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The Assembly of Photosynthetic Proteins

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Abstract:

Photosynthetic energy transduction is performed by oligomeric proteins embedded in the thylakoid membranes. With the exception of the antenna complexes, which are exclusively comprised of nucleus-encoded subunits, all photosynthetic complexes contain both nucleus- and chloroplast-encoded subunits. Thus, the two genetic compartments cooperate to produce these subunits in stoichiometries required for their functional assembly. The nucleus-encoded subunits of a complex are translated in the cytosol, imported into the chloroplast and then targeted to their site of assembly with subunits translated in the chloroplast. Assembly factors assist in folding these polypeptide subunits into an assembly-competent conformation. The pleiotropic loss of all subunits of a photosynthetic protein in *Chlamydomonas* mutants lacking expression of only one of the subunit has indicated that the assembly of each protein complex results from the concerted accumulation of the constituent subunits, which involves two major regulatory processes: proteolytic degradation of some unassembled subunits, and a down-regulation of translation of other chloroplast-encoded subunits when they are not properly assembled. This latter process has been designated CES (for Control by Epistasy of Synthesis). CES is a versatile regulatory process with modalities that may differ from one CES subunit to another, depending on the features associated with the biogenesis of the specific photosynthetic complex.

Here we focus mainly on those studies in which *Chlamydomonas* has contributed new knowledge to the assembly of photosynthetic proteins. Thus, most of this chapter is devoted to the CES process because this microalga has been instrumental in its discovery, but later studies have shown that this process is key to the biogenesis of energy-transducing complexes in higher plant chloroplasts and yeast mitochondria. It probably evolved following the primary endosymbiotic event that led to the evolution of chloroplasts since CES does not occur in cyanobacteria.

Key Words:

photosynthetic protein complexes; concerted protein accumulation; protein degradation; protein targeting; chloroplast translocon; assembly factors; regulation of chloroplast gene expression; CES process.

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I. Introduction

Energy transduction in chloroplasts is performed by membrane-associated oligomeric protein complexes that are localized in the chloroplast within an array of flattened membrane vesicles named thylakoids (Chapter 23). Most polypeptide subunits of these complexes are transmembrane, but in several instances they are peripheral polypeptides attached to the thylakoids on either their stromal or luminal surfaces. With the exception of antenna protein complexes, which consist exclusively of nucleus-encoded proteins (Chapter 15), protein complexes involved in photosynthetic electron transport and inorganic carbon fixation – Photosystem I (PSI) and Photosystem II (PSII) (Chapter 16), Cytochrome *b₆f*, ATP synthase (Chapter 17) and Ribulose-Bisphosphate-Carboxylase-Oxygenase (Chapter 7) – all have subunits that are nucleus-encoded and translated in the cytoplasm of the cell, with others encoded by the chloroplast genome and translated within the organelle. Therefore, one should expect some coordination in expression of proteins in these two genetic compartments, to produce the various subunits of each protein complex in the stoichiometry required for their functional assembly. The biogenesis routes of nuclear and chloroplast encoded subunits are markedly different: nucleus-encoded subunits are translated in the cytosol before being translocated into the chloroplast, often processed and then properly targeted to their site of assembly with their chloroplast-encoded partners that are translated within the organelle. Assembly factors assist in the recognition and folding of these subunits into an assembly-competent conformation (see Chapters 7, 16, 17 for specific factors and Chapter 20 for a detailed presentation of chloroplast chaperons). Based on the observation of the pleiotropic loss of all subunits of the same protein complex in *Chlamydomonas* mutants defective for expression of a single subunit, it was early recognized that the successful assembly of each protein complex results from the concerted accumulation of its constituent subunits (Wollman et al., 1999b; Choquet and Vallon, 2000). This concerted accumulation results from two major regulatory processes - a proteolytic disposal of a subset of unassembled subunits (see Chapter 20 for details concerning chloroplast proteases) and an assembly-dependent regulation of translation of another subset of chloroplast-encoded subunits; this process has been designated CES (Control by Epistasy of Synthesis).

Here we focus mainly on studies in which *Chlamydomonas reinhardtii* has

contributed new knowledge to our understanding of the assembly of photosynthesis proteins. Thus, most of this chapter is devoted to the CES process because this microalga has been instrumental in its discovery based on the ease with which it can be used for both classical genetics and chloroplast molecular genetics (see Introductory Chapter to this volume).

II. Assembly of multisubunit photosynthetic protein complexes.

A. Overview

Bioenergetic membranes are the site of ATP synthesis at the expense of a transmembrane proton-motive force that originates from serial electron transfer reactions coupled to proton translocation across the membrane. Most of these processes develop within, as well as between, membrane-embedded hetero-oligomeric protein complexes. The biogenesis of these molecular machines is particularly complex as they most often comprise more than 10 distinct subunits and accommodate a variety of cofactors - pigments, hemes, iron-sulfur clusters and metal ions - bound either covalently or non-covalently to the protein moiety. For example, PS II, the protein complex that drives the light-induced oxidation of water and initiates photosynthetic electron flow, comprises close to 30 different subunits, most of which are integral membrane polypeptides, and binds a core antenna of 35 chlorophyll *a* molecules, eleven carotenoids, two pheophytin and two quinone molecules, one heme, a non heme-iron and four manganese atoms (Zouni et al., 2001; Ferreira et al., 2004; Tanaka et al., 2017; Sheng et al., 2019), see also Chapter 16).

The assembly of these oligomeric proteins requires a temporal and spatial coordination within the cell to deliver the various components, both protein subunits, and cofactors, in stoichiometry suitable quantities for the assembly of functional complexes. In particular, protein production should be properly controlled to avoid wasteful accumulation of unassembled subunits that might disrupt the integrity of the membrane upon aggregation or become a source of toxic radical species generated in the light and presence of oxygen as a consequence of their associated redox cofactors and/or pigment molecules.

In prokaryotes, the clustering of genes in operons is well suited for fine-tuning coupled transcription/translation rates of the various subunits comprising a given protein complex. In contrast, in chloroplasts, most polycistronic transcription contain

genes encoding subunits belonging to different protein complexes, and polycistronic transcripts are often trimmed to monocistronic mRNAs that are independently translated (Drapier et al., 1998; Cavaiuolo et al., 2017). The biogenesis of oligomeric protein complexes associated with photosynthesis or respiration in the organelles involves an additional level of complexity as their constituent subunits can be encoded on different genomes and synthesized in different cellular compartments. Subunits encoded by the chloroplast genome, and translated on prokaryotic-like 70S ribosomes, are associated in a defined stoichiometry with subunits encoded by nuclear genes that are translated on cytosolic 80S ribosomes and imported into the organelle. Because of the huge imbalance in gene copy numbers for chloroplast-encoded versus nucleus-encoded subunits, which differ by two orders of magnitude in *Chlamydomonas* (Hosler et al., 1989; Eberhard et al., 2002), there must be regulatory mechanisms that allow the stoichiometric accumulation of subunits encoded in the two genetic compartments. The primary level of regulation is provided by nucleus-encoded, gene-specific, organellar trans-acting factors (OTAFs) which are limiting for the expression of their chloroplast mRNA targets, thus providing commensurate expression levels of chloroplast-encoded and nucleus-encoded subunits (Choquet and Wollman, 2002). Consequently, the level of expression of each chloroplast-encoded subunit is under some specific nuclear control (Chapter 13 and 14).

B. Targeting, import and primary localization of neo-synthesized protein subunits in the chloroplast

Nucleus-encoded subunits of photosynthetic proteins are translated in the cytosol as pre-proteins with an N-terminal extension that targets them to the chloroplast. These organelle-targeting sequences, hereafter referred to as targeting peptides (TPs), are rapidly cleaved and degraded after import of the pre-proteins in the chloroplast. TPs differ widely in their primary sequence but share similar physico-chemical properties. Although there are distinctive features between TPs from plants and *Chlamydomonas* that have required the development of a specific targeting prediction program for the latter (Tardif et al., 2012), all TPs display an amphiphilic character and are enriched in positively charged residues (Garrido et al., 2020). In one scenario in the evolution of a chloroplast from a cyanobacterial endosymbiont, these organelle-targeting sequences were proposed to be evolutionary related to a class of antimicrobial peptides (helical amphipathic ribosomally synthesized antimicrobial

peptides: HA-RAMPs) that display similar physico-chemical properties (Wollman, 2016; Caspari and Lafontaine, 2021). *Chlamydomonas* has been instrumental in the demonstration that indeed extant HA-RAMPs still display organelle targeting properties whereas, conversely, organelle targeting peptides display antimicrobial activity (Garrido et al., 2020).

Whether pre-proteins are translated on soluble ribosomes dispersed over the cytosol or localized near the chloroplast envelop, is still a matter of debate (for a review see (Weis et al., 2013)). Most pre-proteins are post-translationally imported across the chloroplast double envelop membranes through protein channels known as the “Translocons of the Outer and Inner Chloroplast membrane” (TOC and TIC, respectively). The actual composition of the chloroplast translocon in vascular plants remains controversial (Nakai, 2015; Li et al., 2020; Ramundo et al., 2020). The composition of its TOC component is agreed upon, with two GTPases, TOC159 and TOC33/34 serving the central TOC75 channel. However the original TIC complex in plants, comprised of three major nuclear-encoded subunits –TIC20, TIC 40 and TIC110-, has been challenged with the identification of an alternative 1 MDa protein complex that contains a chloroplast-encoded subunit, Tic214/Ycf1, which, together with two nucleus-encoded subunits, TIC56 and TIC100, associates with TIC20 as the central TIC channel component (Kikuchi et al., 2013). Recent experiments with *Chlamydomonas* provided strong support for this model (Ramundo et al., 2020), despite the limited sequence conservation between the Ycf1 chloroplast-encoded proteins of *Chlamydomonas* and *Arabidopsis*. The algal TIC complex mirrors the molecular composition of the new version of the TIC complex from *Arabidopsis*, with the same three nucleus-encoded subunits associated with the chloroplast-encoded Tic214/Ycf1 subunit. Ramundo and colleagues also identified another chloroplast-encoded large protein, Ycf2, which is co-expressed with TIC20 and Tic214 during the diurnal cycle of *Chlamydomonas* and interacts with the purified TIC-TOC supercomplex (Ramundo et al., 2020). This chloroplast-encoded protein had been shown previously to be part of an ATP-driven import motor in land plants, physically interacting with Tic214 (Kikuchi et al., 2018). Thus, the chloroplast import machinery is likely a genetic mosaic, as is the case for the chloroplast translation and photosynthesis machineries.

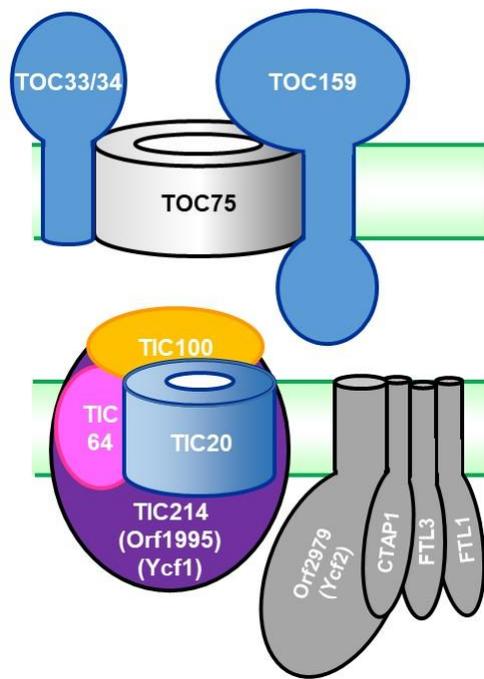


Figure 19.1: **Schematic representation of the TOC/TIC translocon of *Chlamydomonas*.**

Only the subunits with an orthologue in higher plants are represented. Additional *Chlamydomonas*-specific subunits (CTAP2-7) also belong to this translocon but are not shown as their topology and their interaction with other subunits are not known. A putative translocon-associated ATP-driven import motor (equivalent to the 2 MDa Ycf2/FTSHi complex described in higher plants) likely comprising at least the Ycf2, FTL1, FTL3 and CTAP1 subunits is also shown. The two chloroplast-encoded subunits of the import machinery are green filled.

Credit: Yves Choquet

It has been argued that the distribution of the TOC-TIC translocon is not homogeneously distributed along the chloroplast envelop in *Chlamydomonas* (Schottkowski et al., 2012). Based on an imaging approach of the distribution of mRNAs and newly synthesized proteins in *Chlamydomonas* chloroplasts (Uniacke et al., 2011), TOC-TIC were suggested to be enriched in membrane domains where the inner envelop membranes invaginates (Engel et al., 2015), in the vicinity of what has been termed T (for translational)-zones of the thylakoid membranes. Zerges and colleagues defined these T zones as part of a “thylakoid membrane biogenesis center” where the biogenesis of the two photosystems together with chlorophyll biosynthesis occurs (Sun et al., 2019). These results argue for a spatial coordination of the biogenesis of nucleus- and chloroplast-encoded subunits of the photosynthetic proteins in *Chlamydomonas* (Sun et al., 2019). VIPP1, a highly flexible. chloroplast protein that shows a remarkable ability to adopt contrasting oligomerization states and shapes (Rutgers and Schroda, 2013; Gupta et al., 2021) has recently been shown to engulf membranes containing phosphatidylinositol phosphates (Theis et al., 2019).

This capacity may support its role in the biogenesis of the photosynthetic apparatus, which has been documented in several organisms including *Chlamydomonas* (Nordhues et al., 2012). VIPP1 may generate membrane domains displaying the appropriate lipid environment for the recruitment and function of the ALB3 insertases and SEC and TAT translocases, which are central to the biogenesis of photosynthesis proteins (see below).

Although there is currently no identification by tomographic studies of such biogenesis membrane domains in the envelop, which should be particularly enriched in ribosomes next to its stromal surface (see chapter 23), it should be noted that cyanobacterial plasma membranes also were reported to display “biogenic zones”, i.e. sub-domains dedicated to translation (Zak et al., 2001; Rengstl et al., 2011; Stengel et al., 2012; Rast et al., 2019). Whether a similar supramolecular organization for oligomeric protein biogenesis also exists in plant chloroplasts has not been documented.

C. Intra-chloroplast sorting and assembly of photosynthetic proteins

The photosynthesis protein subunits that are transmembrane or localized to the thylakoid lumen must be properly targeted for membrane insertion/translocation. These processes have been characterized mainly by biochemical approaches with isolated thylakoids from plants. Several targeting pathways to the thylakoid membranes were thus identified, which are of bacterial origin (reviewed in (Albiniak et al., 2012)). The GTP-dependent cpSRP route, which is performed by a hetero-oligomeric complex of SRP54 and SRP43, is used by numerous antenna proteins for their stromal migration to the thylakoids where they insert in the membranes via the SRP receptor FTSY and the insertase ALB3, a member of the YidC/Oxa1p family (Chapter 15). In *Chlamydomonas*, there are two ALB3 proteins, of which only ALB3.1 is involved in the antenna biogenesis pathway (Bellafiore et al., 2002). However ALB3.1, together with ALB3.2, also participates in the biogenesis of the photosynthetic reaction centers (RC), although the identity of the RC subunits that require ALB3 for insertion remain to be determined (Ossenbuhl et al., 2004; Gohre et al., 2006).

