

## Chapter 2

# Chloroplast Gene Expression—Translation

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**Abstract** Translation has often been shown to represent the rate-limiting step of chloroplast gene expression. Genetic and biochemical analyses indicate that numerous nucleus-encoded protein factors in concert with their cognate target sites on chloroplast mRNAs are involved in determining protein-specific synthesis rates. In this chapter, we summarize the constituents of the chloroplast translational apparatus as well as the molecular principles underlying its spatiotemporal regulation.

**Keywords** Protein synthesis • Chloroplast translation • Chloroplast translational apparatus • Translation factors • T-zones • Helical repeat proteins

### Abbreviations

CES	Control by epistasy of synthesis
CPSGs	Chloroplast stress granules
HCF	High chlorophyll fluorescence
LDMs	Low density membranes
NTRC	NADPH-dependent thioredoxin reductase C
OPR	octotricopeptide repeat
PABP	Poly(A) binding protein
PET	Photosynthetic electron transport
PPR	Pentatricopeptide repeat
PSI	Photosystem I
PSII	Photosystem II
PSRPs	Plastid-specific ribosomal proteins
RRF	Ribosome recycling factor
SD	Shine-Dalgarno

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TMs	Thylakoid membranes
TPR	Tetratricopeptide repeat
UTRs	Untranslated regions

## 2.1 Introduction

Due to their endosymbiotic origin, chloroplasts contain their own gene expression machinery which is basically of prokaryotic origin. Although this system serves the synthesis of only less than ca. 100 proteins, it is crucial for mainly maintaining photosynthetic functions but also others like, e.g., fatty acid synthesis (see Chap. 1). One intrinsic complication for the control of plastid gene expression is the necessity of coordination with nuclear gene expression because most of the chloroplast protein complexes are assembled from subunits which are encoded either in the nucleus or in the plastid genome. As we know today, this coordination is mainly mediated by nucleus-encoded factors which control almost all steps of chloroplast gene expression ranging from transcription and RNA metabolism to translation and protein complex assembly [for recent overviews see 4 and Chap. 2 of this issue; 101, 115].

Despite the fact that all plastid gene expression steps appear to be dependent/affected by nucleus-encoded trans-acting factors, especially chloroplast translation has been considered to play a key role for determining the levels of photosynthetic proteins. This idea is based on findings that often chloroplast mRNA levels are not limiting for protein synthesis and, moreover, protein levels can vary considerably for instance in response to light, whereas the corresponding mRNA levels remain mostly constant [22, 50]. Furthermore, it should be taken into account that translation per se represents a highly energy consuming process which demands tight regulation to maintain cellular energy economy [123].

In line with this, several aspects of translational regulation in chloroplasts have been addressed in recent reviews [53, 71, 114, 123]. Here, we summarize recent developments and outcomes to provide a state-of-the-art view on the various facets of chloroplast protein synthesis. Aspects of chloroplast translation that are related to the connection between protein synthesis and the biogenesis and assembly of photosynthetic complexes will not be addressed here but are covered in Chap. 13 of this issue. The same holds for the biotechnological aspects of optimized chloroplast translation which will be reviewed in Chap. 20.

## 2.2 Technical Considerations for Measuring Translational Activities in Chloroplasts

The combined use of both genetic and biochemical approaches has dramatically improved our current knowledge on the molecular details of chloroplast translation. This includes the identification of the basic translational machinery, gene-specific

**Table 2.1** Cloned nucleus-encoded factors for chloroplast translation from higher plants and *C. reinhardtii*

Factor	Homologies	Target gene(s)	Organism	Reference
cPDI	Protein disulfide isomerase	<i>psbA</i>	<i>C. reinhardtii</i>	Kim and Mayfield [41]
cPAB1	Poly(A)-binding protein	<i>psbA</i>	<i>C. reinhardtii</i>	Yohn et al. [117]
TBA1	Oxidoreductase	<i>psbA</i>	<i>C. reinhardtii</i>	Somanchi et al. [98]
HCF173	Short Chain Dehydrogenase	<i>psbA</i>	<i>A. thaliana</i>	Schult et al. [90]
DLA2	E2 subunit pyruvate dehydrogenase	<i>psbA</i>	<i>C. reinhardtii</i>	Bohne et al. [9]
TBC2	OPR protein	<i>psbC</i>	<i>C. reinhardtii</i>	Auchincloss et al. [2]
NAC2	TPR protein	<i>psbD</i>	<i>C. reinhardtii</i>	Boudreau et al. [11]
RBP40	–	<i>psbD</i>	<i>C. reinhardtii</i>	Schwarz et al. [91]
AC115	–	<i>psbD</i>	<i>C. reinhardtii</i>	Rattanachaikunsopon et al. [79]
TAB2	ATAB2	<i>psaB</i>	<i>C. reinhardtii</i>	Dauvillée et al. [19]
ATAB2	Tab2	PSI and PSII subunits	<i>A. thaliana</i>	Barneche et al. [5]
CRP1	PPR protein	<i>petA/petD</i>	<i>Z. mays</i>	Fisk et al. [26]
TCA1	–	<i>petA</i>	<i>C. reinhardtii</i>	Raynaud et al. [80]
PGR3	PPR protein	<i>petL</i>	<i>A. thaliana</i>	Cai et al. [15]
PR10	PPR protein	<i>atpH</i>	<i>Z. mays</i>	Pfalz et al. [74]
TDA1	OPR protein	<i>atpA</i>	<i>C. reinhardtii</i>	Eberhard et al. [23]
CRR2	PPR protein	<i>ndhB</i>	<i>A. thaliana</i>	Hashimoto et al. [30]

regulatory protein factors as well as—at least for some of these—their cognate target RNA elements on chloroplast transcripts. Furthermore, the relatively broad spectrum of model systems which have been utilized for analysing chloroplast gene expression has enabled researchers to compare translational processes/principles from single-celled microalgae like *Chlamydomonas reinhardtii* to vascular plants like *Arabidopsis thaliana* or *Zea mays*.

## 2.2.1 Genetic Approaches

### 2.2.1.1 Model Organisms

The identification of nucleus-encoded factors involved in chloroplast translation started with systematic forward genetic screens of photosynthetic mutants from *C. reinhardtii*, *A. thaliana* and maize. Defects in photosynthetic activities are characterized by, e.g., increased chlorophyll fluorescence and, thus, give rise to the so-called *hcf* (high chlorophyll fluorescence) phenotype. Some of these *hcf* mutants were subsequently found to be affected in the synthesis of distinct chloroplast proteins (Table 2.1). The half-a-tetratricopeptide repeat protein HCF107 was the first factor identified by this approach in *A. thaliana* [86]. In *C. reinhardtii*, however, photosynthesis is dispensable provided that cells are fed by a reduced carbon-source, namely

acetate. As a consequence, photosynthetic mutants are fertile and accessible to further genetic manipulations like crossings and/or easy selection of suppressor strains which paved the way for genetic dissection of more complex regulatory networks [123].

