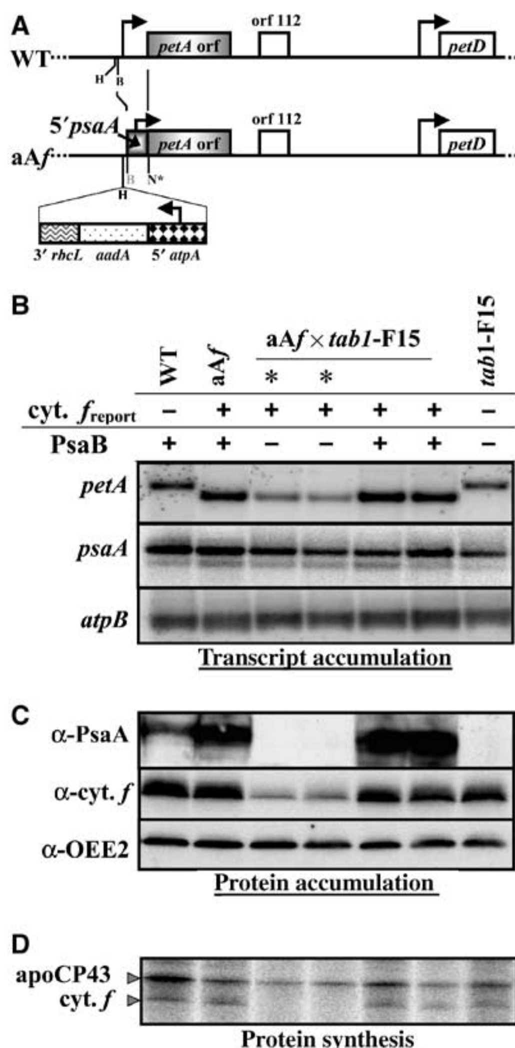

Figure 2 The CES behaviour for the PsaA subunit corresponds to a translational regulation mediated by the *psaA* 5′ UTR. (**A**) Map of the chloroplast *petA* gene in wild-type and aA*f* strains. Relevant restriction sites are indicated: B, *Bgl*II; N*, an *Nco*I site introduced by site-directed mutagenesis around the *petA* initiation codon for cloning purposes; H, *Hin*cII. (**B**) Accumulation of *petA*, *psaA* and *atpB* (as a loading control) transcripts in a representative tetrad progeny (out of seven) of the cross aA*f* × *tab1*-F15 and from parental and wild-type strains, revealed by hybridisation to *petA*, *psaA* (exon 3) and *atpB*-specific probes. (**C**) Accumulation of cytochrome *f*, PsaA and OEE2 (as a loading control), detected with specific antibodies on whole-cell proteins extracted from those strains. Lack of PsaA signs the *tab1* progeny (marked by *). (**D**) Synthesis of cytochrome *f*, determined by 5 min pulse labelling with [^{14}C]acetate in the presence of 8 μg ml^{-1} cycloheximide preventing cytosolic synthesis, in the same strains. Positions of cytochrome *f* and CP43 (providing an incorporation and loading control) are marked.

Since RNA stability determinants can be found in coding sequences (Singh *et al*, 2001), we reproduced the same set of experiments with a third chimeric gene, 5′*psaA*$_C$-*petA*, which contained an extension of 60 nucleotides (nt), corresponding to the first 20 residues of the PsaA protein fused in-frame with the *petA* coding sequence, in addition to the promoter and 5′ UTR of *psaA*. After transformation of the Δ*petA* strain, phototrophic transformants aA$_C$*f* were recovered and crossed to the *tab1*-F15 mutant strain. The expression of cytochrome *f* was similar in strains *aA*$_C$*f* and aA*f* and showed the same decrease in expression in the *tab1* progeny of the crosses. With this construct, the drop in the rate of expression of the reporter protein in the absence of PsaB was not accompanied by any change in the mRNA level (see Supplementary Figure III).

Thus we conclude that both the *aadA* gene product and cytochrome *f* reporters, expressed under control of the 5′*psaA* UTR, showed a drop in translation in the absence of PsaB. In the subsequent experiments, we chose to use the chimeric gene 5′*psaA-petA*, because it contains only sequences from the *psaA* 5′ UTR.

In order to rule out the possibility that the decrease in expression of the chimeric gene in the absence of PsaB resulted from a pleiotropic effect of the *tab1* mutation on translation of both *psaA* and *psaB* messengers, we used the chloroplast mutant strain C3, which expresses a truncated PsaB, but has a wild-type nuclear genome. Upon transformation with the chimeric gene 5′*psaA-petA*, the resulting strains, hereafter named {C3, aA*f*}, were used in pulse-labelling experiments. We observed a low rate of cytochrome *f* translation in all {C3, aA*f*} transformants tested when compared to the aA*f* strain (Figure 3C), similar to that observed in the aA*f*, *tab1* progeny indicated by * in Figure 2D.