Many transmembrane or luminal proteins like cytochrome *f*, plastocyanin and the PSII RC protein D1 use the ATP-dependent SEC pathway for membrane insertion/translocation in their unfolded state. In contrast, the TAT pathway

translocates folded proteins, such as the luminal PSII proteins OEE16 and OEE23, or the transmembrane Rieske protein subunit of the cytochrome *b₆f* complex. These substrates of the TAT pathway have a twin-arginine motif in their N-terminal lumen-targeting domain, which is not present in the luminal proteins using the SEC pathway (Chapter 16-17). *Chlamydomonas*, which is amenable to *in vivo* time-resolved spectroscopic studies (Chapter 18), has been instrumental in demonstrating that the TAT pathway is $\Delta\mu\text{H}$ -dependent *in vivo* and not ΔpH -dependent as had been previously claimed on the basis of *in vitro* studies of plant thylakoids (Finazzi et al., 2003; Theg et al., 2005).

Once the various subunits of each photosynthesis protein get to the thylakoid membranes, their assembly is considered a sequential process with steps that are temporally distinguishable. Each assembly intermediate comprises a subset of subunits of the final complex. Their assembly results from, or is catalyzed by- specific sets of auxiliary proteins, which contribute to the folding and post-translational modification of subunits, or the binding of cofactors. For instance, the biogenesis of PSII core monomers involves more than twenty such proteins that participate in a six-step assembly pathway (chapter 16). Whereas biochemical studies with cyanobacteria and genetic studies of *Arabidopsis* have played a large role in the dissection of the assembly steps for PSII cores (Komenda et al., 2012; Lu, 2016), it is *Chlamydomonas* research, based on gene knock outs through chloroplast transformation, that has been instrumental in elucidating key steps in the assembly of PSI reaction centers. Two chloroplast-encoded PSI assembly factors, Ycf3 and Ycf4, conserved in plants and cyanobacteria, respectively catalyze the assembly of PSI cores and of PSI peripheral antenna with these cores (Boudreau et al., 1997; Ozawa et al., 2009; Ozawa et al., 2010; Nellaepalli et al., 2018), Chapter 16). A detailed description of the assembly steps in the biogenesis of ribulose biphosphate carboxylase-oxygenase (RuBisCO) and antenna proteins- is presented in Chapters 7 and 15, respectively.

D. Concerted accumulation of subunits of photosynthetic complexes

Photosynthesis mutants of *Chlamydomonas*, altered in any of the major complexes - PSI and PSII, the cytochrome *b₆f* complex, the ATP synthase or RuBisCO - are viable when grown in the presence of acetate as a carbon source.

When subsequently characterized at the molecular level, most of these mutants, although defective for expression of a single core subunit of the photosynthetic

complex under study, nevertheless lack accumulation of all the other subunits of that complex. Further studies with mutant strains defective in small subunits of these complexes – most of which span the membrane only once and are peripherally located within the protein complex – have provided a contrasting picture: some small subunits were strictly required for the concerted accumulation of all subunits from the complex, whereas the absence of others still allowed partial accumulation of subcomplexes. For instance, deletion of the *petG* gene, which encodes a small subunit of the cytochrome *b₆f* complex (Berthold et al., 1995), led to the loss of the whole complex as did the deletion of genes encoding any one of the three major chloroplast-encoded core subunits, *petA*, *petB*, *petD*, (Kuras and Wollman, 1994). In contrast, deletion of the *petL* gene still allowed the partial accumulation of a functional protein complex (Takahashi et al., 1996). Tobacco or *Arabidopsis* mutants behave as their *Chlamydomonas* equivalents: mutant lines lacking expression of any of the major cytochrome *b₆f* subunits, but also of PetG, PETM and PetN, show impaired accumulation of the whole complex (Hager et al., 1999; Monde et al., 2000a; Maiwald et al., 2003; Schwenkert et al., 2007; Hojka et al., 2014). By contrast, Δ *petL* lines accumulate the cytochrome *b₆f* complex to almost wild-type levels in young tobacco leaves, but this defective complex, like its *Chlamydomonas* counterpart, is less stable than the wild-type complex and disappears upon aging (Takahashi et al., 1996; Schottler et al., 2007; Schwenkert et al., 2007). As a rule, studies of higher plant mutants led to conclusions very similar to those drawn from the earlier studies of *Chlamydomonas* mutants.

E. Concerted subunit accumulation arose with chloroplast compartmentalization.

In cyanobacteria, in most cases where gene inactivation targeted a major subunit of a photosynthetic protein, there is still a significant – albeit reduced – accumulation of the other subunits of the protein complex. Cyanobacterial mutants deficient for PsaA or PsaB lack the whole set of PSI subunits as do their chloroplast counterparts (Smart et al., 1991; Nyhus et al., 1993; Smart and McIntosh, 1993). But *psaC* deletion strains from *Anabaena* or *Synechocystis* still accumulate PsaA and PsaB that are assembled into chlorophyll–protein complex I (CPI) and able to drive P700 light-induced charge separation (Mannan et al., 1991; Yu et al., 1995). In contrast, the *psaC*

deletion strain from *Chlamydomonas* lacks the whole PSI complex because PsaA and PsaB are rapidly degraded (Takahashi et al., 1991). Also, *psaD* deletion strains from cyanobacteria can still assemble PSI since they grow photoautotrophically (Chitnis et al., 1989; Xu et al., 1994), at variance with the corresponding mutants from higher plants (Ihnatowicz et al., 2004).

In *Chlamydomonas*, the absence of PSII subunits D2 or D1 from the reaction center (RCII) or of the core antenna CP47 leads to the loss of all PSII subunits from the thylakoid membrane (de Vitry et al., 1989). By contrast, D2 shows significant accumulation in the absence of D1 in *Synechocystis*, while RCII can be detected in strains lacking CP47 (Komenda et al., 2004). Furthermore, when minor subunits of PSII were not expressed, the bulk of the protein complex still accumulated at close to wild-type levels, in stark contrast to the situation in *Chlamydomonas*. A mutant of *Synechocystis* 6803 lacking the *psbI* gene retained 70–75% of wild-type levels of oxygen evolution capability and PSII accumulation (Ikeuchi et al., 1995), while in *Chlamydomonas* inactivation of the *psbI* gene decreased oxygen evolution activity and D1 content almost 10-fold (Kunstner et al., 1995). *psbH* and *psbK* deletion strains of *Synechocystis* were still capable of photosynthetic growth even if they showed light sensitivity (Ikeuchi et al., 1991; Mayers et al., 1993; Komenda et al., 2002), while the corresponding mutant strains of *Chlamydomonas* failed to assemble PSII and were not photosynthetic (Takahashi et al., 1994; Summer et al., 1997).

These distinct features of protein accumulation in chloroplasts and cyanobacteria point to an increased quality control of protein assembly in the eukaryotic context. The need for this quality control of photosynthesis protein assembly in the chloroplast is likely due to the intricate biogenesis processes that may be temporarily desynchronized since they are spatially separated between the organelle and the nucleo-cytosol.

F. Proteolytic degradation of unassembled subunits in the chloroplast

The quality control of chloroplast protein assembly is exerted through the action of several proteases which are described in more details in Chapter 20 and in (Nishimura et al., 2016, 2017). Indeed, the chloroplast houses a particularly sophisticated suite of proteolytic enzymes with some protein families having more members in plants and in *Chlamydomonas* than in the cyanobacteria, like the Deg (6 vs. 3) or FtsH (12 vs. 4) families (Sokolenko et al., 2002). These proteases are

responsible for the proteolytic disposal of unassembled, partially assembled, or misassembled subunits. Pulse-chase or immunochase experiments performed on *petA*, *petB*, or *petD* deletion strains have shown that the half-lives of PetD and cytochrome *b₆*, but not their initial synthesis rates, were strongly decreased when another major subunit from the complex was missing. For example, the half-life of PetD drops from almost 2 hours in wild type cells to 45 and 15 minutes in strains lacking cytochrome *f* or cytochrome *b₆*, respectively (Kuras and Wollman, 1994). Unassembled PetD is thus rapidly degraded whereas it is converted upon assembly to a more stable form, probably because the motifs recognized by proteases are shielded by the other subunits. The FTSH protease, maybe in conjunction with others, likely participates in this degradation as mutations that inactivate FTSH restore, to some extent, the accumulation of PetD and cytochrome *f* in mutants originally defective for cytochrome *b₆f* accumulation, or upon nitrogen or sulfur starvation (Malnoe et al., 2011; Malnoe et al., 2014; Wei et al., 2014; De Mia et al., 2019). By contrast, the Clp protease does not have a strong role in eliminating unassembled cytochrome *b₆f* subunits as translational attenuation of the *clpP1* gene did not enhance accumulation of unassembled subunits in deletion strains (Majeran et al., 2000). Similarly, the turnover of the D2 protein strongly increases in mutant strains lacking D1 (de Vitry et al., 1989; Preiss et al., 2001; Minai et al., 2006) or CP43 (de Vitry et al., 1989) expression. These results further illustrate the more active degradation of unassembled subunits in chloroplasts than in cyanobacteria, which do accumulate D2 in the absence of D1 (Komenda et al., 2004).

III. CES, a major control process in the biogenesis of photosynthetic proteins

A. Overview

The ability of chloroplasts to avoid the accumulation of unassembled subunits or assembly intermediates also results from a unique regulatory process, the CES process, which involves assembly-dependent regulation of translation. The CES concept derives from studies that consistently reported that the absence of some core subunits in photosynthesis mutants of *Chlamydomonas* caused a decrease in the rate of synthesis of another chloroplast-encoded subunit from the same protein complex. The latter has been designated as a CES subunit (Wollman et al., 1999a; Choquet and

Vallon, 2000; Choquet et al., 2001; Choquet and Wollman, 2002). CES is a major control step during the biogenesis of the photosynthetic apparatus in *Chlamydomonas*: almost all major chloroplast-encoded polypeptides – those polypeptides whose rate of synthesis can be accurately measured by pulse-labeling experiments – participate in CES, being either CES subunits or assembly partners required for the expression of CES subunits from the same protein complex (Figure 19.2).

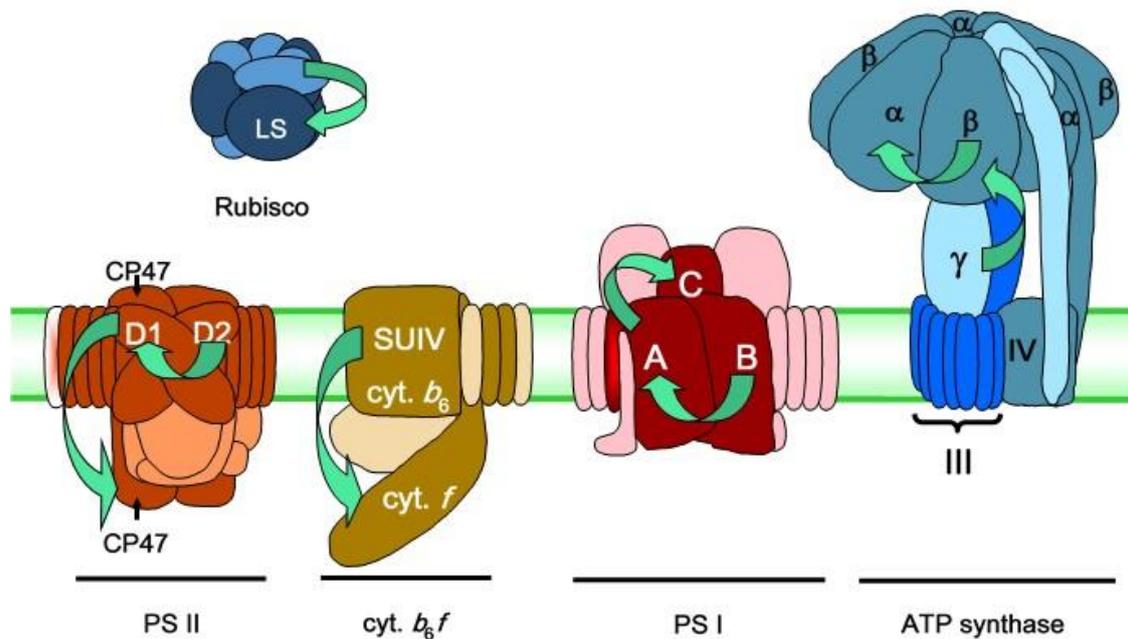


Figure 19.2: *CES contributes to the biogenesis of all major photosynthetic protein complexes in Chlamydomonas chloroplasts.*

Schematic representation of the major photosynthetic proteins. Subunits encoded by the chloroplast genome are filled with dark colors, while those encoded in the nucleus are filled with pale colors. Green arrows indicate epistatic relationships between the subunits of a photosynthetic protein complex and connect an assembly partner whose presence is required for efficient synthesis of a CES subunit, to this CES subunit; the arrows extend from the partner to the CES subunit.

Credit: Yves Choquet

This regulatory process probably arose after the endosymbiotic event that led to the evolution of chloroplasts since it is not observed in the biogenesis of cyanobacterial bioenergetic complexes. Originally discovered in the chloroplast of *C. reinhardtii*, where it has been extensively studied because of its prominent contribution to the biogenesis of photosynthetic proteins, CES later appeared as a general feature of organelle gene expression since it also contributes to the biogenesis of photosynthetic proteins in higher plant chloroplasts (See section IV A) and of respiratory protein complexes in the mitochondria of the yeast *Saccharomyces*

cerevisiae (See section IV B).

B. CES cascades and assembly of multimeric protein complexes in the chloroplast of Chlamydomonas

Cytochrome *f* from the cytochrome *b₆f* complex, encoded by the chloroplast *petA* gene, is a typical CES protein whose rate of synthesis decreases in the absence of cytochrome *b₆* or PetD, to about 10% of that observed in the wild type (Lemaire et al., 1986; Kuras and Wollman, 1994). At variance with cytochrome *b₆* and PetD, which are synthesized at the wild-type rate but rapidly degraded in strains defective for cytochrome *b₆f* complex assembly, the reduced amount of cytochrome *f* synthesized in the absence of its assembly partners, cytochrome *b₆* and PetD, is stable in the membrane (Kuras and Wollman, 1994).