With the comprehensive determination of the entire genome sequences of all abovementioned model systems together with dramatically improved mass spectrometrical detection of low abundant regulatory factors of chloroplast gene expression, nowadays, reverse genetic approaches more-and-more come to the fore to decipher the function of distinct protein factors for chloroplast translation. These include RNAi-based methods as well as the screening of mutant libraries for the identification of knock-out lines [76, 89].

### 2.2.1.2 Chloroplast Reporter Genes

With the development of efficient chloroplast transformation techniques for *C. reinhardtii* as well as tobacco more than 20 years ago, the targeted manipulation of chloroplast genomes at least for these two species became easily feasible [13, 105]. This methodological breakthrough resulted in the identification of numerous cis-acting elements—mainly located within the 5′ untranslated regions (UTRs) of plastid mRNAs—which affect chloroplast translation based on chimeric reporter gene assays. Reporters for measuring plastid translation rates include classical heterologous genes like *aadA*, *uidA*, GFP or *lucCt* (luciferase) as well as endogenous coding regions from *petA* and *atpA* [for an overview see 45, 123].

Unfortunately, no efficient chloroplast transformation systems for either *A. thaliana* or maize are available to date, hence, a genetically combined analysis of both nuclear mutants as well as transgenic chloroplasts harbouring chimeric reporter gene constructs is still hampered. Currently, transplastomic lines in different nuclear mutant backgrounds can only be generated in *C. reinhardtii* and, indeed, this has accelerated the identification of the interplay and fine mapping of regulatory cis-acting elements and the recognizing translational factors.

## 2.2.2 Measurement of *in vivo* Protein Synthesis

### 2.2.2.1 Pulse Labelling of Proteins

To quantitate translational activities more directly, labelling of proteins via the metabolic incorporation of  $^{35}\text{S}$  or  $^{14}\text{C}$  radioisotopes is routinely used for both algae and higher plants [e.g. 22, 67, 72]. Selective inhibition of cytoplasmic translation by cycloheximide treatment prior to labelling ensures detection of only chloroplast-synthesized polypeptides. However, as discussed by Zerges and Hauser [123] several limitations might affect pulse labelling assays. These include analyses of rapidly degraded proteins which might escape detection despite the fact that they are produced at normal rates. Furthermore, secondary effects of starvation phases

prior to addition of radioisotopes or varying uptake kinetics of radiolabel under different culture conditions or between different strains might interfere with the interpretation of data. In addition, efficient incorporation of label during the relatively short pulse phase of ca. 5–30 min. is restricted to those chloroplast proteins with a high synthesis rate such as major subunits of PSII or the Rubisco large subunit RbcL. However, weakly synthesized or very small proteins often are not labelled with sufficient efficiency to allow their unambiguous visualization on autoradiographs. Nevertheless, pulse labelling experiments have been widely used and allow a rapid initial evaluation of molecular phenotypes in a given set of photosynthetic mutants.

### **2.2.2.2 Polysomal Loading**

An alternative approach to estimate the translational status of a certain chloroplast mRNA is represented by so-called polysomal loading experiments [e.g. 90, 91]. These include the separation of high molecular weight polysome-fractions on appropriate sucrose gradients and the subsequent detection of particular transcripts within these fractions by either Northern or RT-PCR analyses. This technique enables solid conclusions on whether the translation of an mRNA is affected at the level of initiation (in this case no polysomal distribution is detected). However, blocks in elongation or termination phases or precise quantification of protein synthesis rates cannot be deduced from such data sets. Therefore, pulse labelling results are often required to complement polysomal analyses.

## **2.2.3 Reconstituted Systems**

### **2.2.3.1 In Vitro Translation Systems**

While the abovementioned methods are applicable to a variety of evolutionary distant model organisms, the analysis of chloroplast translation by in vitro systems is strictly restricted to tobacco chloroplasts. In 1996, Hirose and Sugiura for the first time reported on the successful preparation of translationally active chloroplast extracts which were capable of mediating accurate translation from exogenously added mRNA molecules [32]. In 2007, the same group introduced a non-radioactive improved version of this system which possesses a more than 100-fold higher protein synthesis activity [119]. By using these systems, several burning questions regarding translational processes have been answered as specified below. This underlines the power of a biochemical tool like an in vitro translation system for in-depth analysis of chloroplast translation and strongly argues for further efforts in establishing related systems for the abovementioned model systems.

### 2.2.3.2 RNA Binding Assays

Besides the thorough analysis of cis-acting determinants on plastid mRNAs, in vitro reconstituted systems can also be utilized to identify the regulative trans-acting protein factors by biochemical means [32]. Indeed, the analysis of RNA/protein interactions between chloroplast proteins and in vitro synthesized RNA probes from a huge variety of plastid genes by UV-crosslinking or gel shift assays has revealed the existence of numerous polypeptides interacting especially with the untranslated regions of plastidial mRNAs [for a review see 65]. Some of these proteins have been purified and identified by mass spectrometrical analysis creating the entry point of the abovementioned reverse genetic analysis of the plastid protein synthesis machinery (see Sect. 2.2.1.1).

## 2.3 Constituents of the Chloroplast Translational Apparatus

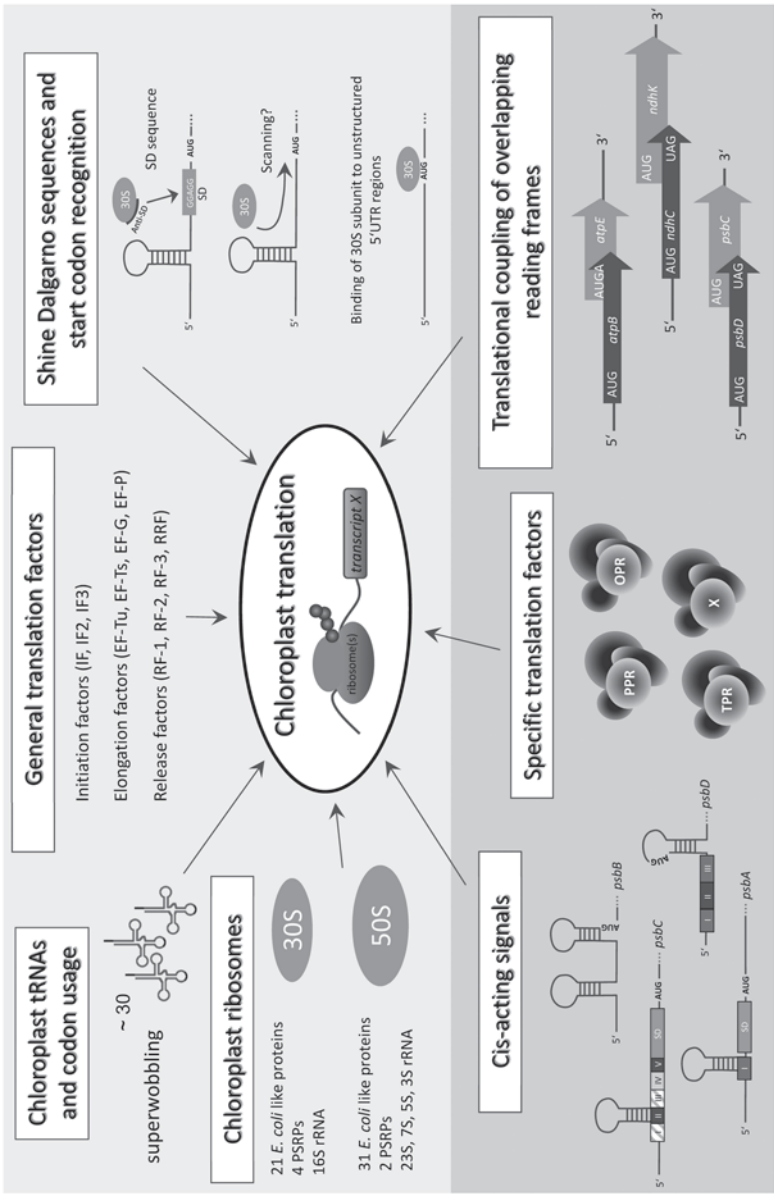
As outlined above the chloroplast translation machinery is of prokaryotic origin and, thus, it is basically assumed that the principle of its working mode and its regulation resembles those of bacteria. Due to evolutionary constraints imposed by the endosymbiotic lifestyle of chloroplasts, then, this machinery has been considerably modified by additional factors and principles which allowed the integration of the former cyanobacterium into an intracellular environment [6, 71]. This view is supported by the fact that many general constituents of this apparatus share strong homologies with their bacterial counterparts but additional—often gene-specific—regulators with novel molecular features entered the scene during endosymbiosis (Fig. 2.1).