Together, these experiments proved that the absence of PsaB causes decreased expression of not only *psaA* but also of chimeric genes translated under the control of the *psaA* 5′ UTR. Therefore, the CES regulation in *psaA* expression occurs

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Table I Transformation experiments

Transformed strains	Recipient strains[a]	Plasmid used	Selection
WT::aAK	WT (Sp^S)	p*f*aAK	Spec. resistance
F15::aAK	*tab1*-F15 (Sp^S), (1)	p*f*aAK	Spec. resistance
C3::aAK	C3 (Sp^S), (2)	p*f*aAK	Spec. resistance
aA*f*	Δ*petA* (Sp^R), (3)	pKaA*f*	Phototrophy
aA_Cf	Δ*petA* (Sp^R), (3)	$pKaA_Cf$	Phototrophy
{C3, aA*f*}	C3 (Sp^S), (2)	pKaA*f*	Spec. resistance
$PsaA_{Tr}$ [b1]	WT (Sp^S)	pK_rPsaA_{Tr}	Spec. resistance
{$PsaA_{Tr}$, aA*f*}	$psaA_{Tr}$ (Sp^S)[b2]	pKaA*f*	Spec. resistance
aC*f*	WT (Sp^S)	pKaC*f*	Spec. resistance
{Δ*psaC*, aC*f*}	Δ*psaC* (Sp^S) (4)	pKaC*f*	Spec. resistance

[a]All recipient strains were *mt*+, and either resistant (Sp^R) or sensitive (Sp^S) to spectinomycin. (1) Stampacchia *et al* (1997), (2) Girard-Bascou *et al* (1987), (3) Kuras and Wollman (1994), (4) Takahashi *et al* (1991).
[b1]The $PsaA_{Tr}$ strain was initially selected for spectinomycin resistance due to the presence of the recycling *aadA* cassette. [b2]After excision of the cassette according to Fischer *et al* (1996), the strain became Sp^S and was used as a recipient strain for a second round of transformation with plasmid pKaA*f*, based on selection for spectinomycin resistance.

before translation elongation or before cotranslational degradation of the nascent PsaA polypeptide, which would both depend on the coding sequence. Rather, some early step in the initiation process is regulated, either an activation step prior to initiation, translation initiation itself or a transition step between initiation and elongation of translation.

Downregulation of PsaA expression in the absence of PsaB is due to autoregulation of translation

In the absence of the PsaB protein, downregulation of *psaA* translation could occur either because PsaB would act as an activator for PsaA translation ('transactivation' hypothesis; Figure 4, right panel) or because unassembled PsaA, which may accumulate to some extent, would expose a translational repressor domain (autoregulation hypothesis; Figure 4, left panel). Because the *psaA* 5′ UTR was sufficient to confer the PsaA CES behaviour to chimeric genes, these two hypotheses could be discriminated by looking at the expression of the 5′*psaA*-driven cytochrome *f* reporter in the absence of both the PsaA and PsaB subunits (Figure 4, bottom). In the case of a transactivation hypothesis, the absence of the positive regulator brought along with the PsaB subunit should result in poor expression of the chimeric gene, either in the presence or in the absence of PsaA. By contrast, in the autoregulation hypothesis, a strain lacking the repressor domain carried by the unassembled CES protein, PsaA, should show a high expression of the chimera, even in the absence of the assembly partner PsaB.

We thus crossed the {C3, aA*f*} strain, which expresses the 5′*psaA-petA* reporter gene, but does not accumulate PsaB, because it expresses only a truncated and unstable polypeptide, with the *maa*-F31 strain, defective for PsaA expression. This mutant is impaired in *trans*-splicing of precursor transcripts carrying exon 1 and exon 2 of *psaA*. Therefore, it lacks full-length *psaA* mRNA but accumulates the exon 1 precursor RNA (Goldschmidt-Clermont *et al*, 1990). As a preliminary control, we first checked that the *maa* mutation did not prevent expression of the chimeric gene by crossing the aA*f* strain with the *maa*-F31 strain. The rates of synthesis and accumulation of the reporter cytochrome *f* were identical in all tetrad progeny (see Supplementary Figure IV). Thus cytochrome *f* expressed from the chimeric 5′*psaA-petA* gene remains insensitive to the *maa* nuclear mutation.

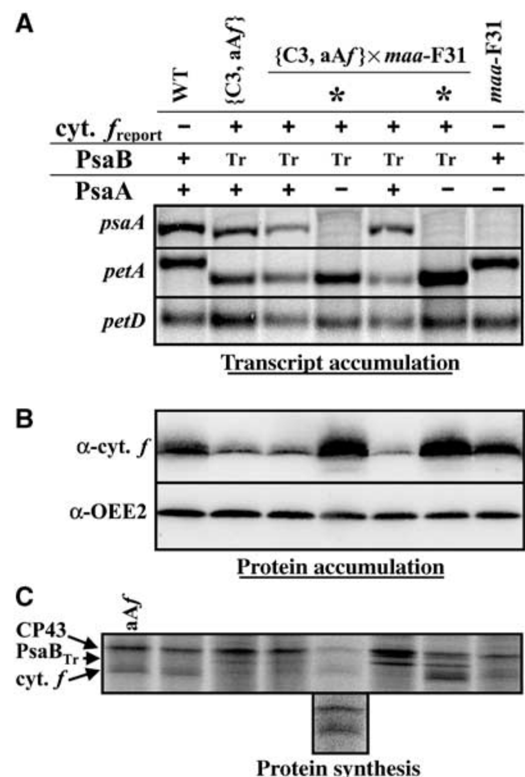

Figure 3 The expression of the 5′*psaA-petA* reporter gene is no longer repressed in the absence of PsaB when the endogenous PsaA subunit is lacking. (**A**) Accumulation of *petA*, *psaA* and *petD* (as a loading control) transcripts in progeny from the cross {C3, aA*f*} × *maa*-F31 and in wild-type and parental strains, detected with probes specific for *petA*, *psaA* (exon 1) and *petD*. Absence of mature *psaA* mRNA signs the *maa* progeny, designated by *. (**B**) Accumulation and (**C**) translation of cytochrome *f* in the same strains. OEE2 accumulation provides a loading control. In (C), the translation of the 5′*psaA-petA* chimeric gene in the presence of PsaB (lane aA*f*) is shown for comparison. (Inset) Longer exposure of the gel for the second progeny of the tetrad, which incorporated poorly radiolabelled ^{14}C. Positions of neosynthesised CP43, truncated PsaB (Tr) and cytochrome *f* are indicated. In the two progeny with a wild-type genome, ^{14}C incorporation is lower in cytochrome *f* than in CP43, while it is higher in the *maa* progeny.