Similarly, PSI mutants defective for PsaB expression show no detectable synthesis of the CES subunit PsaA, whether failure to synthesize PsaB results from mutations in the chloroplast *psaB* gene itself (Girard-Bascou et al., 1987) or in the *TAB1* or *TAB2* nuclear genes, whose products are specifically required for the translation of *psaB* mRNA (Stampacchia et al., 1997; Dauvillee et al., 2003). In contrast, mutants lacking *psaA* gene expression show wild-type levels of *psaB* translation (Girard-Bascou et al., 1987; Goldschmidt-Clermont et al., 1990), but they display a reduced synthesis of the chloroplast-encoded PsaC, which therefore behaves as another CES subunit (Takahashi et al., 1991). The asymmetry in subunit behavior along the biogenesis pathway is further illustrated by the wild type rate of synthesis but rapid degradation of PsaB and PsaA in *psaC* deletion strains (Takahashi et al., 1991). Thus, PsaB is required for sustained synthesis of PsaA, which, itself is required for the translation of the *psaC* mRNA, thus defining a “CES cascade”.

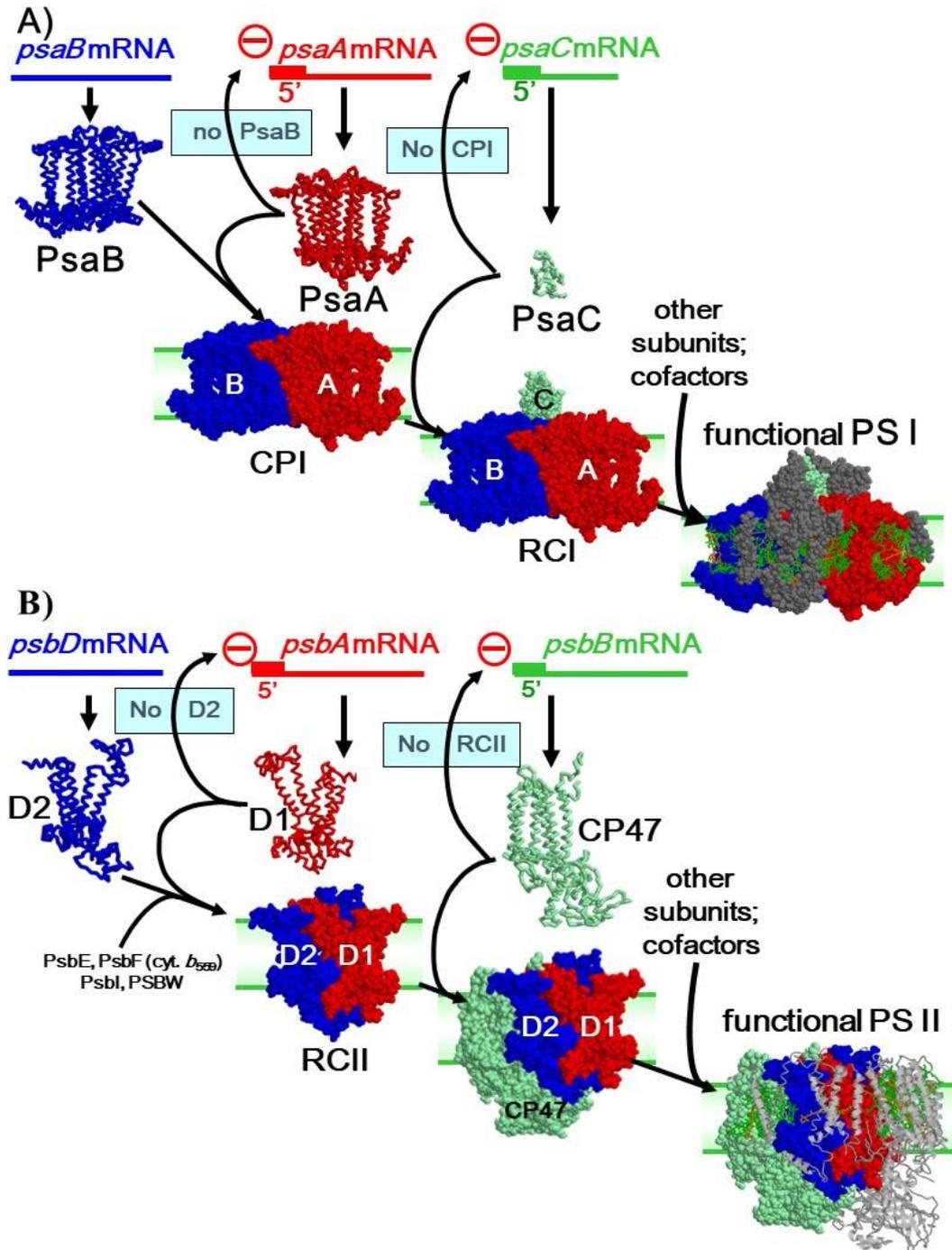


Figure 19.3: *Epistatic cascades contributing to the biogenesis of PS I (A) and PS II (B).*

Assembly pathways of PS I and PS II are schematically depicted. Structures of PS I and PS II are drawn from Jordan et al. (2001) and Loll et al. (2005), respectively. Most upstream assembly partners are drawn in blue, while CES subunits are shown in red and green. When newly-synthesized CES polypeptides, represented as helical backbones, cannot assemble, they repress the translation initiation of their encoding mRNAs, as indicated by the ⊖ symbol in red.

Credit: Yves Choquet

An analogous CES cascade participates in *Chlamydomonas* PSII biogenesis.

Mutants lacking the reaction center subunit D1 show a greatly reduced level of the PSII core antenna CP47 synthesis but maintain synthesis of the other reaction center subunit, D2 (Bennoun et al., 1986; Jensen et al., 1986; de Vitry et al., 1989), while the rate of both D1 and CP47 synthesis is strongly decreased in mutants lacking expression of D2 (Bennoun et al., 1986; de Vitry et al., 1989). Thus, both CP47 and D1 are CES subunits in PSII biogenesis (Minai et al., 2006).

The above-described cascade of autoregulation by unassembled subunits couples the assembly of cytochrome *b₆f*, PSI and of PSII to the expression of their individual subunits. These oligomeric complexes have a core comprised of integral membrane subunits assembled in a 1:1 stoichiometry and their assembly is a stepwise process, with a progressive increase in the stability of assembly intermediates as more subunits are added (reviewed in (Choquet and Vallon, 2000))

Feedback loops working in series prevent excessive production of a CES subunit until all upstream steps in the sequential assembly pathway have been completed. Consequently, sustained expression of CES subunits requires the presence of all subunits that participate in upstream steps in the assembly pathway, but not that of subunits that assemble in downstream steps. In other words, the CES hierarchy necessarily reflects the sequence of the successive steps in the assembly pathway.

CES also contributes to the biogenesis of the soluble CF₁ sector of the ATP synthase complex, which catalyzes ATP synthesis. The α and β subunits, respectively encoded by the chloroplast *atpA* and *atpB* genes, are both CES subunits: mutants defective for the expression of the nucleus-encoded subunit γ show reduced synthesis of subunit β (Drapier et al., 2007), while the rate of translation of *atpA* mRNA is strongly reduced in strains impaired in the expression of the β subunit (Lemaire and Wollman, 1989; Drapier et al., 1992; Drapier et al., 2007).

CES is not restricted to the integral thylakoid membrane proteins: the large subunit of RuBisCO (LS) also is a CES protein, whose rate of synthesis is highly reduced in deletion strains lacking expression of the small subunit (SS) (Khrebtukova and Spreitzer, 1996; Wietrzynski et al., 2021).

CES thus appears as a major control step during the biogenesis of the photosynthetic apparatus in *Chlamydomonas*: almost all major chloroplast-encoded polypeptides – those polypeptides whose rate of synthesis can be accurately measured by pulse-labeling experiments – participate in CES, being either CES subunits or

assembly partners required for the expression of CES subunits from the same protein complex (Figure 19.2).

One exception is the CP43 core antenna of PSII: its rate of synthesis does not depend on the presence of other PSII subunits, even if it is less stable in strains showing compromised PSII assembly. Conversely, its absence does not affect the rate of synthesis of other PSII subunits, even though they are more rapidly degraded in the absence of CP43 (de Vitry et al., 1989).

Whether the small chloroplast-encoded subunits could also be CES subunits remains to be studied. Indeed, being poorly resolved by SDS-PAGE, assessing their actual rates of translation in assembly-defective strains by pulse-labeling experiments is difficult, thus preventing the study of their possible participation in CES cascades. The recently developed ribosome profiling methods (Zoschke et al., 2013; Trösch et al., 2018) will now allow this issue to be addressed.

Efficient translational autoregulation requires that unassembled CES proteins accumulate at least to some extent in the absence of their assembly partners. While we lack experimental data on the behavior of unassembled PsaA and PsaC subunits in strains devoid of PsaB and CPI, respectively, the other CES proteins proved quite long-lived in their fully unassembled forms. D1 and CP47, although synthesized in markedly reduced amounts, resist proteolytic degradation in the absence of D2 and D1, respectively (de Vitry et al., 1989), as does unassembled cytochrome *f* in the absence of cytochrome *b₆* or PetD (Kuras and Wollman, 1994; Choquet et al., 2003) or LS in the absence of SS (Wietrzynski et al., 2021). It is of note that once assembled or partially assembled into protein complexes, CES subunits become more susceptible to degradation than when fully unassembled. The CES subunits D1 and CP47 are prone to proteolytic degradation in mutant strains lacking synthesis of CP43, where they accumulate in a D2/D1/CP47 subcomplex. Once assembled within cytochrome *b₆f* complexes, cytochrome *f* becomes a target for proteases either upon nitrogen or sulfur starvation (Bulte and Wollman, 1992; Wei et al., 2014; De Mia et al., 2019) or in strains expressing a mutated form of the Rieske protein (Majeran et al., 2000). Similarly, LS, although stable for more than 4 h in the absence of SS, is actively degraded upon nitrogen or sulfur starvation (Wei et al., 2014; De Mia et al., 2019; Majeran et al., 2019). Indeed unassembled LS is stabilized by its association with RAF1, a dedicated LS assembly factor (Wietrzynski et al., 2021). Although this remains to be studied for the other CES subunits, the interaction with assembly factors

would confer a protection of the unassembled CES subunits against degradation by shielding the motifs recognized by the proteases. This protection would be lost upon assembly, which releases the assembly factors. In turn, the stability of unassembled CES subunits *in vivo* requires a tight control of their rate of synthesis in order to prevent their over-accumulation.

In contrast to CES subunits, the upstream assembly partners of CES cascades are quite unstable when unassembled, as discussed in sections II.D and II.E. Assembly thus must be more efficient than the competing degradation process. The CES process would optimize the rate of assembly of these partner subunits which, upon thylakoid insertion, make contact with low levels of long-lived unassembled CES subunits, available for a rapid interaction that protect them from degradation. This protective effect of CES subunits may be very efficient when considering that the biogenesis of photosynthetic proteins may be restricted to dedicated membrane domains (Chapter 14; (Uniacke and Zerges, 2007, 2009; Weis et al., 2013; Sun et al., 2019).

Thus, the particular properties of CES subunits offer a unique means to optimize the sequential and multistep processes for protein assembly.

C. Cytochrome *f*, the most highly characterized CES protein in *Chlamydomonas*.

Cytochrome *f*, whose rate of synthesis decreases about 10-fold in absence of its assembly partners (Lemaire et al., 1986; Kuras and Wollman, 1994), is probably the best characterized CES protein in *Chlamydomonas* so far.

1) Regulation of translation initiation

The 2-fold decrease in *petA* mRNA abundance observed in the Δ *petD* strain, defective for cytochrome *f* assembly (Boulouis et al, 2011) cannot alone account for the 10-fold decreased rate of cytochrome *f* synthesis, and thus regulation of cytochrome *f* synthesis occurs at a (post-) translational level. The original observation of a reduced synthesis of cytochrome *f* in cytochrome *b₆f* assembly-defective mutants by short-time pulse-labeling experiments could originate from widely different mechanisms, either an actual regulation of translation or an early degradation process – possibly co-translational – that would prevent the accumulation of full-length

radiolabeled cytochrome *f*. The demonstration that the cytochrome *f* CES process corresponds to a genuine ***assembly dependent regulation of translation*** came with chloroplast transformation experiments with chimeric reporter genes. First, cytochrome *f* no longer behaved as a CES subunit when its coding sequence was translated under the control of the unrelated 5'*atpA*-UTR: its rate of synthesis and accumulation remained constant, irrespective of the presence of its assembly partners (Choquet et al., 2003), excluding a co-translational degradation of unassembled cytochrome *f*. Furthermore, protein reporters expressed under the control of the *petA* 5'UTR mimicked the CES behavior of cytochrome *f* and showed a decreased synthesis in the absence of PetD or cytochrome *b*₆ (Choquet et al., 1998). Thus, the CES process operates at some early step in the process of *petA* translation initiation, either an activation step prior to initiation, translation initiation itself, or a transitional step between initiation and elongation of translation.

2) Auto-regulation of synthesis...

Further clues to the assembly-dependent regulation of cytochrome *f* synthesis came from the study of cytochrome *f* variants generated by site-directed mutagenesis. Cytochrome *f* exposes a large N-terminal luminal domain, on which a *c*-heme is covalently attached by thioester bonds. This globular domain is tethered to the membrane by a C-terminal domain comprising a 20 residue-long transmembrane helix that extends in the stroma by a 15 residues α -helix (see Chapter 17). Two mutant strains lack accumulation of this C-terminal domain, either because it is not synthesized in a strain expressing solely the N-terminal domain, a soluble form of the protein which accumulates stably in the thylakoid lumen (Kuras et al., 1995b), or because it lacks significant accumulation in another strain expressing a highly unstable apocytochrome *f*, barely detectable in the thylakoid membrane (Kuras et al., 1995a). Despite these large differences in cytochrome *f* stability and accumulation levels, both variants behaved similarly: a threefold stimulated synthesis was observed when compared to wild type that became independent of the presence or absence of PetD (Kuras et al., 1995a; Kuras et al., 1995b; Choquet et al., 1998). Thus, the stromal-facing C-terminal domain of cytochrome *f* appears responsible of the down-regulated translation of the *petA* mRNA, a hypothesis further confirmed upon characterization of the CES translational repressor domain by site-directed mutagenesis. Substitutions of the residues within a critical tetrapeptide cluster

K₃₀₅QFE₃₀₈ in the stromal extension of cytochrome *f*, or of two K₃₀₂ and Q₂₉₇ upstream residues, lying respectively at the interface between the membrane and the stroma or buried in the thylakoid membrane (Figure 19.3A), led to enhanced translation of cytochrome *f*, which became independent of, or poorly dependent on, the presence of its assembly partners. In contrast, substitutions of other residues within the C-terminal domain had no impact on the regulation of cytochrome *f* translation (Choquet et al., 2003). Thus, unassembled cytochrome *f* exerts a negative feedback on its own synthesis by exposing a C-terminal CES repressor domain. Upon assembly, this motif, shielded by the other cytochrome *b₆f* subunits [(Stroebel et al., 2003) and see (Figure 19.3B)], would allow cytochrome *f* synthesis to resume.