### 2.3.1 Basic Elements and Factors

#### 2.3.1.1 Chloroplast tRNAs and Codon Usage

Chloroplasts like mitochondria do not encode an entire minimal set of 32 tRNAs which would be required to read all codons according to Crick's wobble rules. For instance the tobacco chloroplast genome contains 30 tRNA genes while the one of *C. reinhardtii* harbours only 29 [54, 96]. Recently, it has been demonstrated that superwobbling mechanisms compensate for this apparent deficiency, i.e., tRNAs with a non-modified U residue in the wobble position are capable of recognizing all four nucleotides in the third codon position [83]. However, superwobbling is accompanied by reduced translational efficiencies as indicated by targeted manipulations of tobacco plastid glycine tRNA genes [83].

Hence, tRNA levels and concomitant codon usage appear to represent crucial determinants for manifesting synthesis levels of distinct chloroplast proteins. Indeed,



**Fig. 2.1** Schematic illustration of factors and elements constituting the chloroplast translation machinery. Basic elements and general factors are delineated in the *upper* part. Gene-specific elements and factors involved in the translation of particular chloroplast transcripts are exemplified in the *lower* part. Illustration of PSII-specific cis-acting signals was adapted from Marin-Navarro et al. [53]. For further explanation see text

the codon usage in chloroplasts is different from that in prokaryotic and eukaryotic nuclear genomes. The question on a connection between codon usage and translational efficiencies has been further addressed by utilizing the above mentioned in vitro translation system from tobacco (2.2.3.1). In some cases, a clear correlation between translation rate and the usage of synonymous codons was observed, but surprisingly, frequent exceptions were also dismantled [63, 64]. This led to speculations that the frequent use of weakly translated codons like the chloroplast arginine codon CGA represent a mechanism to regulate translation, possibly in concert with other yet to be identified factors [64].

### 2.3.1.2 Chloroplast Ribosomes and General Translation Factors

As mentioned above chloroplast ribosomes—like mitochondrial ones—are of the bacterial-like 70S type and besides the ribosomal RNAs contain 58 proteins, 33 of these form part of the large 50S subunit and 25 build the small 30S subunit [8, 123]. 52 of them are orthologues to *E. coli* ribosomal proteins while six are chloroplast-specific and thus were named PSRPs (plastid-specific ribosomal proteins) [116]. Another plastid-specific feature of the otherwise prokaryotic ribosomes are extensions at the N- and C- termini of some of the ribosomal proteins, and consequently, plastid ribosomes are larger than their bacterial counterparts.

The very limited set of ca. 100 mRNAs being translated in chloroplasts together with the structural specialities of the ribosomal machinery led to speculations whether these apparent differences as compared to the bacterial translation systems reflect plastid-specific functions of ribosomes, e.g., light-regulated protein synthesis or thylakoid membrane associated translation of integral membrane proteins. Detailed cryo-electron microscopic analysis of ribosomes from spinach and *C. reinhardtii* has then revealed that PSRPs appear to fulfil more structural roles, e.g., by compensating for missing 16S rRNA elements [51, 94]. A recent reverse-genetic study with *A. thaliana* supports this notion in part and shows that PSRP3, 4 and 5 behave like typical classical ribosomal proteins. In contrast, a knock-down of PSRP2 and 5 does not result in a detectable phenotype in ribosome synthesis and translation [106].

On the other hand, plastid-specific extensions of the conserved ribosomal S21 protein from spinach or the S2 protein from *C. reinhardtii* were shown to be localized at the mRNA exit channel of the 30S subunit [51, 94]. Here, the initial interactions—like the recognition of the Shine-Dalgarno sequence that is located in the 5'UTR of mRNAs—take place and, thus, this region would be ideally suited to mediate chloroplast-specific translation initiation mechanisms. Supportive of that idea, UV-crosslinking experiments using purified ribosomes revealed that the plastid S2 protein directly interacts with RNA whereas the bacterial one does not [51]. Another ribosomal protein directly binding to 5' UTR sequences in both bacteria and plastids, namely S1, is closely localized to S2 at the mRNA exit channel. However, the plastid S1 is much smaller than the bacterial one and exhibits different RNA binding affinities thereby potentially enabling a plastid-style positioning of the ribosome on the mRNA [56, 97].



Additional outcomes of structural analyses of chloroplast ribosomes are factors that form transient interactions with ribosomes. These include a ribosome recycling factor (RRF) or the plastid-specific PSRP1 protein which shares homologies with the *E. coli* cold-shock protein pY [94]. Both inhibit translation by binding within the intersubunit space of 70S ribosomes where they block the mRNA and tRNA binding sites and are involved in storage and stabilization of ribosomes under stress conditions [95]. It has been proposed that PSRP1 might be involved in overall chloroplast translational regulation by balancing the pools of stored and actively translating ribosomes in a light-dependent manner [95].

In *C. reinhardtii*, two other proteins, named RAP38 and RAP41, have been identified which co-sediment with 70S particles [116]. These two proteins, exhibit homologies with the higher plant plastid RNA binding proteins CSP41a and CSP41b which were implicated with functions during diverse processes such as transcription, RNA stabilization and ribosome assembly [7, 10, 78]. However, neither CSP41a nor CSP41b from *A. thaliana* are associated with polysomal fractions leaving open the question on a direct function of these factors during translation [10].