Analysis of cytochrome *f* expression in one out of five tetrads from the cross {C3, aA*f*} × *maa*-F31 is shown in Figure 3A–C. Each tetrad progeny harboured both the chi-

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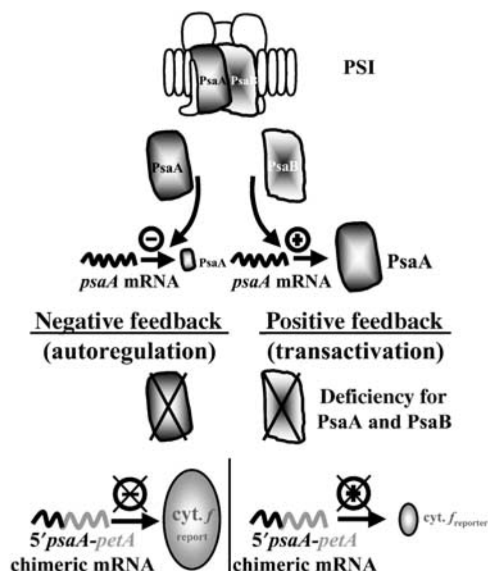

Figure 4 The two models for the repression of *psaA* mRNA translation in the absence of the dominant protein PsaB can be discriminated by looking at the expression of a 5′*psaA*-driven reporter gene in the absence of both PsaA and PsaB.

meric gene, as demonstrated by the higher mobility of *petA* mRNAs (Figure 3A), and the *psaB* mutation, as revealed by the synthesis of a truncated PsaB polypeptide (Figure 3C). Only half of the progeny, identified by the absence of mature *psaA* mRNA (Figure 3A), inherited the *maa* nuclear mutation and are indicated by * in Figure 3. The two progeny with a wild-type nuclear genome were similar to the parental strain {C3, aA*f*}: they showed low synthesis and accumulation of the *psaA*-driven cytochrome *f* (Figure 3B and C) due to the absence of PsaB. In contrast, the *maa* progeny, which express neither full-length PsaB nor PsaA, recovered a high expression of the reporter gene, similar to that observed in the aA*f* strain.

Therefore, translation of the 5′*psaA*-driven cytochrome *f* is no longer decreased in the absence of the PsaB subunit, when the CES protein PsaA cannot accumulate in the thylakoid membranes. These experiments strongly support an autoregulation of *psaA* translation rather than a transactivation hypothesis.

A truncated PsaA polypeptide escapes the psaA CES control

We noted that accumulation of chimeric 5′*psaA-petA* transcripts was higher in the *maa* nuclear background (Figure 3A). This has been observed previously with other 5′*psaA*-containing transcripts in various *psaA trans*-splicing mutants, for example with the *psaA* exon 1 precursor itself (Choquet *et al*, 1988). The high expression of the 5′*psaA*-driven cytochrome *f* in the *maa* progeny from the cross {C3, aA*f*}, observed in the above experiment, may be due to the overaccumulation of the chimeric messenger. In addition, some nucleus-encoded factors, required for the translation of *psaA* mRNA, may not be able to bind to the precursor transcript of *psaA* exon 1 and thus become fully available for translation of the sole chimeric 5′*psaA-petA* mRNA. For a critical assessment of these alternative hypotheses, we caused a premature termination of translation 155 residues after the initiation methionine by introducing a frameshift in the third exon of *psaA*, thereby preventing accumulation of full-length PsaA (Figure 5A). In this case, the putative nuclear regulatory factors participate normally in the translation of a truncated *psaA* product, which should be rapidly degraded because of its inability to assemble into a PSI complex.

A two-step procedure was used to introduce both the *psaA* frameshift and the 5′*psaA-petA* reporter into the same strain. We first associated the *psaA* mutation with a 'recycling' spectinomycin resistance cassette that allowed us to select transformants on spectinomycin-supplemented TAP medium before losing specifically the cassette—but not the mutant *psaA* allele—once the selective pressure is released (Fischer *et al*, 1996). The resulting strains, $PsaA_{Tr}$, were screened for PSI deficiency by fluorescence kinetics and showed normal accumulation of the mutated *psaA* messenger (data not shown). A truncated PsaA polypeptide of about 16 kDa was detected by a pulse-labelling experiment, between two CF0 subunits of the ATP synthase (Lemaire and Wollman, 1989). Its rate of synthesis—as quantified by a PhosphorImager scan of the ^{14}C labelling of the gel and corrected for the number of carbon atoms—is similar to that of the PsaB and PsaA polypeptides in a wild-type strain. In a pulse-chase experiment (Figure 5B), the truncated form of PsaA proved to be very unstable, with a half-life of less than 10 min. Therefore, it does not accumulate to any significant extent in thylakoid membranes.