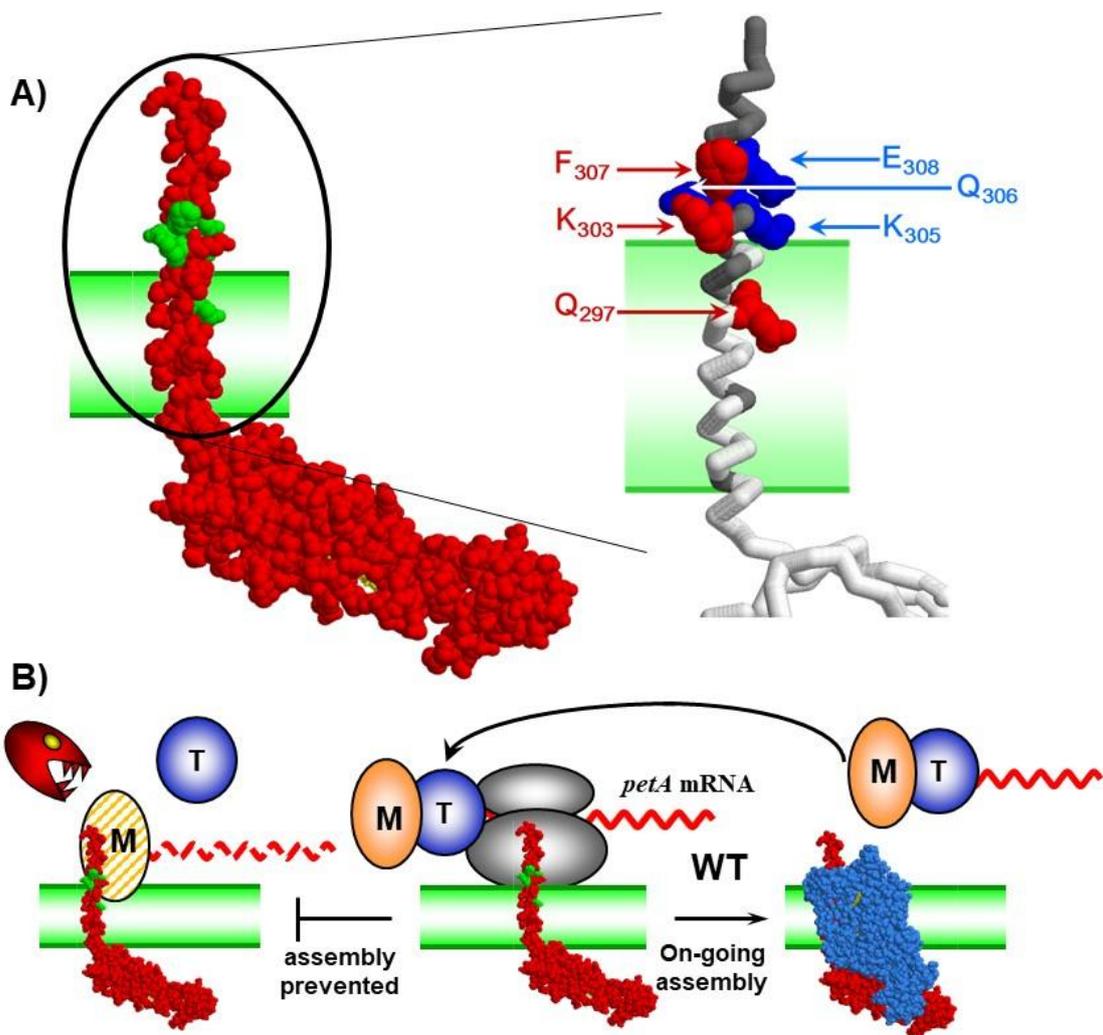


Figure 19.4: **CES control of cytochrome *f* synthesis**

A) The translation repressor domain from cytochrome *f*.

Schematic representation of the cytochrome *f*, with zooming in on its C-terminal domain (drawn from structure of Stroebel et al., 2003). Residues shown in space-fill contribute to the repressor domain of cytochrome *f*. They are in red when substitutions completely abolish the CES process and in blue if substitutions result in significant, but not a

total loss of CES. Substitution of the residues represented as backbones have no effect on the CES control of cytochrome *f* (in dark grey) or have not been tested (in pale grey).

B) *Model for the autoregulation of petA mRNA translation in Chlamydomonas.*

In the wild type, with on-going assembly in the cytochrome *b₆f* complex, MCA1 and TCA1 control the stabilisation and translation of *petA* mRNA. When assembly is prevented (or delayed), the C-terminal repressor motif (in green) exposed in unassembled cytochrome *f* (in red) traps MCA1 and targets it for degradation, preventing further cytochrome *f* synthesis.

Credit: Yves Choquet

3) CES is mediated by *petA*-specific organelle trans-acting factors

Located within a single α -helix extending from the membrane to the stroma (Stroebel et al., 2003), this regulatory motif does not show any feature typical of an RNA-binding protein. It also comprises a critical residue, Q₂₉₇, that is unlikely to interact directly with the *petA* 5'UTR, being embedded in the membrane bilayer. Rather, this interaction would rely on ternary effector(s), competitively binding to unassembled cytochrome *f* and to the *petA* 5'UTR. Bound to cytochrome *f*, effectors would decrease the rate of cytochrome *f* synthesis, but would behave, once released, as translation activator(s).

Extensive genetic analyses of many independent mutants specifically defective for cytochrome *f* expression have shown the involvement of two, and probably only two, *petA*-specific OTAFs: MCA1 primarily controls the accumulation of the transcript (Gumpel et al., 1995; Lown et al., 2001; Loiselay et al., 2008) while TCA1 is required for its translation (Wostrikoff et al., 2001; Raynaud et al., 2007). Their possible involvement in the CES behavior of cytochrome *f* required further understanding of their function at the molecular level. Chloroplast transformation with modified *petA* genes showed that MCA1 acts on the very first 21 nucleotides of the *petA* transcript to protect it from 5'→3' exo-nucleases which are particularly active in the chloroplast (Drager et al., 1998; Loiselay et al., 2008). MCA1 and TCA1 recognize adjacent but distinct targets in the very 5' end of *petA* 5'UTR (Loiselay et al., 2008) and interact together, as shown by two-hybrid experiments in yeast and by co-immuno-precipitation, to form *in vivo* a high molecular mass complex that also contains the *petA* mRNA (Boulouis et al., 2011). They display partially overlapping functions in stabilization and translation of the *petA* mRNA. Indeed, TCA1 contributes to the stabilization of the transcript through its interaction with MCA1. Conversely, a modified *petA* transcript whose stability does not require the presence of MCA1 still relies on TCA1 for translation but also shows a 10-fold reduced translation in the absence of MCA1. Thus, MCA1, although not strictly required for

translation, behaves as a *translational enhancer*, presumably by assisting the binding of TCA1 to its own target. MCA1 and TCA1, together with the *petA* transcript, can be regarded as the *petA gene expression system* controlling the rate of cytochrome *f* synthesis (Loiselay et al., 2008; Boulouis et al., 2011).

In transformed strains expressing varying amounts of MCA1 or TCA1, *petA* mRNA accumulation and translation were correlated with the concentration of MCA1 and TCA1, respectively, suggesting that these two proteins are limiting for cytochrome *f* expression (Raynaud et al., 2007). In exponentially growing cells, the abundance of MCA1 sets the size of the pool of *petA* transcripts, which can be further selected for cytochrome *f* synthesis upon formation of the MCA1/TCA1/*petA* ternary complex. While TCA1 is quite stable with a half-life longer than the doubling time of the culture (8 h), MCA1 displays a half-life of ~2 h. Thus, MCA1, as a short-lived translation enhancer whose abundance varies rapidly depending on physiological conditions (Raynaud et al., 2007; Wei et al., 2014), is a key factor in the regulation of cytochrome *f* expression. Major clues on its regulatory role in the CES process came from the study of the signal leading to its degradation. MCA1 proteolysis is triggered by its interaction with unassembled cytochrome *f* that transiently accumulates during the biogenesis of the cytochrome *b₆f* complex. Strikingly, the interaction between MCA1 and unassembled cytochrome *f* relies on the very same residues that form the CES repressor motif. Mutations that disrupted the CES repressor motif also prevented the degradation of MCA1, which became as stable as TCA1 and over-accumulated by ~10-fold compared to strains expressing wild-type cytochrome *f*. Thus, the CES process for cytochrome *f* synthesis results from the regulated proteolysis of its translation enhancer, MCA1, triggered by its interaction with unassembled cytochrome *f*, thereby preventing sustained re-initiation of *petA* mRNA translation (Figure 19.4B; (Boulouis et al., 2011)). The regulatory role of MCA1 in the expression of its target gene suggests a wide participation of OTAFs to the other CES processes.

D. General mechanism for the CES process?

Besides being a well-characterized CES protein, cytochrome *f* is also archetypal as the regulation of its synthesis recapitulates the behavior of most CES processes:

1) Regulation of translation initiation

In all instances reported so far, changes in CES protein synthesis were not

correlated with changes in mRNA levels (Drapier et al., 1992; Khrebtukova and Spreitzer, 1996; Choquet et al., 1998; Wostrikoff et al., 2004; Minai et al., 2006; Drapier et al., 2007; Wietrzynski et al., 2021), suggesting that the regulation operates at a (post-)translational level. Experiments performed with reporter genes translated under the control of the 5'UTRs of CES genes consistently led to the same observations: 5'CES-driven reporters were less expressed upon compromised assembly of the CES subunit, even though the reporter protein was unrelated to the protein complex whose assembly was investigated. Thus, as for cytochrome *f*, regulation of some early step of translation initiation is responsible for the reduced synthesis of the CES subunits from PSI (PsaA and PsaC: (Wostrikoff et al., 2004)), PSII (D1 and CP47: (Minai et al., 2006)), ATP synthase CF₁ (subunits α and β : (Drapier et al., 2007)) or RuBisCO (LS: (Wietrzynski et al., 2021)) upon defective protein assembly and the presence of the unassembled CES subunit.

2) Autoregulation of synthesis: a general mechanism for the CES process.

At variance with cytochrome *f*, most CES subunits in *Chlamydomonas* are polytopic membrane proteins, thus identifying the putative repressor motif is not straightforward. Nevertheless, two different hypotheses could account for the reduced synthesis of a CES protein in the absence of its assembly partner. Either the unassembled CES subunit exerts a *negative feedback* on its own translation, as described above for cytochrome *f*, or the assembly partner is required – directly or indirectly – to stimulate the synthesis of the CES subunit, in a *transactivation* mechanism. As the protein expressed from a reporter gene that is fused to a CES-5'UTR recapitulates the behavior of the CES protein itself, i.e. reduced expression in absence of assembly partners, one can discriminate between the two hypotheses by assessing their expression in the combined absence of the CES subunit and of its assembly partner. In the case of a transactivation mechanism, translation of the chimeric gene should be low in absence of the trans-activating partner, irrespective of the presence of the CES protein. By contrast, in case of a negative feedback mechanism, the reporter gene should remain highly expressed when the CES protein carrying out the autoregulation is missing, whether the assembly partner is present or not.

The expression of the CES subunit can be prevented by replacing its coding sequence by a variant encoding a mutated or truncated version of the CES protein that

is prone to rapid proteolytic degradation (Choquet et al., 1998; Wostrikoff et al., 2004; Minai et al., 2006; Drapier et al., 2007; Wietrzynski et al., 2021), rather than by deletion of its full gene or mutation of its cognate OTAF. This helps to avoid artifacts due to the titration of OTAFs present in limiting concentration (as observed for the accumulation of 5' *atpA*-driven endogenous and chimeric transcripts (Drapier et al., 2007)) and precludes possible dual effects of these OTAFs on several subunits of the same complex (see section III F1b).

In strains expressing these unstable protein versions, reporter genes translated under the control of the 5'UTRs for PSI or PSII CES subunits were consistently highly expressed, whether the assembly partners (D2/D1 or PsaB/PsaA, respectively) were present or not (Wostrikoff et al., 2004; Minai et al., 2006). Thus, as was the case for cytochrome *f*, synthesis of CES subunits from PSI or PSII is not trans-activated by their assembly partners, but is down-regulated when CES subunits accumulate in an unassembled state.

E) CES, a versatile regulatory process that depends on the particular traits of each biogenesis pathway.

While CES processes controlling the biogenesis of PSI, PSII and cytochrome *b₆f* are mediated by the unassembled CES protein itself, assembly intermediates, rather than the unassembled CES subunit alone mediate the translational repression during the biogenesis of the ATP synthase (Drapier et al., 2007) (see chapter 17) or RuBisCO (Wietrzynski et al., 2021) (see chapter 7).

1) ATP synthase.

Unlike PSI, PSII and cytochrome *b₆f*, whose assembly proceeds, as described above, by sequential accretion of subunits in a 1:1 stoichiometry to the growing protein complex, the biogenesis of ATP synthase involves the formation of two independent modules, the soluble CF1 that catalyzes ATP synthesis, and CF_o, a membrane-embedded proton-specific channel; these modules each contain subunits in unequal stoichiometry.

a) CES and CF1 biogenesis

CF1 comprises five subunits, α , β , γ , δ , and ϵ , present in a 3:3:1:1:1 stoichiometry. Among these, subunits α and β are both CES proteins whose rates of

translation, respectively, depend on the presence of β and of γ subunits. Down-regulation of their synthesis upon defective assembly relies on a control of translation initiation mediated by the *atpA* and *atpB* 5'UTRs, respectively (Drapier et al., 2007). However, in contrast to the CES subunits described above, the synthesis of the α subunit is not auto-regulated, but *trans*-activated by its assembly partner, subunit β . Indeed, a reporter gene translated under the control of the *atpA* 5'UTR remains poorly expressed in the combined absence of both α and β subunits. This unique regulatory mechanism probably originates from the propensity of the unassembled α subunit to form aggregates (Ketchner et al., 1995; Du and Gromet-Elhanan, 1999; Lefebvre-Legendre et al., 2005; Drapier et al., 2007; Rak et al., 2011), which makes it unavailable for autoregulation.

As another unusual characteristic, translation of *atpB* mRNA is not merely auto-regulated. Fully unassembled β subunit does not down-regulate its own synthesis: despite a significant accumulation of unassembled β subunit, translation of the *atpB* mRNA (or of 5'*atpB*-driven reporters) remained high in mutant strains lacking expression of both subunits α and γ (Drapier et al., 2007). A negative feedback on the synthesis of subunit β is only observed when a CF₁ assembly intermediate comprising both α and β subunits (either the catalytic hexamer $\alpha_3\beta_3$ or α/β dimers in equilibrium with the hexamer) accumulates. This regulatory mechanism sets the accumulation of subunits α and β to a level three times higher than that of their assembly partners in CF₁ since the negative feedback exerted by α/β assembly intermediates on the translation of subunit β is only released upon assembly of subunit γ with $\alpha_3\beta_3$ hexamers.