In addition to 70S ribosomes, chloroplasts contain an entire set of 11 bacterial-type non-ribosomal translation factors which are involved in initiation of translation (IF, IF2 and IF3), elongation (EF-Tu, EF-Ts, EF-G and EF-P) of polypeptide chains and RF-1, RF-2, RF-3 and RRF for release/recycling of ribosomes [for an overview see 8, 123]. Interestingly, both RRF and EF-G have been postulated to act in concert with the abovementioned PSRP1 factor during reactivation of ribosomes after stress/dark-phases at least in spinach [95]. Expression profiles of these factors in various organisms suggest that they might also be involved in the light/redox-dependent induction of general chloroplast translational activities [53].

Mutations in the genes encoding the plastidial release factor 2 (*hcf109/Atp<sub>prf</sub>B2*) lead to a pleiotropic photosynthetic phenotype, since the synthesis of several plastid-encoded subunits belonging to different thylakoid membrane protein complexes is disturbed [57, 58]. In contrast, the knock-out of *Atp<sub>prf</sub>B3*, a PrfB-like protein, affects specifically the biogenesis of the cytochrome *b<sub>6</sub>f* complex. This protein/gene can only be found in the nuclear genomes of vascular plants and appears to be involved in the light, redox and stress control of this thylakoid membrane complex [102].

Plastid translational activity is essential for photosynthesis but other cellular functions can also depend on chloroplast-encoded proteins like, e.g., the D subunit of the acetyl-CoA carboxylase (AccD) which is required for fatty acid synthesis. Nevertheless, single ribosomal components/subunits might be dispensable as suggested by the absence of some of the ribosomal protein genes from non-photosynthetic parasitic plants. Recent systematic reverse genetic approaches using transplastomic tobacco lines now revealed that many but not all of these genes are essential. For instance, the ribosomal proteins from the large subunit, namely Rpl20, Rpl22, Rpl23, and Rpl32 as well as Rps2, Rps3, Rps4, Rps16, and Rps18 from the small subunit cannot be inactivated whereas deletion strains lacking Rpl33, Rpl36 and Rps15 can be generated [27, 82, 83]. The resulting phenotypes of knockout lines revealed a specialized function for Rpl32 during acclimation of tobacco plants to cold stress and for Rpl36 in control of leaf morphology [27, 83]. This suggests that chloroplast

translation influences nuclear gene expression presumably via retrograde signalling pathways documenting a tight integration of chloroplast translation into the overall cellular gene expression activity [27, 73].

### 2.3.1.3 Shine-Dalgarno Sequences and Start Codon Recognition

As outlined in detail by Zerges and Peled-Zehavi and Danon translation initiation, especially, the recruitment of the small ribosomal subunit and the subsequent recognition of the AUG start codon fundamentally differ between prokaryotes and eukaryotes [71, 121]. In the cytosol of eukaryotic cells, translation initiation follows the so-called scanning model which predicts the 40S ribosomal subunit to be bound immediately downstream of the mRNA's 5' cap structure via an interaction between the cap-binding eIF4F complex and other eIFs associated with the 40S subunit. This complex formation is assisted by a further interaction of the eIF4F complex with the poly(A) binding protein (PABP) bound to the mRNA's 3' poly A tail. Subsequently, the 40S subunit—with the help of an RNA helicase—scans the 5' UTR for the first appropriate AUG start codon in a 5' to 3' direction.

In contrast to this scenario, the small 30S ribosomal subunit of bacteria directly recognizes the AUG start codon. This recognition is mediated by the Shine-Dalgarno (SD) ribosome binding site, a short purine-rich stretch (GGAGG) ca. 10 nt upstream of the start codon which base pairs with the 3' end of the 16S rRNA moiety of the 30S ribosomal subunit. As a consequence, translation initiation does not require any scanning mechanisms and, thus, allows the simultaneous translation of multiple reading frames on polycistronic transcripts.

Chloroplasts with their cyanobacterial history were assumed to utilize the second, bacteria-like mechanism for translation initiation. In agreement with this, one third of chloroplast genes contain bona fide SD-sequences at an appropriate distance to the AUG start codon [103]. However, two thirds do not contain SD-elements what has provoked a long-standing discussion on the role of chloroplast SD-like sequences as well as of alternative cis-acting determinants during translation initiation [for recent overviews see 3; as well as references herein; 53, 71]. In brief, the targeted mutagenesis of chloroplast genes in *C. reinhardtii* and tobacco revealed transcript-specific effects of SD-mutations. For instance in case of the *psbA*, *psbD* and *psbC* mRNAs from *C. reinhardtii*, altered SD-sequences clearly affect protein synthesis rates while this does not hold for other transcripts like those from the *petD*, *atpB*, *rps14*, and *rps7* genes [24, 55, 67, 85, 126]. Similarly, in vitro translation assays with tobacco chloroplast extracts revealed a non-homogeneous picture with regard to the functionality of SD-sequences [34–36, 75].

In conclusion, the available data suggest multiple alternative pathways for translation initiation in chloroplasts apart from the “classical” SD-element-mediated one. Basically, these include three different mechanisms which might also occur in prokaryotes. First, it has been proposed that other regions of the 16S rRNA apart from those complementary to the SD-sequence base pair with elements in 5' UTRs of chloroplast mRNAs [48]. For instance in tobacco, the *psbA* 5' UTR contains two crucial elements for translation initiation (AAG and UGAUGAU) which are

positioned close to the start codon and have the capacity to base pair with the 3' end of the 16S rRNA [32]. Secondly, a scanning procedure related to the eukaryotic one described above was suggested by the preferential use of upstream start codons during the analysis of chloroplast reporter gene constructs [21, 34]. Finally, recent analyses revealed that the absence of secondary structure elements at translation initiation sites represents a critical determinant for enabling access of the translational apparatus to the start codon in the absence of SD-sequences in both bacteria and chloroplasts [87]. This is illustrated by the *hcf107* mutant of *A. thaliana* which is affected in *psbH* synthesis and hence deficient in photosystem II [25]. The defect in *psbH* translation is associated with a lack of all *psbH*-containing transcripts that are processed and have *psbH* as their leading cistron; only non-processed *psbH* transcripts accumulate in the *hcf107* mutant background. These non-processed *psbH* transcripts contain a stable stem and loop structure just in front of *psbH* that encompasses the translational start site. The translational start site becomes only available when the stem loop structure is cleaved and/or when the secondary structure is unfolded by the half-a-tetratricopeptide repeat protein HCF107 [25, 29]. Thus, unstructured single-stranded RNA regions in 5' UTRs might serve as landing pads for the 30S ribosomal subunit, and thus circumvent the need of precise base pairing of the 16S rRNA pairing via SD-elements.