Next, we allowed spontaneous excision of the recycling cassette from the chloroplast genome of $PsaA_{Tr}$ transformants, and used them as recipient strains for a second round of transformation with the 5′*psaA-petA* reporter gene, associated with a new spectinomycin resistance cassette. Transformants {$PsaA_{Tr}$, aA*f*}, selected on antibiotic-supplemented TAP medium, were crossed with the nuclear mutant strain *tab1*-F15 to compare expression of the reporter gene in the absence of PsaA alone or in the absence of both PsaA and PsaB. All tetrad progeny from that cross inherited the *psaA* 5′ UTR-driven cytochrome *f* (Figure 5C) and the truncated *psaA* allele carried by the chloroplast genome, because of the uniparental inheritance from the mt+ parent (Figure 5E). Half of the progeny inherited the *tab1* nuclear mutation and were identified in pulse-labelling experiments by their lack of PsaB synthesis (Figure 5E), while the other half had a wild-type nuclear genome and translated PsaB normally. The rate of synthesis of the truncated PsaA polypeptide was identical in the four progeny ($PsaA_{Tr}$, indicated by a dot in Figure 5E). Therefore, the translation of this truncated and unstable PsaA was no longer dependent on the presence of PsaB. Similarly, the 5′*psaA*-driven *petA* reporter gene was expressed at the same high level in all progeny of the cross {$PsaA_{Tr}$, aA*f*} × *tab1*-F15 (Figure 5D and E), in contrast to what was observed among the progeny of the cross aA*f* × *tab1*-F15 (where translation was repressed in the *tab1* members of the tetrad; Figure 2D). Therefore, when PsaA cannot accumulate in thylakoid membranes, translation of the 5′*psaA-petA* transcript remains high, even in the absence of the assembly partner, PsaB. That the presence of PsaB does not stimulate any 5′*psaA*-driven translation fully excludes the transactivation hypothesis. Our data point to an autoregulation of translation where unassembled PsaA exerts a negative feedback on the translation of *psaA* mRNA.

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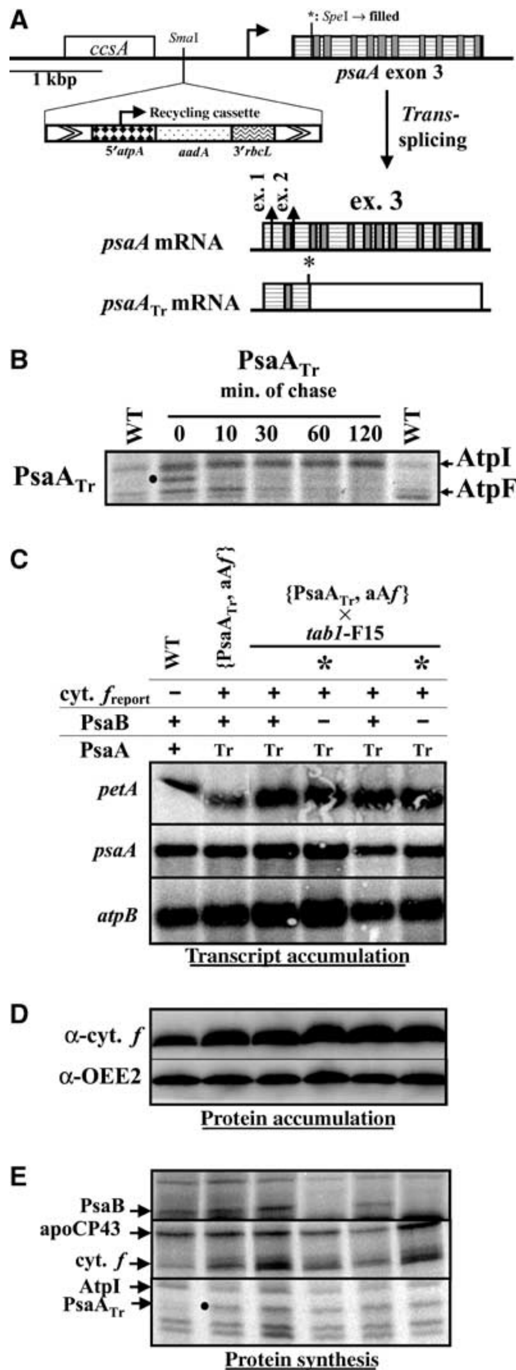

Figure 5 Expression of the 5′*psaA-petA* reporter gene in strains expressing a truncated PsaA. (**A**) Strategy used to introduce a mutation (*) in the third exon of *psaA*. Relevant restriction sites are shown, as well as transmembrane helices coding regions (grey boxes). Coding sequences are indicated by horizontal hatches. Due to the mutation *, most of the *psaA* mRNA (after *trans*-splicing) is not translated in the mutant strain (white rectangle). (**B**) Pulse labelling of the $PsaA_{Tr}$ strain (time 0) followed by a chase for the indicated times in the presence of an excess of nonradioactive acetate and 200 μg ml^{-1} chloramphenicol. The position of truncated PsaA is marked with a dot. As already described (Delepelaire, 1983), the AtpF protein is short-lived. (**C**) Accumulation of *petA*, *psaA* and *atpB* (as a loading control) transcripts in a representative tetrad from the cross {$PsaA_{Tr,}$ aA*f*} × *tab1*-F15 and in wild-type and {$PsaA_{Tr,}$ aA*f*} strains, detected using probes specific for *petA*, *psaA* (exon 1) and *atpB*. (**D**) Accumulation of cytochrome *f* (and OEE2 as a loading control) in the strains. (**E**) Chloroplast translates in the same strains. Lack of PsaB synthesis signs the *tab1* tetrad progeny, designated by *.

Synthesis of the PsaC CES subunit is controlled at the level of translation initiation

As indicated in Introduction, PSI contains another CES protein, PsaC, whose synthesis is decreased in the absence of PsaA. To determine whether translation initiation of PsaC was indeed regulated by the availability of PsaA, we constructed a 5′*psaC-petA* gene (including the first 30 nt of PsaC coding sequence), associated with a spectinomycin resistance cassette (Figure 6A). Transformants containing this reporter, named aC*f*, were selected for growth on spectinomycin–TAP plates and were found to grow phototrophically despite accumulating only ∼10% of the wild-type level of cytochrome *f* (see Figure 6C, lane aC*f*). This low expression of cytochrome *f* when driven by the *psaC* 5′ UTR prevented its detection in pulse-labelling experiments.