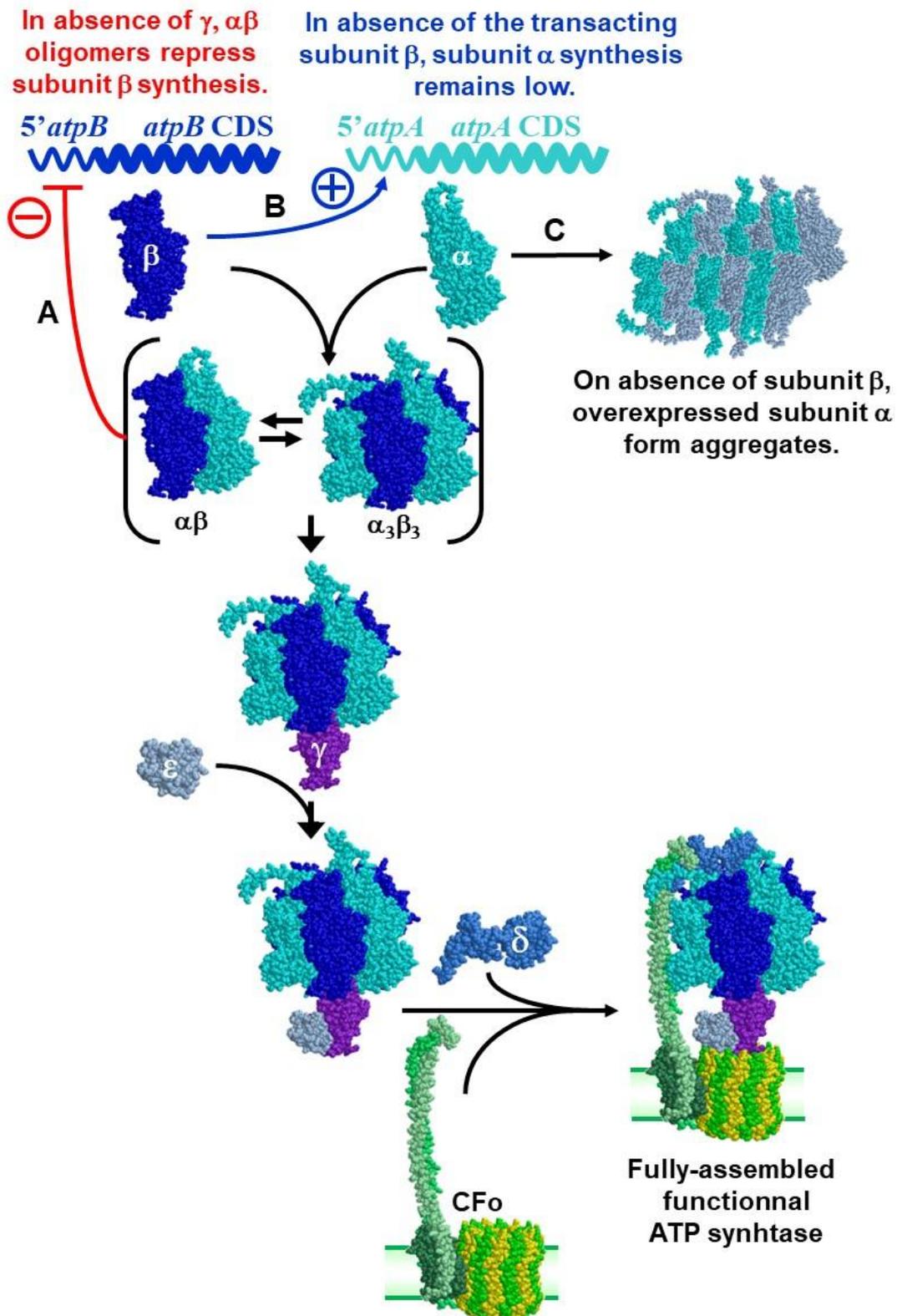


Figure 19.5: CES processes in the biogenesis of ATP synthase CF1

A) In the absence of subunit γ , $\alpha\beta$ oligomers accumulate and repress the translation of *atpB* mRNA. B) In the absence of subunit β , the synthesis of subunit α is reduced because subunit β is required to activate in *trans* the translation of *atpA* mRNA. C) Indeed, in the absence of subunit β , subunit α , when expressed over a certain

threshold forms aggregates and would thus become unavailable to repress its own synthesis.

Credit: Yves Choquet

These intertwined translational feedback loops (see Figure 18.2) are well suited to control the uneven accumulation of CF₁ subunits by allowing a tight control of the relative α/β stoichiometry: an excess of subunit β will *trans*-stimulate translation of subunit α until stoichiometry is reached again, while reduced accumulation of subunit β will prevent new synthesis of subunit α and deplete the pool of α/β assembly intermediates, increasing the rate of synthesis of subunit β . These regulatory features also ensure tight control of the nucleus on the biogenesis of CF₁ solely through the synthesis of subunit γ .

b) Biogenesis of CF_o

The ATP synthase CF_o sector also contains subunits present in unequal stoichiometry since it is formed of a tetradecameric ring of AtpH subunits, associated with one AtpI subunit to form the selective proton channel that binds a peripheral arm comprised of one copy each the AtpF and ATPG subunits (Seelert et al., 2003; Meyer Zu Tittingdorf et al., 2004; Hahn et al., 2018; Yang et al., 2020). This raises question about the mechanism ensuring the relative stoichiometry of the various subunits.

At variance with cyanobacteria and higher plants, where the chloroplast *atpI* and *atpH* genes are adjacent, in *Chlamydomonas*, they belong to distinct transcription units but nevertheless still show some co-regulation in expression as exemplified by the phenotype of the nuclear mutant *ac46* (Levine, 1960), lacking the ATP synthase because of the absence of the *atpH* mRNA (Majeran et al., 2001). However, besides the absence of the *atpH* gene product, this mutant also shows a specific reduction in the rate of synthesis of AtpI (Lemaire and Wollman, 1989), suggesting a possible CES relationship between the synthesis of the two proton hemi-channels. That these two subunits were not part of a CES regulation process came with the demonstration that the synthesis of the AtpI subunit remained unaffected in a Δ *atpH* deletion strain from *Chlamydomonas* (Ozawa et al, 2020). The phenotype of the *ac46* strain was then explained by the nature of the mutated gene, *MTHII* (for Maturation and Translation of the *atpH* and *atpI* mRNAs), which encodes a bifunctional protein required to stabilize the *atpH* mRNA and activate its translation, but also to increase the translation of the *atpI* transcript, while contributing to its stability (Ozawa et al.,

2020). This is a very atypical property for a *Chlamydomonas* OTAF, which, in general, controls the expression of only a single chloroplast gene.

The MTH1 protein is an OPR protein, which recognizes a nonanucleotide motif on its *atpH* and *atpI* mRNA targets (see chapter 13). Thus the *atpH* and *atpI* transcripts compete for binding with MTH1 and this common dependence for a single OTAF as well as their different affinity for MTH1 allow for close coordination of their rates of synthesis, in the appropriate stoichiometry (Ozawa et al., 2020).

Both the nonanucleotide motif and the MTH1 protein are remarkably conserved through evolution in Chlorophyceae and the Ulvaceae clade of Ulvophyceae. This is at variance with most other OTAFs, which evolve rapidly and are only conserved in species closely related to *C. reinhardtii* (the *reinhardtii* clade). It suggests that the development of an OTAF with two chloroplast mRNA targets as a strategy for concerted expression of two chloroplast-encoded subunits of the same oligomeric protein represents an efficient alternative to the CES process. This strategy may be used in other instances or organisms such as in maize chloroplasts where the concerted synthesis of two cytochrome *b₆f* subunits, cytochrome *f* and subunit IV - which are part of a CES process in *Chlamydomonas*-, is explained by the action of CRP1, a bifunctional OTAF (Schmitz-Linneweber et al., 2005).

2) RuBisCO

Although RuBisCO is the simplest photosynthetic complex in terms of subunit composition - being made of only two subunits, LS and SS, respectively encoded by the *rbcL* chloroplast gene and by a small family of two adjacent nuclear genes, *RBCS1* and *RBCS2* - its assembly, and especially the folding and oligomerization of LS, is a complex process that requires several dedicated chaperones. As detailed in Chapter 7, RuBisCO biogenesis involves several steps, some of which remain to be established with certainty in *Chlamydomonas*: nascent LS, first taken in charge by the CPN60/20/10 chaperonin, then undergoes a head-to-head dimerization possibly assisted by the RBCX assembly factor. The dimer then interacts with RAF1, another LS specific chaperone and further oligomerizes into the LS₈ octamer, still associated with RAF1. In a last step, this octamer assembles with SS, which may itself be stabilized by its interaction with the RAF2 chaperone to form the biologically active LS₈SS₈ hexa-decameric holoprotein, with the concomitant release of the RAF1 and RAF2 chaperones.

LS is a CES subunit, whose rate of synthesis and accumulation level decrease

significantly in the absence of its SS assembly partner (in the $\Delta rbcS$ genetic context, where both adjacent *RBCS* genes are deleted), but the residual amount of LS (about 1 %) is stable over 4 h. It is found in three high molecular mass complexes. The large one (>720 kDa) corresponds to the CPN60 chaperonin-bound LS, while two smaller complexes (~200 and ~720 kDa) contain the RAF1 chaperone and are stable over 4 h in the $\Delta rbcS$ genetic context, accounting for the stability of unassembled LS in absence of SS (Wietrzynski et al., 2021). Based on their apparent molecular masses determined from immunoblots and on analyses of LS mutants, these species were attributed to LS₂-RAF1₂ and LS₈-RAF1_x assembly intermediates. Indeed, the LS₂-RAF1₂ complex was absent in a mutant where the head-to-head dimerization of LS was prevented because of mutation of two residues, E109 and R253, involved in the formation of stabilizing salt bridges between the two monomers. It was still present in another site-directed mutant, where LS can still dimerize but cannot further oligomerize into the LS₈ octamer because of mutations that change residues A₁₄₃, R₂₁₅ and D₂₁₆ compromise binding at the interface between adjacent LS dimers. This mutant, in turn, lacks the LS₈-RAF1_x complex.

Here again, down-regulation of LS synthesis in the absence of SS relies on the *rbcL* 5'UTR and thus operates at the level of translation initiation. In strains expressing a truncated and unstable version of LS, the synthesis of this short LS version, or of the protein encoded by a 5' *rbcL*-driven reporter gene were high, even in absence of SS, excluding a trans-activation mechanism and pointing to an autoregulation mediated by LS unassembled forms: either the monomer, the dimer or the octamer. In the above-described octamerization mutant, the synthesis of LS remained high in the absence of SS showing that the repression of LS synthesis is not mediated by the LS₂ dimer but by the LS₈-RAF1_x complex (Wietrzynski et al., 2021). This involvement of the last assembly intermediate before the binding of SS ensures tight coordination of the synthesis of the two RuBisCO subunits, even though encoded in two distinct genetic compartments.

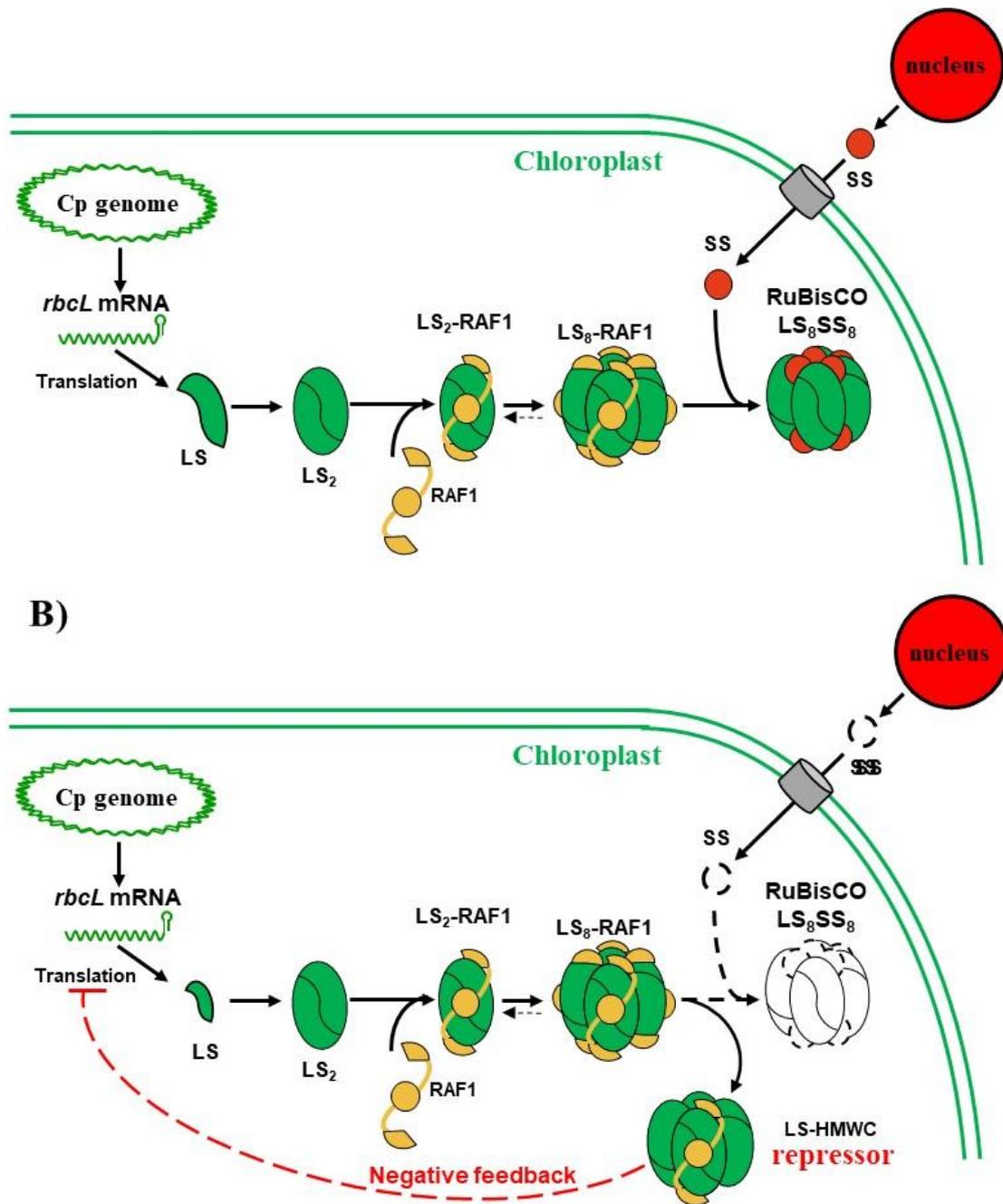


Figure 19.6: *CES control of rbcL mRNA translation*

A) Simplified pathway of RuBisCO biogenesis.