### 2.3.2 *Gene Specific Elements and Factors for Translation Initiation*

The abovementioned general principles of translational control in chloroplasts already open a wide area for regulatory control possibilities which might resemble those in cyanobacteria. However, one particular outcome of recent scientific work on chloroplast biology is that the synthesis of many if not all chloroplast-encoded proteins is dependent on gene-specific translational regulators. These appear to be new inventions having embellished the basic prokaryotic apparatus during the evolutionary development of an organelle as discussed by Barkan [4]. Thus, these factors and their molecular working modes are likely to provide new insights of how gene expression machineries can be modified by the creation of new molecular principles in evolutionary terms, and thus represent a driving force for the development of eukaryotic cells. The phylogenetic development of chloroplast-specific translational mechanisms is likely to have occurred by a co-evolution of the two basic components of every gene expression system, i.e., the chloroplast-encoded cis-acting elements on mRNAs and their cognate interactors which are normally represented by nucleus-encoded polypeptides.

#### 2.3.2.1 *Cis-acting Signals on Chloroplast RNAs*

Translation initiation is generally considered as the rate-limiting step determining individual protein synthesis rates independent of the system under investigation

[46]. Therefore, most attention has been attracted by the 5' UTRs of chloroplast mRNAs where initiation takes place. However as mentioned above, the 3' UTRs of cytoplasmic mRNAs interact with their 5' regions and thereby promote translation initiation. Interestingly in some cases, also chloroplast translation appears to depend—at least to some extent—on 3' RNA regions suggesting that related principles of translational regulation occur also in the organelle [40, 84]. Whether this indeed represents a considerable general phenomenon remains to be shown by future scientific work.

The targeted manipulation of many 5' UTR regions from chloroplast genes in both *C. reinhardtii* and tobacco, chloroplast in vitro translation assays as well as RNA binding studies have revealed a complex picture involving various different cis-regulating RNA elements for plastid protein synthesis [for comprehensive reviews see 53, 71]. Intriguingly, these elements are often found close to elements required for the stabilization and/or processing of chloroplast mRNAs suggesting a tight correlation of these processes via 5' UTRs of plastid messages (see also III.B.2 and IV.C).

Basically, these regulatory elements fall into three categories, i.e., the above described prokaryotic elements like SD-sequences, elements which are recognized by specific trans-acting factors and elements that define secondary RNA structures which positively or negatively affect the entry of the translational apparatus, namely the ribosomal subunits. Often it cannot be clearly distinguished between these possibilities because the underlying molecular mechanisms involve a mixture of different determinants. Especially, stem-loop structures within 5' UTRs have attracted much attention. The first evidence for the role of such a hairpin-like element was obtained for the *C. reinhardtii psbC* gene encoding the inner antenna protein CP43 of PSII. The stem region of the *psbC* translational element contains bulged residues due to imperfect complementarity which are critical for translational activity. Increased stability of the stem region by the introduction of mutations or a vice versa reduced stability in suppressor mutants led to reduced or rescued CP43 synthesis, respectively [81, 125].

Furthermore, conserved RNA stem-loop structures in the vicinity of the AUG start codon as well as an unstructured region located upstream have been shown to be critical for the translation of the *petD* mRNA in *C. reinhardtii* [31, 47]. Similar to a stem-loop close to the *psbA* SD-sequence in *C. reinhardtii*, these structures appear to serve as recognition signals for trans-acting regulatory factors. Other stem-loops appear to constitute negatively acting elements due to the fact that they incorporate crucial basic translational elements like the SD-sequence (if present) or the AUG start codon into their double stranded stem region thereby abolishing ribosomal access to the mRNA. These cases include, e.g., the *C. reinhardtii psbD* and *psbB* mRNAs with structured AUG start codons and the *C. reinhardtii psaB* and *Z. mays atpH* mRNA with affected SD-sequences [44, 77, 99, 113]. The stem-loop structure within the 5' UTR of *psbH* genes of higher plants is another example for a structured AUG codon [25, 29]. It is generally assumed that the conformation of these regions is released by directly or indirectly interacting protein factors. Hammani et al. provide convincing evidence that the binding of the he half-a-tetratricopeptide peptide repeat protein

HCF107 to its binding site within that stem-loop structure is required to open the secondary structure and thereby to enhance translation of *psbH* [64]. This view is further supported by genetic suppressor analyses affecting the *psbD* stem loop and by in vitro RNA binding data of the *atpH* 5' UTR as specified below [44, 77]. In case of the *psbD* and *petD* mRNA also unstructured elements were experimentally mapped which are required for translational activities. The same holds for distinct elements in the tobacco *psbA* 5' UTR which have been postulated to interact with the 16S rRNA thereby substituting for a lacking SD element [32].

Besides the signals in 5' UTRs, other crucial cis-acting determinants have been identified which are involved in the translation of overlapping reading frames of polycistronic plastid RNA transcripts. This appears particularly interesting because this gene organization raises questions as to whether a translational coupling exists between the reading frames similar to some bacterial or viral cases [39]. This would imply that the downstream cistron is translated exclusively by those ribosomes that completed the translation of the upstream cistron. Such a translational coupling usually leads to a rather low translation efficiency of the downstream cistron because many ribosomes are released at the stop codon of the upstream reading frame [37]. Recently three out of four cases of overlapping protein coding regions from the tobacco chloroplast genome, namely *ndhC-ndhK*, *atpB-atpE* and *psbD-psbC*, have been investigated in detail by using the tobacco in vitro translation system [1, 104, 120]. Interestingly, in all three cases the gene pairs encode subunits of the same chloroplast multiprotein complex, i.e., the NAD(P)H dehydrogenase, the ATP synthase and PSII, respectively. Since these subunits should accumulate to stoichiometrically related amounts, a translational coupling mechanism alone would not guarantee proper synthesis rates of both polypeptides. Indeed, different molecular mechanisms for the translation of these cistrons were revealed including a translational coupling between the *ndhC-ndhK* and *psbD-psbC* units [1, 120]. However, additional—yet to be dismantled—translational pathways are acting on these overlapping genes which allow meeting a 1:1 stoichiometry of the respective subunit pairs. It has been proposed that a rigid stem-loop structure encompassing the *psbC* SD-sequence as well as the AUG start codon is unwinded by ribosomes translating the upstream *psbD* cistron thereby facilitating translation initiation from these sites [1]. In case of *atpB-atpE*, AtpE was shown to be synthesized independently of AtpB via cis elements located ca. 25 nt upstream of its start codon within the *atpB* coding region [104].