To study the expression of the chimeric 5′*psaC-petA* gene in the absence of PsaA, we crossed the aC*f* strain with the *maa*-F31 strain. The whole progeny carried the chloroplast chimeric gene, uniparentally transmitted, as demonstrated by the faster migration of its transcript upon electrophoresis (Figure 6B). Half of the progeny, identified by the lack of mature *psaA* mRNA (Figure 6B), inherited the *maa* nuclear mutation (indicated by * in Figure 6). Cytochrome *f* accumulation was nearly undetectable in those progeny (Figure 6C), whereas offspring harbouring a wild-type nuclear genome displayed the same accumulation of cytochrome *f* as did the parental strain aC*f*. Since RNA hybridisation analysis showed

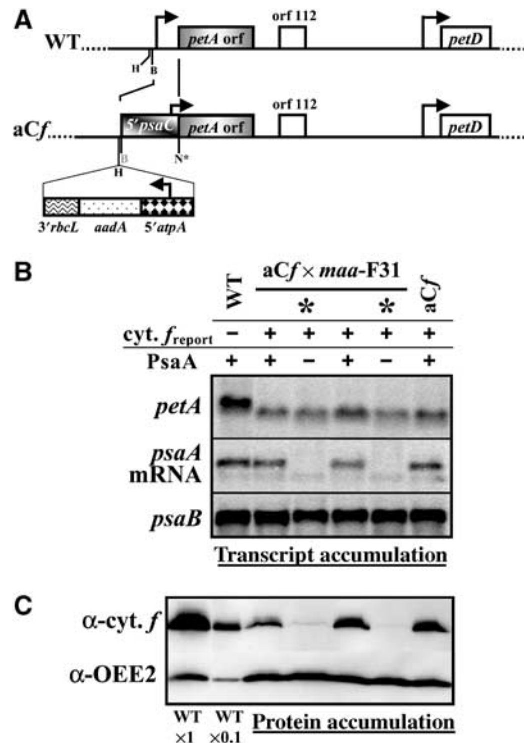

Figure 6 The CES behavior for the RCI subunit PsaC corresponds to a translational regulation mediated by the *psaC* 5′ UTR. (**A**) Maps of the *petA* gene in wild-type and aC*f* strains. Same conventions as in Figure 2A. (**B**) *petA*, *psaA* and *psaB* (as a loading control) transcripts accumulation in a tetrad of the cross aC*f* × *maa*-F31 and in wild-type and parental strains, detected using probes specific for *petA*, *psaA* (exon 3) and *psaB*. Progeny lacking the mature *psaA* transcript (indicated by *) bear the nuclear *maa* mutation. (**C**) Cytochrome *f* (and OEE2, as a loading control) accumulations in the same strains.

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no correlation in the tetrad progeny between changes in chimeric mRNA content and changes in cytochrome *f* accumulation (Figure 6B and C), we conclude that translation of the chimeric 5′*psaC-petA* gene was decreased in the absence of the PsaA subunit. Thus, the *psaC* 5′ UTR is able to confer CES behaviour of the PsaC subunit on a *petA* reporter gene. PsaC is a bona fide CES subunit, whose synthesis is regulated at some early step of the translation process.

The decreased PsaC synthesis is due to autoregulation of translation mediated by the unassembled PsaC subunit

To test the mechanism of PsaC translational downregulation in the absence of the PsaA protein, we followed the same rationale as we did for PsaA. Expression of the 5′*psaC-petA* gene was examined in the absence of both PsaA, here the assembly partner, and PsaC, the CES subunit.

We introduced the chimeric 5′*psaC-petA* gene, associated with the spectinomycin resistance cassette, in the chloroplast genome of a Δ*psaC* deletion strain (Takahashi *et al*, 1991), in the place of the endogenous *petA* gene. Transformants {Δ*psaC*, aC*f*} were selected for spectinomycin resistance and crossed to the *maa*-F31 nuclear mutant. All progeny that lacked PsaC, but had either a wild-type nuclear genome or a *maa* mutant background (indicated by *, see Figure 7A), displayed the same rates of cytochrome *f* synthesis and accumulation (Figure 7B and C) as the parental strain {Δ*psaC*, aC*f*}. It amounted up to about 50% of the wild-type rate. This is a much higher rate than that observed in transformants expressing the chimeric gene in the presence of the endogenous PsaC protein (about 10% of the wild-type level; Figure 7D).

The expression of the chimeric 5′*psaC-petA* gene is thus increased in strains lacking PsaC, independent of the assembly partner PsaA. This excludes the transactivation hypothesis, where the PsaA subunit would activate *psaC* translation. Rather, PsaC translation is autoregulated by its ability to assemble with PsaA.