Only the participation of the RAF1 chaperone, involved in the LS CES process, is shown in this simplified assembly pathway (for a more detailed description of RuBisCO assembly see Chapter 7 and Fig. 7.1 therein). After translation of the *rbcL* mRNA, the nascent LS subunit dimerizes and recruits RAF1, then further oligomerizes into the LS₈ octamer still associated with RAF1, which is released upon assembly with SS to form the holoenzyme.

B) The CES control of *rbcL* mRNA translation.

In absence of SS, the last intermediate in the assembly pathway before the binding of SS, the LS₈-RAF1 octamer, is converted into a high molecular weight complex repressing, by a mechanism which remains to be elucidated, the translation of the *rbcL* mRNA.

Credit: Yves Choquet

CES is thus a versatile regulatory process, whose actual molecular mechanism

depends on the specific features of the biogenesis of each photosynthetic oligomeric protein.

IV. Is CES central to organelle protein expression?

Originally discovered and studied in *Chlamydomonas*, CES has now been shown to be a general feature of organelle gene expression since it contributes to the biogenesis of PSII and RuBisCO in higher plants and also of cytochrome oxidase, cytochrome *bc*₁ and ATP synthase in yeast mitochondria.

A. CES in the chloroplasts of vascular plants.

In higher plants, technical limitations in the determination of the rate of synthesis of chloroplast-encoded polypeptides as well as the lack of plant mutant lines specifically defective for expression of a single chloroplast-encoded subunit, have long hampered a systematic assessment of the contribution of CES processes to the biogenesis of chloroplast proteins.

1) CES regulation of LS synthesis.

Only in 2007 was Rubisco LS shown to be a typical CES protein in higher plant, as its synthesis is reduced in RNAi or antisense tobacco plants with decreased SS expression (Rodermeil et al., 1996; Wostrikoff and Stern, 2007). Although accumulating to the same level as in wild-type plants, there was less *rbcL* mRNA loaded on polysomes, suggesting a regulation of translation initiation (Wostrikoff and Stern, 2007). Similarly, there was less *rbcL* mRNA associated with polysomes in maize RNAi lines expressing a much lower amount of SS (Wostrikoff et al., 2012), showing that LS is a CES protein in C4 as in C3 plants. As in *Chlamydomonas*, the CES regulation for LS relies, in tobacco, on a negative feedback mechanism. In plant lines in which the stable accumulation of LS was compromised (because they expressed a truncated and unstable version of LS), and the expression of SS was reduced by RNAi, the rate of translation, and the polysomal association of *rbcL* mRNA remained high (Wostrikoff and Stern, 2007). Thus, the combined absence of the CES protein and of its assembly partner leads to high translation rates of the *rbcL* mRNA which, as explained in III.D.2 excludes the trans-acting model and supports a negative feed-back regulation. Considering the conservation across species of most

molecular actors in play (RBCX, RAF1, RAF2, but not BSD2), it is timely to understand whether the mechanism described for the LS CES process in *Chlamydomonas* also operates in land plants.

Whether CES would explain the differential expression of LS in bundle sheath and mesophyll cells of C4 plants is still unclear. In mesophyll cells, where *RBCS* mRNA is barely detectable, *rbcL* mRNA accumulation and LS expression are low, possibly because of the occurrence of CES in absence of the SS assembly partner. In agreement with this hypothesis, in the maize *bsd2* mutant, the accumulation and polysome association of *rbcL* mRNA were increased in mesophyll cells, compared to the wild-type line (Brutnell et al., 1999), probably because improperly folded LS could not properly repress its own synthesis, as observed in tobacco. However, LS synthesis could not be restored by ectopic expression of SS in mesophyll, nor does ectopic expression of both SS and a nucleus-encoded version of LS result in RuBisCO accumulation (Wostrikoff et al., 2012). Additional work is needed to understand whether the inhibition of *rbcL* translation in C4 mesophyll cells is due to a CES-related process.

2) CES controls of PSII biogenesis.

In higher plants, a possible CES contribution to PSII biogenesis was first suggested by the phenotype of the *vir*¹¹⁵ mutant of barley which primarily affects, in a developmentally regulated manner, the translation of D1 (Kim et al., 1994), but also shows a down-regulation of CP47 expression (Gamble and Mullet, 1989). Furthermore, an *Arabidopsis* mutant lacking the HCF173 *psbA* translation activator showed a reduced loading of *psbB* mRNA onto polysomes (Schult et al., 2007). Similarly, in *Arabidopsis*, CP47 synthesis was decreased in the absence of HCF107, which is required for the maturation of a translatable form of the *psbH* mRNA (Felder et al., 2001; Hammani et al., 2012). Interestingly, expression of a nucleus-encoded PsbH protein in the *hcf107* mutant background released the inhibition of CP47 synthesis, indicating that the defect in CP47 synthesis is not due to a dual action of HCF107 on both *psbH* and *psbB* mRNA, but rather that CP47 is a CES protein in plant whose translation requires the presence of PsbH (Levey et al., 2014).

The recent development of ribosome profiling methods, by providing genome-wide information on the translational status of individual transcripts (Zoschke et al., 2013), has overcome the previous limitations of studies investigating translation. It

has allowed a more systematic assessment of CES processes in plants and, for example, it confirmed the CES status of RuBisCO LS in both tobacco and *Arabidopsis* (Ghandour, 2020). Most interestingly, these methods also revealed a reduced translation efficiency of *psbA*, *psbB*, and *psbH* mRNA in a tobacco *psbD* knock-down mutant (Ghandour, 2020), further suggesting that the PSII CES cascade described in *Chlamydomonas* has been conserved through evolution.

The regulation of D1 synthesis has attracted attention for a long time because, even under low light conditions, the conversion of light energy by PSII damages the D1 subunit (Keren et al., 1995); this is the price paid for generating the highly reactive molecular species required to split water and generate dioxygen. D1 damage is proportional to the intensity of incident light and leads to photoinhibition (Chapter 25) and, in plants exposed to high light, its high rate of turnover leads *psbA* to be, by far, the most actively translated chloroplast transcript.

Activation of *psbA* mRNA translation depends only on one RNA-binding protein, HCF173 (Schult et al., 2007; McDermott et al., 2019; Watkins et al., 2020; Gawroński et al., 2021). The newly synthesized D1 interacts with SRP54 (Nilsson and van Wijk, 2002; Hristou et al., 2019) which delivers it to the ALB3 insertase, and upon insertion it interacts with the luminal HCF136 chaperone (Meurer et al., 1998; Plucken et al., 2002; Komenda et al., 2008; Nixon et al., 2010). The later transfers it to the OHP1/OHP2/HCF244 complex, which is involved in pigment binding to nascent D1 and the assembly of D1 into a D1-less PSII core (Link et al., 2012; Hey and Grimm, 2018; Li et al., 2019; Hey and Grimm, 2020). This OHP1/OHP2/HCF244 complex, also involved in the initiation of *psbA* mRNA translation (Chotewutmontri et al., 2020), interacts not only with the D1-less PSII core but also with the HCF136/D1 complex on the luminal side and with the HCF173/*psbA* mRNA complex on the stromal side of the thylakoid membranes (Myouga et al., 2018).

The above knowledge of the main actors in D1 biogenesis proved instrumental in a better understanding of PSII repair upon photoinhibition. In plants, the *psbA* mRNA ribosome occupancy drops in the dark, and is restored within a few minutes after a dark to light shift, indicating regulation at the level of translation initiation (Chotewutmontri and Barkan, 2018). The translation of *psbA*, poorly induced by photosynthetic-active wavelengths, is highly stimulated by low doses of UVA, which destroys the oxygen-evolving complex and highly damages D1 (Chotewutmontri and Barkan, 2020). In *hcf136* mutants, which are impaired at an early step of D1

assembly, the ribosome occupancy of *psbA* mRNA is constitutively high, even in the dark (Chotewutmontri and Barkan, 2020; Chotewutmontri et al., 2020). The OHP1/OHP2/HCF244 transient complex, which cannot bind D1 in the *hcf136* mutant and is required for *psbA* translation activation appears central in this regulated coupling of D1 synthesis to the extent of photodamage. By a mechanism which remains to be elucidated, the OHP1/OHP2/HCF244 complex would, when loaded with D1, prevent activation of *psbA* mRNA translation by HCF173. This inhibition would be released when the bound D1 is delivered to a D1-less PSII core; these features are typical of a CES process, which is dependent on a protein assembly-dependent negative-feedback (Chotewutmontri and Barkan, 2020; Chotewutmontri et al., 2020). Interestingly, mutants with compromised *psbA* translation (*ohp1*, *ohp2*, *hcf244*, and *hcf173*) also showed lower *psbB* mRNA ribosome occupancy than in wild-type cells (Trösch and Willmund, 2019; Chotewutmontri et al., 2020). Even if this effect is much milder than the 10-fold decrease of *psbB* translation observed in *Chlamydomonas*, this suggests, as evoked in the above discussion, a conservation in higher plants of D1 CES control over CP47 synthesis.

The above-described experiments were conducted on differentiated chloroplasts, where D1 synthesis mostly serves the PSII repair cycle, but this mechanism likely accounts as well for the coupling between D1 synthesis and PSII biogenesis during chloroplast maturation. Most molecular actors in play, HCF136 (Cre06.g273700 in *Chlamydomonas*, Ycf48 in cyanobacteria), OHP1 (Cre02.g109950, Light-harvesting-like Lil2 in cyanobacteria), OHP2 (Cre06.g251150, Lil6), HCF244 (Cre02.g142146: CGL102, Ycf39) and HCF173 (Cre13.g578650: CPLD10) are conserved through evolution in algae and land plants as well as in cyanobacteria. However, among them, HCF173, the *psbA* translation activator which is central to the autoregulation of D1 translation, while well conserved between plants and algae, does not seem to have a true orthologue in cyanobacteria. It probably appeared and acquired its regulatory properties after the endosymbiotic event.

Whether this autoregulatory mechanism fully accounts for the CES control of *psbA* translation in *Chlamydomonas* remains to be studied. Indeed, in this alga, previous studies of the up-regulation of D1 synthesis upon photoinhibition did not support a regulatory mechanism involving translation initiation, as the expression of 5'*psbA*-driven reporters do not increase under high light exposure (Minai et al., 2006). This may be due to the spatial separation of D1 synthesis for PSII repair relative to de novo

PSII assembly (Uniacke and Zerges, 2007), which would take place in the T zones near the pyrenoid (see section II.B, this chapter). By contrast, repair of D1 during the recovery from photoinhibition occurs in stromal or margin thylakoids, which is required for D1 assembly within D1-less PSII cores (Kettunen et al., 1997; Uniacke and Zerges, 2007). Similarly, *de novo* biogenesis of PSII complexes in cyanobacteria occurs in plasma membranes or in specialized “biogenic centers” closely associated to it (Zak et al., 2001; Rengstl et al., 2011; Stengel et al., 2012; Rast et al., 2019), while repair of photodamaged complexes takes place in the thylakoid membrane (Sacharz et al., 2015). To our knowledge, such spatial segregation of PSII repair and biogenesis has not been observed in plants, where the two phenomenon are rather temporally separated in developing and mature chloroplasts (Chotewutmontri and Barkan, 2016).

3) CES control of the biogenesis of other photosynthetic complexes.

Whether cytochrome *f*, which is a CES protein in *Chlamydomonas* (section III C), behaves similarly in higher plant chloroplasts has been investigated using transplastomic tobacco lines harboring deletions of *petD* or *petB* genes. These plants fail to accumulate the whole set of cytochrome *b₆f* subunits and accumulate cytochrome *f* at very low levels. It was expected that in these mutants, the *petA* coding sequence, which is the last one of a polycistronic transcript that also contains *psaI*, *ycf4*, and *cemA*, would be loaded onto polysomes. Nevertheless, in these strains, the *petA* mRNA was shifted to polysomes of smaller size when compared to wild-type plants (Monde et al., 2000b), but still larger than those observed in the *crp1* maize mutant, fully defective for *petA* mRNA translation (Barkan et al., 1994). A definitive conclusion regarding the CES status of cytochrome *f* in higher plants would probably require the characterization of transplastomic lines that express a cytochrome *f* variant insensitive to the CES process because of a mutation in its repressor domain, which is fully conserved between *Chlamydomonas* and higher plant cytochrome *f* (Choquet et al., 2003).

It is of note, however, that several CES processes described in *Chlamydomonas* are not conserved in plants. As an example, maize mutants lacking expression of the β subunit of ATP synthase only display a very minor (less than two-fold) reduction in the translation of the *atpA* mRNA (Zoschke et al., 2013), as compared to its strong reduction in a similar *Chlamydomonas* mutant.

B. CES and the biogenesis of respiratory proteins in yeast mitochondria

CES also contributes to the biogenesis of respiratory complexes in the mitochondria of yeast, as shown by studies of the assembly of the cytochrome oxidase (COX), cytochrome *bc*₁ and ATP synthase complexes (see below). Thus, as in the chloroplast of *Chlamydomonas*, the biogenesis of all bioenergetics complexes that contain organelle-encoded subunits involves CES processes in the mitochondria of yeast.

1) The Cox1p CES subunit

The assembly of the COX complex involves the formation of three independent modules, organized around the three mitochondrion-encoded core subunits Cox1p, Cox2p and Cox3p that bind the metal and redox cofactors of the complex. Each complex comprises a specific and distinct subset of nucleus-encoded COX subunits (McStay et al., 2013c; McStay et al., 2013b; Su et al., 2014; Franco et al., 2018). The Cox1 module appears central in COX assembly and acts as a seed, onto which the Cox2, then Cox3 modules assemble. Cox1p is a *bona fide* CES subunit, whose rate of synthesis is strongly decreased in yeast strains deficient in other COX subunits (Cabral and Schatz, 1978; Poutre and Fox, 1987; Calder and McEwen, 1991; Nakai et al., 1995; Barrientos et al., 2004; Fontanesi et al., 2010; Fontanesi et al., 2011), or expressing the full complement of COX subunits but lacking COX assembly factors (Barrientos et al., 2004). Furthermore, Cox1p, although synthesized in a markedly reduced amount, is stable when COX assembly is compromised, while Cox2p and Cox3p are synthesized at wild-type levels but are rapidly degraded (Calder and McEwen, 1991; Nakai et al., 1995; Barrientos et al., 2004). Strikingly, newly synthesized Cox1p interacts physically with Mss51p, one of the *cox1* translational activators, acting at both initiation and elongation steps (Perez-Martinez et al., 2003; Zambrano et al., 2007) and with Cox14p and Coa3p (Mick et al., 2010; McStay et al., 2013a; Mayorga et al., 2016), two COX assembly factors. Cox14p and Coa3p stabilize the interaction between Mss51p and Cox1p (Perez-Martinez et al., 2003; Barrientos et al., 2004) and *cox14* mutants show a normal rate of Cox1p synthesis, even in the absence of COX assembly. Once sequestered in this complex, as depicted in Figure 19.6B, Mss51p is prevented from initiating new rounds of Cox1p synthesis until it is released in a free, translationally-active, form upon further Cox1p assembly catalyzed by Shy1p, another COX assembly factor (Mick et al., 2007). This is

reminiscent of the autoregulation of *petA* translation by the C-terminal domain of cytochrome *f* in *Chlamydomonas* chloroplasts. Indeed, deletion of the last 11 matrix-exposed, C-terminal residues of the polytopic Cox1p protein did not prevent its assembly into a functional COX complex but loosened the interaction between Cox1p and Mss51p and abolished the assembly-controlled regulation of *cox1* translation (Shingú-Vázquez et al., 2010). Site-directed mutagenesis more precisely identified residues P₅₂₁, P₅₂₂ and V₅₂₄, conserved through evolution between yeast and mammals, as essential for the interaction with Mss51p and for the CES control of Cox1p synthesis (Figure 19.6A; (García-Villegas et al., 2017).