### 2.3.2.2 Nucleus-Encoded Factors for Chloroplast Translation

Besides the nature of the regulatory chloroplast-encoded elements, especially, the identification and characterization of often gene-specific translational regulator proteins have been in the focus of research work on plastid protein synthesis. Both, genetic and biochemical approaches have led to the identification of some of such factors which are listed in Table 2.1 [for an overview see also 53, 114]. One interesting outcome of this work is that similar to the situation for control of plastid

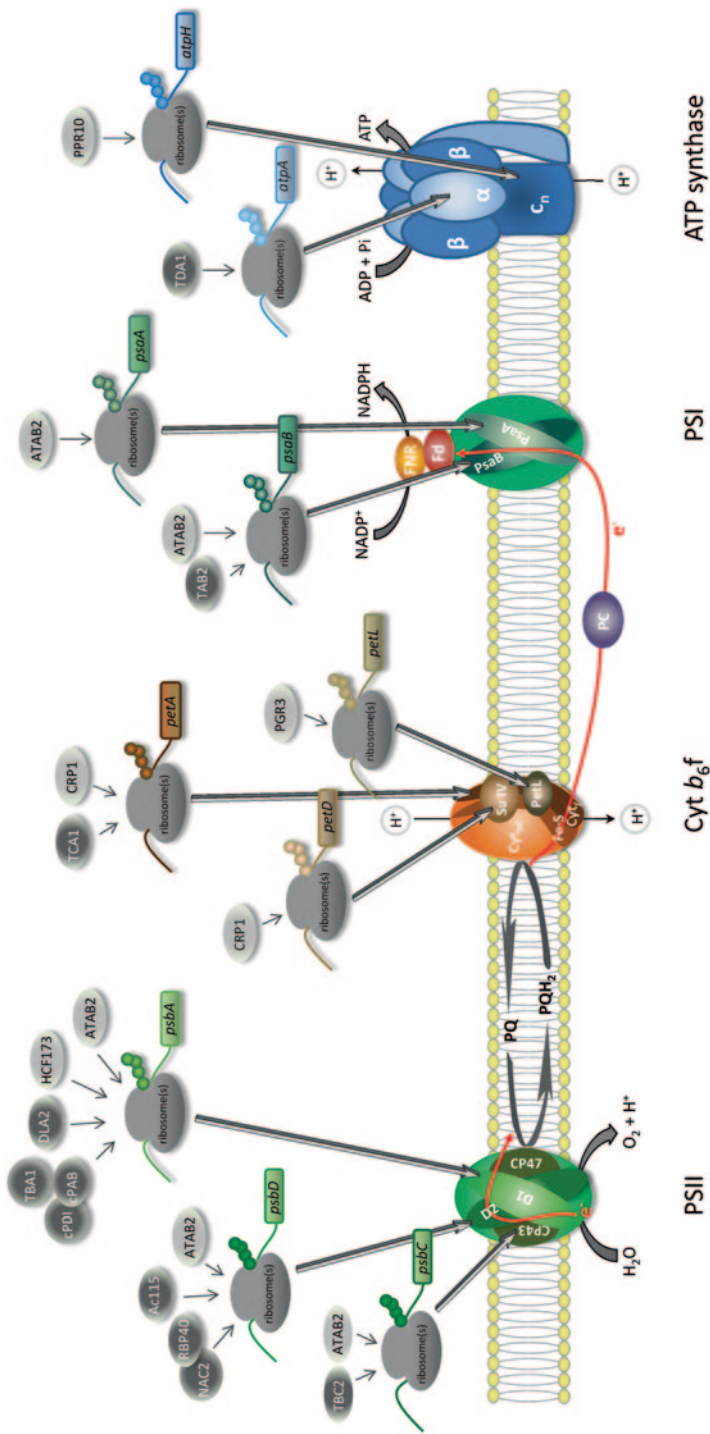
RNA metabolism many of the trans-acting factors belong to the family of so-called “helical repeat proteins” [4, 101; Table 2.1]. This family comprises proteins containing the TPR (tetratricopeptide repeat), PPR (pentatricopeptide repeat) or OPR (octatricopeptide repeat) motifs which show a different evolutionary distribution amongst photoautotrophic organisms. Whilst TPR proteins mediating mainly protein/protein interactions are ubiquitously found from cyanobacteria to higher plants, the PPR family of RNA-binding proteins is not present in prokaryotes and contains only few members in eukaryotic algae but is predominantly present in higher plant genomes. In contrast, RNA recognizing OPR motifs are mostly identified in algal genomes but are absent or almost absent in cyanobacteria and higher plant genomes, respectively [23]. Thus, it is tempting to speculate that OPR proteins substitute for PPR proteins with regard to their organellar functions in algal systems.

The helical repeat proteins include, e.g., CRP1 and PPR10 from *Z. mays* as well as PGR3 from *A. thaliana* and Tda1 from *C. reinhardtii* which are involved in the translation of *petA/petD*, *atpH*, *petL* and *atpA* mRNAs, respectively [15, 77, 86, 87]. Moreover, factors sharing homologies with redox-sensitive or C-metabolic enzymes like protein disulfide isomerases (cPDI), oxidoreductases (TBA1), short chain dehydrogenases (HCF173) and the E2 subunit of the plastid pyruvate dehydrogenase (DLA2) all being involved in *psbA* mRNA translation were discovered supporting the idea of a tight coupling of chloroplast translation and the redox and/or the C-metabolic status of the cell [12, 41, 90, 98]. Finally, so-called “pioneer proteins” with no obvious similarities to any other described protein motif suggest that novel molecular principles of plastid protein synthesis still await their discovery (Table 2.1). However, for some of these factors like RBP40 or TCA1 recent work provides some initial insights into their precise working modes as outlined below. Both genetic analyses as well as biochemical assays detecting a high number of chloroplast RNA binding activities suggest that the list of known trans-acting translational factors still is very short and awaits dramatic extensions in the near future [65] (Fig. 2.2).

## 2.4 Regulatory Principles of Chloroplast Translation

Chloroplast translation is known to be highly regulated especially in response to different environmental stimuli with light being the most recognized one. However, developmental and metabolic signals must also be integrated into the translational regulatory system for instance during gametic differentiation, circadian rhythms or changing nutrient availability [121]. Putative mediators for regulatory processes are the abovementioned gene-specific trans-acting factors which have the potential not only to enable but also to modulate and thereby fine-tune chloroplast gene expression in response to various stimuli. Since these factors are usually identified and characterized by analysing knock-out mutants it remained an open question whether they are merely constitutive or fulfil regulatory functions. However, in an elegant work Raynaud et al. [80] showed that the RNA stability factor MCA1





**Fig. 2.2** Illustration of cloned nucleus-encoded factors involved in translation of subunits of thylakoid membrane complexes. Shown are photosystems I and II (PSI and PSII), the Cytochrome *b*<sub>6</sub>f complex (Cyt *b*<sub>6</sub>f), and the ATP synthase. Transcript specific factors involved in synthesis of respective proteins are depicted in *light grey* (higher plants) and *dark grey* (*C. reinhardtii*) circles. For further explanation see text and Table 2.1

and the translational activator TCA1 (Table 2.1) both acting on the *petA* 5' UTR indeed regulate cytochrome *f* synthesis in *C. reinhardtii*. The authors constructed a set of strains with varying levels of these factors and found that both factors limit *petA* gene expression as would be expected for a regulatory factor. Moreover, both MCA1/TCA1 and Cyt*f* levels decreased dramatically under different environmental conditions, i.e., stationary growth phase and nitrogen limitation, substantiating the idea that the levels of trans-acting nucleus-encoded factors determine and regulate chloroplast gene expression levels [80]. In line with this, also the RNAi-mediated silencing of the *NAC2* gene encoding a *psbD* mRNA stability factor from *C. reinhardtii* suggests that NAC2 is rate-limiting for D2 synthesis (C. Schwarz and J. Nickelsen, unpublished data). Thus, the emerging picture is that gene-specific translational activators do not only complement for the lack of genetic autonomy of chloroplasts like, e.g., nucleus-encoded ribosomal proteins, but alternatively, they mediate regulatory functions for adaptive cellular processes. Some of the better characterized examples are outlined in the following sections.