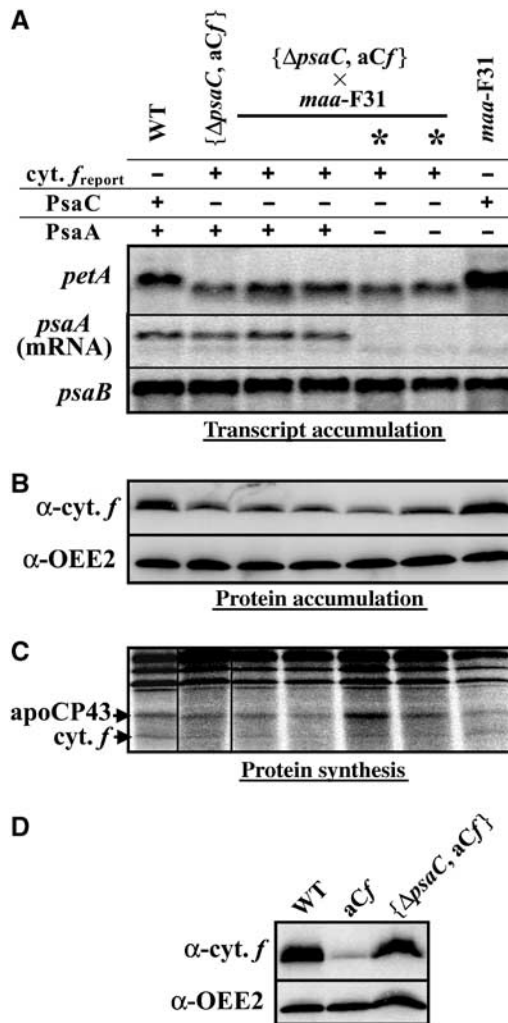

Figure 7 : Cytochrome *f* translated under the control of the *psaC* 5′ UTR is no longer repressed in the absence of the PsaA subunit, when PsaC is lacking. (**A**) Accumulation of *petA*, *psaA* and *psaB* (as a loading control) transcripts in a representative tetrad (out of six) from the cross {Δ*psaC*, aC*f*} × *maa*-F31 and in the wild-type and parental strains. * designates the *maa* members of the tetrad, lacking *psaA* messenger. (**B**) Cytochrome *f* and OEE2 (as a loading control) accumulation in the same strains. (**C**) Rate of translation of the chimeric gene 5′*psaC-petA* in these strains. The positions of the neosynthesised cytochrome *f* and CP43 (as a loading and incorporation control) are indicated. The lane {Δ*psaC*, aC*f*} is boxed because it originates from another region from the same gel. (**D**) Cytochrome *f* expressed from the 5′*psaC-petA* chimeric gene (normalised to that of the loading control OEE2) accumulates to 9 and 54% of the wild-type level, respectively, in strains expressing (aC*f*) or lacking PsaC ({Δ*psaC*, aC*f*}).

Discussion

Critical assessment of the reporter constructs used to study the CES process

Our attempt to characterise the molecular mechanism of the CES processes at work in PSI biogenesis critically depends on the expression of reporter proteins, translated under the control of the 5′ UTR of putative CES genes, in *C. reinhardtii* chloroplasts. The expression of these 5′ UTR CES-driven reporters should faithfully reflect the behaviour of the original subunits. We used two constructs, one expressing the aminoglycoside adenine transferase (*aadA*), a heterologous, soluble protein from *Escherichia coli*, and the other cytochrome *f*, a thylakoid-bound protein from *C. reinhardtii*. The level of *aadA* translation can be measured only indirectly through the level of resistance to antibiotics. Indeed, changes in the level of expression of a resident CES protein can be mimicked with *aadA* when driven by the corresponding CES 5′ UTR (this study and Choquet *et al*, 1998). In order to measure directly changes in the rates of protein synthesis, we also used cytochrome *f*, which is particularly well suited for use as a reporter protein: (i) At variance with other reporters, such as *aadA* gene product, GUS or GFP, its rate of translation can be accurately determined *in vivo* by pulse-labelling experiments and its accumulation can be measured by immunodetection with specific antibodies. (ii) Whether assembled or not into the cytochrome b_6f complex, cytochrome *f* is a very stable protein (Kuras and Wollman, 1994; Choquet *et al*, 1998). Its accumulation is therefore a faithful measure of its rate of translation, even when the latter is too low to be measured directly. (iii) Finally, cytochrome *f*, synthesised as a precursor protein with a lumen-targeting sequence of 31 amino acids, can accommodate, at least, 35 extra amino-acid residues (Y Choquet and K Wostrikoff, unpublished observations). As the presequence is cleaved

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after membrane insertion, these extra residues are not part of mature cytochrome *f* and do not alter its stability.

The fact that cytochrome *f* is itself a CES protein (Choquet *et al*, 1998) does not interfere with the study of other CES processes since it is conferred only by the 5′ UTR of the *petA* gene, which is no longer present in the reporter constructs. Indeed, the two reporter genes used in this study (*petA* and *aadA*) gave similar results.

CES processes in the biogenesis of PSI are born by the 5′ UTR of PsaA and PsaC

Previous work clearly established that PsaA was a CES protein, which requires the presence of the PsaB protein to be synthesised at a wild-type level (Girard-Bascou *et al*, 1987; Stampacchia *et al*, 1997). Here we show that the *psaA* 5′ UTR is sufficient to confer a PsaB-dependent rate of synthesis to the *aadA* reporter gene. In a previous study, Stampacchia *et al* (1997) failed to observe a decrease in expression of the chimeric 5′*psaA-aadA* gene, in the absence of PsaB. However, the antibiotic concentration they used (1000 μg ml^{-1} of spectinomycin *alone*) was too low to observe the regulation. We confirmed our finding using *psaA*-driven *petA* genes. In preliminary experiments, we observed that indeed the loading of *psaA* mRNA on polysomes was drastically reduced (at least four-fold) in the C3 strain compared to the wild type. We observed a similar reduction in mRNA loading on polysomes in a Δ*psaB* deletion strain (data not shown). This appears conflicting with the observation of Dauvillée *et al* (2003) that *psaA* mRNA was only slightly less (70%) associated with polysomes in the *tab2*-F14 nuclear mutant strain, specifically deficient for the translation of the *psaB* mRNA. However, these results may be difficult to compare since different protocols were used for the isolation and analysis of polysome fractions in the two studies.