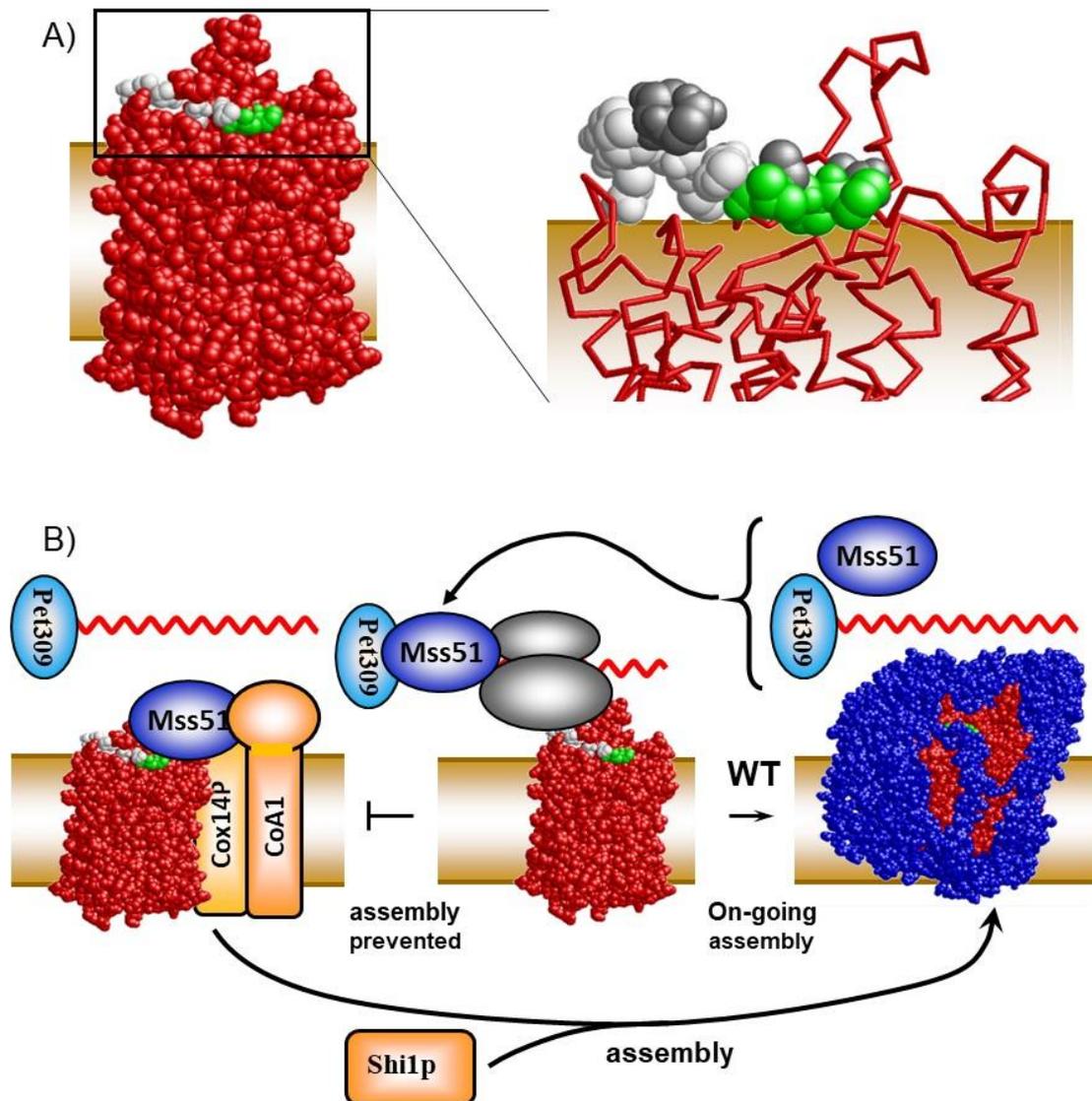


Figure 19.7: *CES control of Cox1p synthesis in the mitochondria of yeast.*

A) *The translation repressor domain from Cox1p.*

Schematic representation of the Cox1p protein, that also zoom in on its C-terminal domain (drawn from PDB entry 6YMY). Residues shown in space-fill are in green when substitutions

abolish the Cox1p translational regulation, in dark grey if substitutions have no effect on the CES control of Cox1p synthesis or in pale grey when not tested.

B) *Model for the autoregulation of cox1 mRNA translation.*

cox1-specific translational activators (Mss51 and Pet309) are shown in purple and blue, while COX assembly factors are represented in orange. Mss51 binds to the repressor motif exposed by unassembled Cox1p to form a complex stabilized by the assembly factors Cox14p and CoA1 and becomes unavailable for initiating new rounds of translation. Upon assembly with other COX subunits, catalyzed by Shy1, Mss51 becomes free to activate translation, together with Pet309. For the sake of simplicity, the effect of Mss51 on translation elongation has not been represented in this figure.

Credit: Yves Choquet

2) CES control of the biogenesis of ATP synthase

Despite its limited coding capacity, the mitochondrial genome of yeast encodes other CES proteins. Indeed, the synthesis of the mitochondrion-encoded subunits Atp6p and Atp8p is highly reduced in mutants lacking expression of Atp9p (Jean-Francois et al., 1986; Ooi et al., 1987; Payne et al., 1991; Bietenhader et al., 2012). Furthermore, the rate of synthesis of both Atp6p and Atp8p is also strongly reduced, despite unaltered accumulation of the bicistronic *atp8-atp6* mRNA, in mutants defective for F1 assembly whether lacking the Atp1p (α) or Atp2p (β) subunits, or the Atp11p and Atp12p nucleus-encoded chaperones that are required for F1 assembly (Rak and Tzagoloff, 2009). An *arg8^m* reporter gene recoded for mitochondrial gene expression, substituting either *atp6* or *atp8* coding frames conferred arginine auxotrophy in a wild type background, but was too poorly expressed in absence of F1 to sustain the growth of yeast in the absence of arginine in the medium, demonstrating a regulation of *atp6* and *atp8* expression at the level of translation initiation (Rak and Tzagoloff, 2009). The expression of both subunits in the absence of F1 can be restored either by overexpression of Atp22p, the translation activator of *atp6* or by deletion of Smtp1p, a ribosome-associated protein that, in its free form binds to the *atp8-atp6* mRNA to prevent its translation, presumably by competing with Atp22p for binding to the transcript (Rak and Tzagoloff, 2009; Rak et al., 2016). The authors proposed that F1 would be required to activate in *trans* the synthesis of Atp6p and Atp8p since expression of Atp6p and Atp8p remained high in the *atp8* and *atp6* deletion strains respectively (Rak et al., 2007; Rak and Tzagoloff, 2009). However, the down regulation of *atp6* and *atp8* translation in the absence of F1 could alternatively result from a negative feedback mediated by a complex comprising both Atp6p and Atp8p. ATP synthase assembly involves an early assembly intermediate containing, in addition to Atp6p and Atp8p, the Atp10p and Atp23p chaperones, with

the latter being also involved in proteolytic processing of the Atp6p precursor bound to the INA Complex (Innner membrane Assembly Complex), which is composed of two integral proteins, Ina22 and Ina17. Interestingly, the absence of any of the Ina subunits increases the translation of *atp6* and *atp8* more than two fold (Naumenko et al., 2017), which is consistent with a negative feed-back on their translation from the assembly intermediate complex.

Whatever the actual mechanism(s) involved in these down-regulations of Atp6/8 expression, it prevents the formation of an unassembled Atp9p/Atp6p proton channel, which, in the absence of F1, would be lethal due to proton leakage and cancelation of the membrane potential (Duvezin-Caubet et al., 2006; Godard et al., 2011). The mechanisms ensuring the stoichiometric expression of CFo subunits in the mitochondria of yeast and in the chloroplast of *Chlamydomonas*, which have evolved independently after two different endosymbiotic events, thus exhibit very distinct features. This may be due to a higher proteolytic activity in chloroplasts than in mitochondria. Indeed, in *Chlamydomonas*, CF1 mutants synthesize CFo subunits at a wild-type rate, but do not accumulate them at any detectable level, thereby preventing proton leaks. In contrast, *Chlamydomonas* mutants lacking expression of any ATP synthase subunit, but α and δ , show a reduced rate of synthesis of subunit β , and to a lesser extent, of subunit α (YC, DD: unpubl. obs.). This regulatory step, which could not have a counterpart in the mitochondria of yeast since both subunits α and β are nucleus-encoded, probably evolved to limit the hydrolysis of ATP by partially assembled CF1.

3) Autoregulation of cytochrome *b* synthesis.

The synthesis of cytochrome *b*, the central subunit of complex III (cytochrome *bc*₁), also is autoregulated by its status of assembly in yeast mitochondria. Expression of the mitochondrial *cob* gene encoding cytochrome *b* relies on several OTAFs: Cpb1p binds to the 5' end of the mature *cob* transcript to protect it from exonucleolytic degradation (Chen and Dieckmann, 1997), while Cbs1p and Cbs2p, required for its translational activation, interact with the *cob* 5'UTR and also with the large subunit of the mitoribosome (Krause-Buchholz et al., 2004; Krause-Buchholz et al., 2005). In addition, Cbp3p, a cytochrome *bc*₁ assembly factor, and Cbp6p, another *cob* translational activator, form a complex which plays a dual role in cytochrome *b* biogenesis and partitions between the mitoribosome and cytochrome *b* assembly

intermediates (Gruschke et al., 2011; Gruschke et al., 2012). Newly synthesized apocytochrome *b* is co-translationally loaded on this complex, bound to the exit channel of the ribosome, where it undergoes a sequential two-step hemylation process. It first binds heme *b*₁ and Cbp4p, another cytochrome *bc*₁ assembly factor, then heme *b*_h, with the concomitant release of the Cbp3p/Cbp6p complex, displaced by the nucleus-encoded Qcr7 small subunit of the cytochrome *bc*₁ complex. The Cbp3p/Cbp6p complex thus becomes available again to activate *cob* mRNA translation. When one of these hemylation steps fails, either because of heme depletion or because of a mutation of the histidine axial ligands of the *b*-type hemes, then the Cbp3p/Cbp6p complex remains trapped on cytochrome *b*, which arrests complex III biosynthesis (Hildenbeutel et al., 2014). Although required for *cob* mRNA translation, Csb1p also acts as a *cob* translational repressor: bound to the ribosome peptide exit tunnel it sequesters the *cob* mRNA away from the ribosome small subunit, thereby preventing translation initiation. *cob* mRNA translation can only resume when it is displaced by the Cbp3p/Cbp6p complex, released from the fully hemylated cytochrome *b*. These translational regulation processes, thus, have the genuine features of a CES process, whereby translation of apocytochrome *b* is driven by downstream assembly steps requiring its conversion to holocytochrome *b*.

Most interestingly, mutations of the UQCC2 proteins, the human orthologue of Cbp6p, which interacts with UQCC1, the Cpb3p orthologue, also cause complex III deficiency in humans and a severe defect in cytochrome *b* synthesis and/or stability, as shown by pulse labeling experiments. This suggests that at least some of these assembly-controlled translational autoregulation processes are conserved in mammals as well (Tucker et al., 2013).

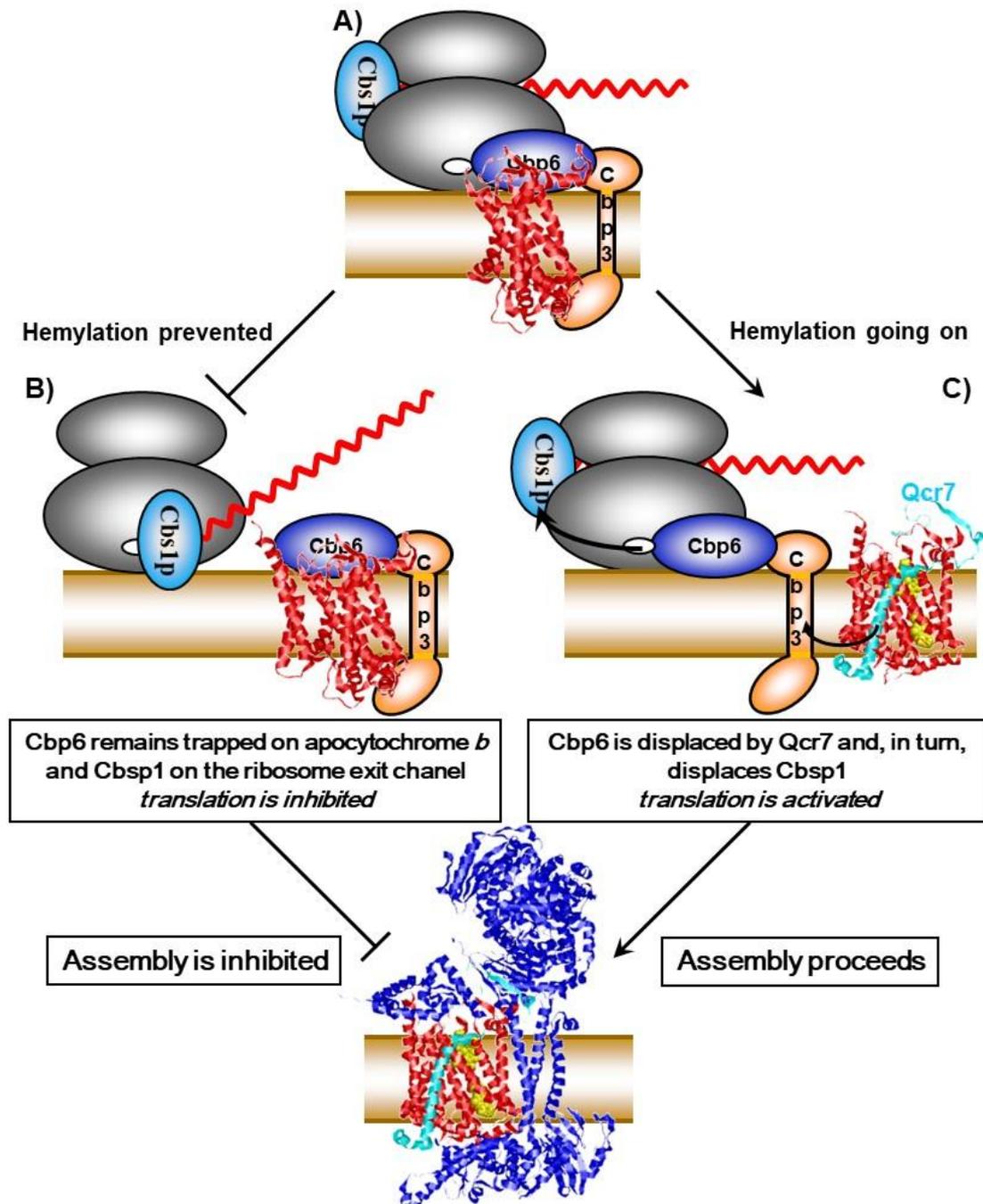


Figure 19.8: *CES control of cob mRNA translation in yeast mitochondria.*

A) Cytochrome *b* translated from the *cob* mRNA is co-translationally taken in charge by a complex made of the Cbp6 translational activator and of the Cbp3 chaperone.