### 2.4.1 Regulation by Light and Redox/Metabolic Status

It has been known for a long time that light plays a crucial role for chloroplast protein synthesis levels based on findings that synthesis rates of chloroplast-encoded photosynthetic proteins like the large subunit of the Rubisco or the reaction center proteins D1 and D2 of PSII increase upon illumination despite the fact that their respective mRNA levels stay mostly constant [for an overview see 122]. When light regulation is addressed two principally different aspects have to be considered which are connected to the light intensities under investigation. Under normal light in growth phases of cells, the subunits of photosynthetic complexes are made in a concerted fashion to guarantee their stoichiometric synthesis for complex de novo assembly. This situation fundamentally differs from high light conditions (>700  $\mu$ E) when the chloroplast translation machinery is mainly engaged in the repair synthesis of the PSII D1 protein which rapidly turns over in a light-dependent manner. Thus, especially for the D1 protein two different modes of protein synthesis must be considered.

Another important question concerns the nature of the chloroplast light signaling pathway influencing chloroplast translation. In the most favoured model, a redox signal generated by the photosynthetic electron transport chain is transferred to the translational apparatus via the redox status of the plastoquinone pool or via the chloroplast thioredoxin system [for a review see 6].

#### 2.4.1.1 *psbA* mRNA Translation

Chloroplast D1 synthesis represents probably the most extensively studied case for light regulation and has been comprehensively reviewed [53, 62, 71, 123]. In



brief, light regulated *psbA* mRNA translation has been shown to be mediated via its 5' UTR in both *C. reinhardtii* and tobacco [55, 100], however, the involved cis-elements appear to be different. In *C. reinhardtii*, a stem-loop region preceding the putative SD-sequence was shown to be critical for translation whereas in tobacco mutation of the SD-sequence had no effect but instead three other elements within the 5' UTR appeared to be critical for D1 synthesis [32, 100]. These include two sequences (RBS1 and RBS2) that were proposed to base-pair with the 3' end of the 16S rRNA and an AU rich box located exactly between RBS1 and RBS2. The AU-box has been postulated to be looped out upon interaction with the small ribosomal subunit thereby serving as the recognition site for (a) yet to be cloned translational factor(s) [32].

However, the most elaborate—but also the most controversial—model has been proposed for *psbA* mRNA translation for D1 repair synthesis in *C. reinhardtii* [6, 123]. This model predicts that high-light induced regulation is mediated via redox reactions coupled to the photosynthetic electron transport (PET). Biochemical isolation of a multisubunit complex (the so-called RB complex) by affinity chromatography using the *psbA* 5' UTR as affinity ligand revealed four associated proteins, i.e., a 60 kDa protein disulfide isomerase (cPDI), a 47/70 kDa poly (A) binding protein (cPAB1), a yet to be identified 55 kDa protein (RB55) and RB38, an RNA binding protein of 38 kDa (Table 2.1). The in vitro binding of the RB complex to the *psbA* 5' UTR via RB47 has been shown to be sensitive to redox reagents like DTT or reduced thioredoxin in vitro and parallels the level of *psbA* mRNA translation under different light/redox conditions or in different genetic backgrounds in vivo [19, 107, 117]. This led to a model for *psbA* mRNA translational regulation which involves a redox-controlled regulation of cPDI and subsequently RB47 via thioredoxin which becomes reduced by the PET chain. Thus, PET would function as the light sensor in this scenario.

An additional player in this redox control network has been identified by isolation of the *TBA1* locus from *C. reinhardtii* using a forward genetic approach (Table 2.1). *TBA1* encodes an oxidoreductase which has been hypothesized to play an opposing role to cPDI by mediating the re-reduction of RB47 upon illumination after its former oxidation by cPDI during dark phases [98]. Interestingly, a distinct mechanism coupled to the energy status of the chloroplast appears to act in addition to redox reactions on the RB complex, i.e., its inactivation via ADP-dependent phosphorylation of cPDI in the dark. This primary repression is then relieved via PET in the light and, as a consequence, makes the RB complex accessible to redox control via PET, a step termed “priming” [108]. While this model provides an attractive explanation of how light can be signalled to the translational apparatus via the PET, some of its details are at odds with other published data as discussed in detail by Zerges and Hauser [123]. One particular point concerns the specificity of the RB complex formation on the *psbA* mRNA which is questioned by the fact that its RB38-component has recently been shown to be involved in *psbD*—instead of *psbA*—mRNA translation [see below and 91]. Secondly, the model would predict an activation of the RB complex under reducing conditions in the chloroplast, however, high light stress resulting in preferential D1 repair synthesis results in

inactivation of PSII and PSI activities and, thus, oxidizing conditions which would inactivate the RB complex rather than activating it [28, 68].

One reason for difficulties in developing a coherent model for *psbA* mRNA translation might be the fact that two distinct but co-existing translational systems involving different trans-acting factors mediate D1 synthesis, one operating during D1 synthesis for de novo synthesis of PSII and the other one during D1 repair synthesis. This idea is mainly supported by two lines of evidence mainly obtained from work on *C. reinhardtii*. First, the analysis of appropriate chloroplast reporter gene constructs revealed that D1 repair synthesis, in contrast to de novo synthesis, is not controlled by the *psbA* 5' UTR suggesting that not initiation but elongation of translation represents the regulatory target [60, 61, 111]. Secondly, de novo and repair synthesis of D1 were demonstrated to be spatially separated in *C. reinhardtii* [109; see also V.]. Whereas de novo D1 synthesis takes place at centered regions around the pyrenoid of algal chloroplasts named T-(translation) zones, repair synthesis is distributed all over the thylakoid membrane lobes of *C. reinhardtii* constituting a clear separation between both processes [109]. Furthermore, based on FISH data, a directed light-dependent transport of mRNAs, ribosomes, and translational activators like RBP40 to T-zones was observed, raising questions on the molecular mechanisms determining mRNA and translation factor localization. In case of the *psbA* mRNA, a putative candidate for an RNA targeting factor has been identified by in vitro RNA binding analyses which was named RBP63 [70]. RBP63 recognizes a stretch of consecutive A residues in the 5' UTR which is critical for translation of the *psbA* message [14, 70]. Interestingly, RBP63 is associated with stromal thylakoid membranes and, thus, was proposed to be involved in targeting of the *psbA* mRNA to distinct sites where PSII biogenesis takes place. Intriguingly, recent mass spectrometrical analyses identified RBP63 as the E2 subunit (DLA2) of the chloroplast pyruvate dehydrogenase (cpPDC) from *C. reinhardtii* [12]. Furthermore, molecular characterization of RBP63/DLA2 revealed that it is indeed involved in targeting of *psbA* mRNA to T-zones around the pyrenoid and thereby fine-tunes D1 synthesis in response to chloroplast C-metabolism and light conditions [12].