Any significant contribution of pretranslational steps to the regulation of *psaA* expression can be excluded because the accumulation of the endogenous *psaA* mRNA is not decreased in strains lacking PsaB, such as C3 or *tab1* strains, that show no detectable expression of the PsaA protein and because we found no decrease in the accumulation of the 5′*psaA-aadA* or 5′$psaA_C$-*petA* messengers in the absence of PsaB, despite their decreased expression. A correlation between accumulation and translation of the chimeric messenger was only observed with the 5′*psaA-petA* reporter gene. The 5′*psaA-petA* messenger could be less stable than *psaA* or *petA* transcripts and protected from degradation by translating ribosomes. The decreased accumulation of the 5′*psaA-petA* transcript in strains lacking PsaB would thus be an effect, rather than a cause, of the decreased synthesis of the *psaA*-driven cytochrome *f*.

We also demonstrated that the *psaC* 5′ UTR (together with the first 30 nt downstream of the *psaC* initiation codon) is sufficient to confer a PsaA-dependent regulation to the reporter protein cytochrome *f*. The 5′*psaC-petA* chimeric gene is less translated in the absence of PsaA than in its presence, with no changes in the mRNA level, as was confirmed by the drastic decrease of *psaC* mRNA loading on polysomes (data not shown). These experiments account for the observation by Takahashi *et al* (1991) that PsaC synthesis is decreased in mutants lacking PsaA. Since PsaC interacts with residues from both PsaA and PsaB in assembled PSI (Jordan *et al*, 2001), CPI, rather than PsaA alone, is likely to be the actual protein partner whose assembly with PsaC controls its rate of translation initiation.

CES processes in PSI biogenesis are due to autoregulation of translation mediated by unassembled PsaA and PsaC subunits

The PsaA and PsaB subunits are in close contact through their last five transmembrane helices in the PSI (Jordan *et al*, 2001). The downregulation of *psaA* translation in the absence of PsaB could be due to a negative feedback from a protein domain exposed by unassembled PsaA or it could result from the absence of a positive feedback by the PsaB subunit. The latter possibility can be excluded since the expression of the 5′*psaA-petA* reporter gene was high in strains lacking expression of both PsaA and PsaB, as was that of the truncated PsaA in strains lacking the PsaB protein. Translation of PsaA is therefore autoregulated by its unassembled form. Similarly, unassembled PsaC autoregulates its own synthesis since expression of the 5′*psaC*-driven *petA* reporter gene was stimulated—and not repressed—in the absence of both PsaC and PsaA subunits.

A critical point in these regulation mechanisms is the extent of accumulation that can be reached by the non-assembled *CES* proteins in the absence of their assembly partners. We definitely lack direct information of the concentrations of unassembled PsaA and PsaC subunits in strains lacking PsaB and PsaA, respectively. It proved difficult to assess the rate of PsaA synthesis in the absence of PsaB, since it migrates as a diffuse band in gels. Strains lacking expression of PsaB were found to accumulate less than 0.5% of wild-type levels of PsaA (Dauvillée *et al*, 2003). In a wild-type chloroplast, the accumulation of PSI (or PsaA) is in large excess over that of the *psaA* messenger (10^6 proteins versus 10^3 copies of the mRNA) (Rapp *et al*, 1992). Thus, the concentration of PsaA in strains devoid of PsaB, even reduced below detection level, remains probably high enough to prevent translation of *psaA* messenger. Unassembled PsaC could not be detected in the absence of PsaB (Boudreau *et al*, 1997), but the antibody failed to detect PsaC in a 20% dilution of wild-type cells. In the *tab1* mutant, which lacks PsaB, the two peripheral subunits PsaD and PsaE, which assemble coordinately with PsaC, still accumulate to about 20% (Boudreau *et al*, 1997), whereas they are completely absent in a Δ*psaC* deletion strain (Fischer *et al*, 1999). Therefore, strains lacking the PsaA/PsaB heterodimer probably accumulate some unassembled PsaC that would be associated with PsaD and PsaE in a protease-resistant form.

As the syntheses of the CES subunits PsaA and PsaC are autoregulated, we might expect an increased expression of the reporter genes in strains lacking the corresponding CES proteins, that is, either PsaA or PsaC, compared to a wild-type context. These strains should indeed be fully devoid of the repressor domain counteracting expression of the reporter gene, whereas in wild-type chloroplasts one may expect the presence of a subset of unassembled subunits, as was observed for the CES control of cytochrome b_6f biogenesis (Choquet *et al*, 1998). This was indeed observed for *psaC*, because the 5′*psaC-petA* reporter gene is more highly expressed in Δ*psaC* deletion strains than in a wild-type genetic context. However, in the case of PsaA, expression of the 5′*psaA-petA* reporter gene was similar in strains expressing or lacking PsaA, but dropped in the absence of PsaB. We

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conclude that the concentration of unassembled PsaA, available to repress its own translation, must be kept very low in the wild type. This subunit may bind up to 40 chlorophyll molecules, an efficient source of triplet chlorophyll, hence of damaging radicals, in the absence of energy transfer to the primary PSI donor P700.