B) As long as apocytochrome *b* is not fully hemylated (or when hemylation is prevented) the Cbp3/6 complex remain bounds to apocytochrome *b*, while Cbsp1, a *cob* translation activator is trapped on the ribosome exit channel, sequestering the *cob* mRNA away from the translation initiation site. Cytochrome *b* synthesis is arrested and further steps of cytochrome *b* assembly are prevented.

C) Following hemylation (hemes are shown in yellow), Qcr7, a structural component of the cytochrome *bc*₁ complex, binds to holocytochrome *b* and releases the Cpb6/3 complex, which, in turn, displaces the Cpsb1 factor, allowing *cob* mRNA to be translated again and assembly of cytochrome *bc*₁ proceeds.

Credit: Yves Choquet

V. Physiology of the CES process

A. Specificity of CES autoregulation and roles of OTAFs

Apart from CES proteins, the majority of translational autoregulations described in the literature involve RNA-binding proteins, such as ribosomal subunits, translation factors or viral proteins (reviewed in (Kozak, 2005; Bachand et al., 2006; Babitzke et al., 2009; Betney et al., 2010; Mikhaylina et al., 2021)). In their unassembled state, these proteins most often bind a structural element of their own 5'UTR that mimics their target RNA, thereby preventing the formation of the translation initiation complex.

The only chloroplast-encoded CES subunit that interacts with RNA is the RuBisCO LS subunit, whose N-terminal domain shows structural similarities with “ferredoxin-like” RNA-binding domains (Yosef et al., 2004). Highly conserved, from purple bacteria to higher plants (Cohen et al., 2006), this domain, normally buried in assembled RuBisCO, becomes exposed upon a conformational change triggered by oxidative stress conditions (Yosef et al., 2004). Whether it could be involved in the CES control of LS synthesis is unclear. Indeed, this domain does not show sequence-specificity (Yosef et al., 2004), a requirement for the CES process. However, since *rbcL* translation is restricted to the periphery of the pyrenoid (Uniacke and Zerges, 2009; Zhan et al., 2018), in a region different from the T zones where the initial steps of PSI and PSII biogenesis occur, the local enrichment of unassembled LS and *rbcL* mRNA in this specialized compartment may still allow an interaction that would inhibit further LS synthesis.

All other chloroplast or mitochondrial CES subunits have no known RNA-binding properties. Their sequences, constrained by their function in light capture, electron transfer and energy conversion, are highly conserved from cyanobacteria lacking CES processes to CES-proficient algae and higher plants. By contrast, the nucleotide sequences of the CES 5'UTRs show considerable variation between organisms, arguing against their specific recognition by the highly conserved CES proteins. Moreover, the regulatory motifs identified in *Chlamydomonas* cytochrome *f* (Choquet et al., 2003) or yeast Cox1p (Shingú-Vázquez et al., 2010; García-Villegas et al., 2017) definitely lack RNA-binding features. More generally, the repressor motifs that signal the unassembled state of CES subunits should be exposed in the

stroma (matrix), where they can interact with the chloroplast (mitochondrial) translation machinery and be shielded upon assembly. Indeed, MCA1, the trans-acting factor that has a bi-functional action of mRNA protection and translation activation for the *petA* gene, is targeted for degradation in absence of cytochrome *f* assembly within cytochrome *b₆f* complexes, due to its interaction with the translational repressor motif at the C-terminus of unassembled cytochrome *f* (Boulouis et al, 2011).

Based on this example, it is tempting to suggest that CES processes would most often rely on trapping, by translational repressor motifs exposed by unassembled CES subunits, of OTAFs, eventually associated with assembly factors required for the biogenesis of the CES subunit. Indeed MCA1, HCF173/OHP1/OHP2/HCF244, Mss51p/Cox14p and Cbp3p/Cbp6p, whose action on cytochrome *f*, D1, yeast Cox1 and cytochrome *b* synthesis have been described above, provide the best examples of such molecular mechanisms. Repressor motifs, as well as the OTAFs/assembly factors that are components of the regulatory mechanism, remain to be identified for the large and polytopic CES subunits in PSI and PSII. We hypothesized that they would preferentially localize to the C-terminus of the polypeptide, as do those identified in *Chlamydomonas* cytochrome *f* (Choquet et al., 2003) and in yeast COX1 (Shingú-Vázquez et al., 2010; García-Villegas et al., 2017), to provide a “quality control” for full-length synthesis of the CES subunits, preventing premature translation products from inhibiting the synthesis of active CES subunits.

B. Contribution to cellular fitness?

The contribution of CES to cellular fitness has not yet been properly assessed. Mutations in CES repressor motifs that abolish CES without preventing complex assembly or function, such as those identified in *Chlamydomonas* cytochrome *f* (Choquet et al., 2003) and in yeast COX1 (Shingú-Vázquez et al., 2010; García-Villegas et al., 2017), should allow this point to be addressed in the future. However, it should be stressed that the CES process, most easily observed in assembly-defective mutants, also operates in a wild-type context to fine-tune the synthesis of the CES proteins to the availability of their assembly partners. This has been documented in *Chlamydomonas* by the threefold overexpression of cytochrome *f* in variants of cytochrome *f* lacking the C-terminal repressor domain, when compared with the level of cytochrome *f* synthesized in the wild type cells (Kuras et al., 1995b; Choquet et al.,

1998; Choquet et al., 2003), or by the higher expression of 5'-CES-UTR-driven reporter genes in the absence of assembly competent CES subunits regardless of the presence of their assembly partners (Minai et al., 2006; Drapier et al., 2007; Wietrzynski et al., 2021). The absence of unassembled CES subunits prevents the repressed expression of the reporter gene. As an exception, the expression of *psaA* 5'UTR-reporter genes was similar in strains expressing or lacking PsaA. Most probably, the concentration of unassembled PsaA, which can bind up to 40 chlorophyll molecules, has to be kept very low in the wild type to avoid the production of damaging radicals.

However, the physiological advantage conferred by some of the above-described cases of CES regulation is clear. This is most obvious for the regulatory mechanism that sets the synthesis of D1 to the extent of photodamage during the recovery of photoinhibition (Chotewutmontri and Barkan, 2020). The fitness of a strain that escapes the CES regulation of RuBisCO LS synthesis, because *rbcL* coding sequence was expressed under the control of the *psaA* 5'UTR has not been tested. However, in strains that escape LS CES control of synthesis because they express truncated and unstable LS subunits, the truncated subunits remain bound in a high molecular weight chaperonin complex comprising pn60/Cpn20/Cpn10 (Majeran et al., 2019). Strikingly, the chloroplast *clpP1* gene encoding a major subunit of the Clp protease cannot be attenuated in these strains, suggesting that these truncated subunits, if not properly degraded, poison the Cpn60 chaperonin. By contrast, *clpP1* can readily be attenuated in the $\Delta RBCS$ nuclear context (Majeran et al., 2019), showing that the CES-reduced synthesis of LS prevents this poisoning.

CES represents an optimal strategy for the tight control of the rate of production of chloroplast-encoded subunits in order to facilitate their assembly within a functional complex. Depending on light quality and physiological conditions, the relative cellular demand for *de novo* biogenesis of photosynthetic proteins should vary (Chow et al., 1990). With the here described regulatory mechanism, the nucleus needs only to control the rate of expression of the most upstream assembly partner in a biogenesis pathway, with CES cascades then ensuring the stoichiometric production of the other subunits. For example, an increased expression of *psbD*, encoding D2 should trigger synthesis of D1 and CP47 *via* the hierarchical control of *psbD* translation over that of *psbA*, and *psbB* mRNAs. Indeed, a mutation of the *psbD*

5'UTR that destabilizes a secondary structure sequestering the initiation codon, increased the rate of synthesis and the accumulation of D2. Since unassembled D2 is rapidly degraded *in vivo* (de Vitry et al., 1989), its increased accumulation in the mutant indicates it should be protected from degradation by assembly. As a result, that strain shows a moderate, but significant, increase in the photoautotrophic growth rate (Klinkert et al., 2006).

VI. CES is a post-endosymbiosis innovation

At variance with the extensive contribution of CES processes to protein biogenesis in bioenergetic organelles, currently there is no evidence for CES processes in cyanobacteria.

Because cytochrome *b₆f* and ATP synthase complexes are essential in cyanobacteria, deletion strains are not available to assess the participation of CES in their biogenesis. However, in contrast to what is observed in *Chlamydomonas*, unassembled and unprocessed pre-apo-cytochrome *f* accumulates to a high level in the thylakoid of a *Synechococcus elongatus* mutant which shows decreased heme binding to apo-cytochrome *f*, with no indication of autoregulation of translation (Tichy and Vermaas, 1999). Congruent with this lack of autoregulation in cyanobacteria is the finding that, the aromatic F₃₀₇, which is a critical residue of the CES repressor domain in cytochrome *f* from *Chlamydomonas*, is highly conserved in cytochrome *f* from green lineage organisms, but it is absent in the cyanobacterial sequences where it is replaced by aliphatic residues (V or I) (Choquet et al., 2003). In *Chlamydomonas*, substitution of F₃₀₇ by a valine prevents the CES process (Y. C.: unpubl. result). This supports the view that cytochrome *f* acquired its CES regulatory properties following the initial endosymbiosis event.

Similarly, mutations preventing cyanobacterial PSII assembly because of defective expression of one core subunit had no effect on the rate of transcription or translation of the remaining subunits (Yu and Vermaas, 1990; Komenda et al., 2004). Evidence includes the findings that a *Synechocystis* deletion strain lacking expression of D2 still showed significant synthesis of D1, and mutations preventing *psbA* expression did not down-regulate expression of *psbB* (Komenda et al., 2004). Still, a *Synechocystis* mutant lacking cytochrome *b₅₅₉* because of a deletion of the *psbEFLJ* operon -PsbE and PsbF being the split cytochrome *b₅₅₉* apo-protein - also lacks

detectable synthesis of D2, while expressing D1 normally (Komenda et al, 2004). In *Chlamydomonas* as well, the synthesis of D2, D1, CP47 and CP43 may be much reduced in absence of PsbE (Morais et al., 1998), even though this result (Minai et al., 2006) and the status of cytochrome *b₅₅₉* in the CES cascade remain to be confirmed.

CES most likely arose as a post-endosymbiosis *de novo* innovation because of the primary role of nucleus-encoded OTAFs, which allowed the host cell to take control over organelle gene expression. These OTAFs rarely have a counterpart in cyanobacteria, where gene expression is mainly – although not exclusively – regulated at the transcriptional level. Still CES may have expanded in organelles from some ancestral cyanobacterial features. In this case, we would expect the original translational regulations in cyanobacteria to obey a somewhat different mechanism, for example a direct binding of the unassembled protein to the cognate mRNA instead of an assembly-dependent conditional trapping of a translational activator. Whether the synthesis of RuBisCO LS, the only CES protein showing RNA-binding properties (see below section V.A), is controlled by assembly has not been studied yet in cyanobacteria.

VII. Conclusion and Future Directions.

The properties of the CES process further confirms the prevalent role of translation as a major regulatory step in the expression of chloroplast genes, as was previously proposed (Eberhard et al., 2002). CES probably relies extensively on nucleus-encoded gene-specific factors, the OTAFs. Their participation in CES remains to be further substantiated in most cases, which represents a major research goal for the future. The actual regulatory function of some of these OTAFs has been demonstrated only recently (Raynaud et al., 2007; Boulouis et al., 2011; Schwarz et al., 2012; Lefebvre-Legendre et al., 2015); see also Chapter 14). Many should turn out to be critical for the assembly-controlled translational autoregulation in organelles.

The examples of RuBisCO LS in *Chlamydomonas*, of D1 in higher plants, of Cox1p and cytochrome *b* in the mitochondrion of yeast also suggest that assembly intermediates comprising assembly factors, rather than the CES subunits alone, mediate the negative feedback on the translation of their own mRNA. A number of assembly factors have been characterized in higher plants, with more than 20 assembly factors required for the biogenesis of the PSII complex (Lu, 2016).

Comparatively, only a few assembly factors have been identified in *Chlamydomonas* (Park et al., 2007; Heinnickel et al., 2013; Heinnickel et al., 2016; Wittkopp et al., 2018; García-Cerdán et al., 2019; Cecchin et al., 2021; Nellaepalli et al., 2021; Spaniol et al., 2021), probably because research has for long focused on stringent phenotypes, such as an absolute requirement for acetate, while mutations of assembly factors often result in leaky phenotypes.

The study of such assembly factors in *Chlamydomonas* should provide not only a better understanding of the assembly of photosynthetic proteins, but also provide clues on the molecular mechanism of CES processes. A proper description of the landscape of protein assembly factors across the green lineage should offer new perspectives regarding the biogenesis of photosynthesis proteins. Indeed, the complement of assembly factors may differ in green algae and plants: the recently identified PSII associated factor PBA1 (Cre01.g042200) is specific to Chlorophyceae (Spaniol et al., 2021), while the plant PSII assembly factors PD16 (Wittenberg et al., 2014), and HCF243 (Zhang et al., 2011) have no counterpart in the current version of the *Chlamydomonas* genome. Even the conserved assembly factors may have a different function in plants and algae, such as Ycf4, strictly required for PSI biogenesis and phototrophic growth in *Chlamydomonas* (Boudreau et al., 1997) but dispensable in plants (Krech et al., 2012).

Finally, one should consider CES as reflecting one of the modes of nuclear control of chloroplast gene expression. Not only would it have co-evolved with the production of nuclear *trans*-acting factors in organelle gene expression, but it also couples the expression of chloroplast genes to that of nucleus-encoded subunits. This is particularly striking for the biogenesis of Rubisco and of CF₁ from the ATP synthase, where delivery of the nucleus-encoded subunits – SS and subunit γ , respectively – turns out to act as rate limiting in the expression of the other chloroplast-encoded subunits of the same protein complex.

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