The residues that form the PsaA or PsaC repressor domains have not been identified. For PsaA, they are likely located in the C-terminal part of the protein, which interacts with the corresponding region of PsaB. PsaA and PsaC are highly conserved from cyanobacteria to higher plants, especially in the C-terminal region of PsaA (Baymann *et al*, 2001). Since there is no evidence for a CES process in cyanobacteria (Choquet *et al*, 2003), it seems unlikely that these two proteins have evolved a protein domain capable of sequence-specific interactions with the 5′ UTR of their mRNA in *C. reinhardtii*. 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 RuBisCo. However, its RNA-binding property was not sequence specific (Yosef *et al*, 2004), a requirement if it is the cause of a CES behaviour. Therefore, we would favour a model based on an indirect interaction involving a ternary effector, capable of competitive binding to both the 5′ UTR of the transcript and the unassembled subunit, as we have previously proposed for cytochrome b_6f biogenesis (Wostrikoff *et al*, 2001; Choquet *et al*, 2003). Depending on its binding to the unassembled CES subunit, the ternary factor would modulate translation of the corresponding transcripts, thereby behaving as a translational activator. This class of nucleus-encoded factors is a major feature of chloroplast gene expression in *C. reinhardti* (Zerges, 2000). Recently, we identified a nuclear mutant specifically deficient for translation of *psaA* mRNA (unpublished results), the molecular characterisation of which is now in progress.

In the mitochondrion of the yeast *Saccharomyces cerevisiae*, it is likely that Cox1p is a CES subunit, because its apparent rate of synthesis decreases in mutants lacking Cox2p or Cox7p, with no decrease in the stability of the newly synthesised polypeptide (Calder and McEwen, 1991). Most interestingly, it was recently shown (Perez-Martinez *et al*, 2003) that one of the translational activators required for the expression of Cox1p, Mss51p, is also able to interact physically with newly synthesised Cox1p. The authors conclude that ‘Mss51p could tightly coordinate Cox1p synthesis with downstream events leading to cytochrome oxidase assembly’. These are the very properties one would expect for a CES ternary effector.

A CES cascade follows photosystem I assembly steps

The present study defines a ‘CES cascade’ among the chloroplast-encoded subunits of PSI: PsaB is required for significant PsaA synthesis, which, in turn, is required for PsaC translation (Figure 8). Whether the other chloroplast-encoded PSI subunit, PsaJ, is also a CES protein is unknown. This CES hierarchy (PsaB > PsaA > PsaC) is reminiscent of the sequence of polypeptide assembly during PSI biogenesis. Since the CES process for PSI subunits relies on autoregulation mediated by the unassembled CES subunits, the translation of a given subunit can reach significant levels only when the substrate for its assembly is present, that is, when the previous steps in the sequential assembly of PSI have been completed.

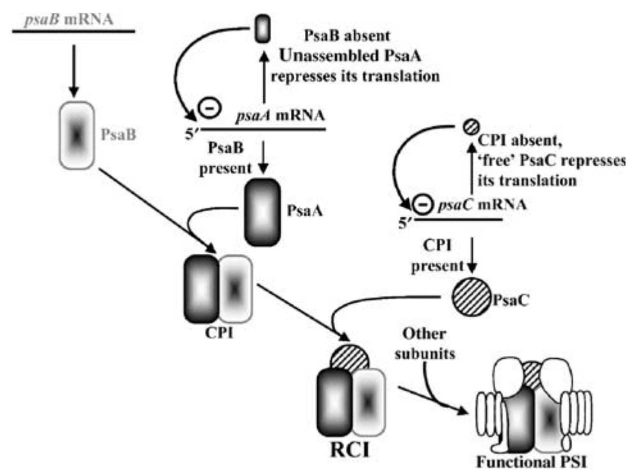

Figure 8 The CES cascade involved in PSI biogenesis.

Depending on light quality and physiological conditions, the amount of PSI (relative to PSII) may vary in chloroplasts (Chow *et al*, 1990). Translation, the major regulatory step of chloroplast gene expression in *C. reinhardtii* (Eberhard *et al*, 2002), is likely involved in this process. One benefit of the CES cascade over independent regulation of translation of each subunit is that only the rate of production of the most upstream subunit, PsaB, has to be controlled in order to determine the stoichiometric expression of all subunits of the protein complex.

Materials and methods

Media, culture conditions and strains

Wild-type and mutant strains of *C. reinhardtii* were grown on TAP medium, pH 7.2, at 25°C under dim light (5–6 $\mu E\,m^{-2}\,s^{-1}$). Recipient strains (all *mt*+) for chloroplast transformation experiments are listed in Table I. *mt*− parental strains *tab1*-F15 (Stampacchia *et al*, 1997) and *maa*-F31 (Goldschmidt-Clermont *et al*, 1990) were used for crosses, performed according to Harris (1989). All tetrad progeny were tested for a 2:2 segregation of mating types. Antibiotic resistance tests were performed according to Choquet *et al* (1998).

Plasmids and nucleic acid manipulations

Standard nucleic acid manipulations were performed according to Sambrook *et al* (1989). Details about the construction are presented in Supplementary data. Northern analyses were carried out as described previously (Drapier *et al*, 1998). DNA probes were as described by Eberhard *et al* (2002).

Transformation experiments

Cells were transformed by tungsten particle bombardment as previously described (Kuras and Wollman, 1994). Transformants were selected on spectinomycin-supplemented medium (100 $\mu g\,ml^{-1}$) under dim light (5–6 $\mu E\,m^{-2}\,s^{-1}$) or for photoautotrophic growth on minimum medium under high light (80 $\mu E\,m^{-2}s^{-1}$). After several rounds of subcloning, correct insertion of transforming DNA and homoplasmy were checked by RFLP analysis of specific PCR amplification products. At least three independent transformants were analysed for each transformation.

Protein isolation, separation and analysis

Pulse-labelling and pulse-chase experiments, protein isolation, separation and immunoblot analysis were carried out on cells grown to a density of 2×10^6 cells ml^{-1} as described by Kuras and Wollman (1994). Cell extracts were loaded on an equal chlorophyll

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basis. Quantification of protein synthesis or accumulation was performed according to Choquet *et al* (2003).

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

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