In line with this, a similar *psbA* mRNA tethering function has been proposed for the HCF173 protein of *A. thaliana* [90]. The HCF173 protein is attached to membranes and is associated with the *psbA* RNA. In its absence, D1 synthesis is drastically impaired and *hcf173* mutants are not able of photoautotrophic growth. HCF173 protein is part of a higher molecular weight complex suggesting that other factors are involved in controlling D1 synthesis [90]. However, their molecular identification has not been achieved yet.

#### 2.4.1.2 *psbD* mRNA Translation

A second, well characterized example for translational control in *C. reinhardtii* is represented by the *psbD* mRNA encoding the immediate D1 assembly partner D2 in the reaction center of PSII. Similar to D1, D2 synthesis is regulated via the

5' UTR of its message [67]. In contrast to the A-rich element within the *psbA* 5' UTR recognized by DLA2, the *psbD* message reveals an U-rich element located 14–25 nt upstream of the AUG start codon which has been proven to be required for its translation [67]. This element is recognized by the abovementioned RBP40 factor both in vitro and in vivo. Interestingly, this interaction requires the mRNA stabilization factor NAC2 which together with RBP40 forms part of a high molecular weight complex suggesting a tight interrelationship between processes of RNA stabilization and translation [69, 91]. Based on the analysis of suppressor mutations and in vitro RNA mapping experiments it could be shown that binding of this NAC2/RBP40 complex induces a conformational change of the downstream RNA region at the AUG start codon. As a consequence, a stem-loop structure containing the start codon within its stem region is melted and the AUG codon is exposed thereby enabling access of the small ribosomal subunit and the initiator tRNA to the mRNA and subsequent initiation of D2 synthesis [44; Fig. 2.1]. It is well established that similar to D1 also D2 synthesis is induced by light [50]. Recent analyses now provided first evidence that the light signal is transmitted by the NAC2/RBP40 complex [92]. In the light, this complex forms in a redox-dependent manner, i.e., it is sensitive to treatment with reduced DTT or glutathione. In the dark, RBP40 detaches from the complex and consequently D2 synthesis is turned down. Intriguingly, a single Cys residue in RBP40 forms an intermolecular disulfide bridge with NAC2 which becomes reduced in the dark thereby coupling *psbD* gene expression to the redox status of the chloroplast [92]. However, in contrast to the above mentioned view that reducing power generated by the PET activates translation in the light, in case of *psbD* mRNA translation reduction of the NAC2/RBP40 disulfide bond causes inactivation. Even more surprising, reduction occurs in the dark raising questions on the source of the reducing electrons. In vitro studies now suggest that the disulfide bond is reductively released by the chloroplast NADPH-dependent thioredoxin reductase C (NTRC) which transfers electrons from NADPH generated by the oxidative pentose phosphate pathway [42]. This suggests a crosstalk between chloroplast gene expression and chloroplast carbon metabolism during dark adaption of algal cells [92]. Together with previous reports on the induction of chloroplast translation by acetate treatment of *C. reinhardtii* cells in the dark and the abovementioned function of the DLA2 subunit of the plastid PDC for D1 de novo synthesis, this adds another piece of evidence that plastid C-metabolism has severe impacts on the regulation of translation at least in *C. reinhardtii* [12, 59].

#### 2.4.1.3 *rbcL* mRNA Translation

The synthesis of the chloroplast-encoded large subunit of the CO<sub>2</sub> fixing Rubisco enzyme (RbcL) is also highly regulated by light. Especially under oxidative stress, *rbcL* mRNA translation was found to be repressed [38, 93]. This repression appears to be induced by the redox state of the chloroplast glutathione pool which serves as a redox sensor under high light conditions [38]. Interestingly, the RbcL

protein itself contains an intrinsic non-specific RNA binding activity located within its N-terminus [118]. It has therefore been hypothesized that the binding of RbcL to its own mRNA inhibits its translation when either its redox-controlled interaction with the chloroplast chaperone system or the assembly of the Rubisco subunit is disturbed [17]. The precise molecular mechanism of repression remains elusive but it has been shown that the early translational elongation phase appears to be affected under high light conditions because *rbcL* mRNA then shifts from polysomes to monosomes [17].

#### 2.4.1.4 *psaB* mRNA Translation

Another interesting example of light controlled translational regulation is represented by the case of the *psaB* gene encoding the reaction center PsaB protein of PS I. PsaB synthesis has been shown to be controlled by the nucleus-encoded *Tab2* locus in *C. reinhardtii*. TAB2 represents a novel type of RNA binding protein with a characteristic WLL motif at its C-terminus which directly interacts with the *psaB* 5' UTR [19]. Interestingly, this translation factor shares homologies with several orthologues in both eukaryotic and prokaryotic organisms performing oxygenic photosynthesis which range from *A. thaliana* to *Synechocystis* sp. PCC 6803 [19]. This structural evolutionary conservation is at least partially paralleled by a functional conservation of TBA2 since the *C. reinhardtii* TAB2 gene can partially complement a mutant of the *A. thaliana* homolog ATAB2. However, the *A. thaliana* factor has at least two targets—one for each PS—indicating a broader function for thylakoid membrane biogenesis in higher plants. A particularly interesting finding was that nuclear ATAB2 expression is tightly controlled by low fluence blue light via the photoreceptors CRY1 and CRY2 thereby providing an entry point to explain how blue light influences chloroplast—or more precisely thylakoid membrane—biogenesis during plant development [5].

### 2.4.2 *Regulation of Translation via Interconnected Steps of Gene Expression*

Besides external stimuli like light, internal regulatory principles underlie translational control in chloroplasts. The best described example is represented by the so-called CES (control by epistasy of synthesis) principle which links chloroplast translation to the assembly status of multisubunit photosynthetic complexes via feedback control mechanisms at least in *C. reinhardtii*. The underlying molecular details will be described in Chap. 13.

Another gene expression step connected to translation is the processing/stabilization of chloroplast transcripts (see Chap. 1). The long standing question whether RNA processing—especially of polycistronic transcripts—is required for translation or its regulation is still lively discussed, but current new insights into the dual

role of some trans-acting factors in both RNA metabolism and translation start to shed some more light onto this issue [4]. It was principally believed that the reason for extensive RNA processing in chloroplasts is the acceleration of translational events via monocistronic mRNAs. Recent comparative analyses on the selection of SD-sequences suggested that at least in some cases internal SD-sequences are inefficiently recognized favouring a scanning model for translation initiation [21, 33]. This indeed would argue for a critical role of polycistronic transcript processing for translational control. However, other lines of evidence support the idea that polycistronic chloroplast transcripts are well translated [3, 119].

In the context of this question, one interesting observation during the analysis of several nuclear mutants affected in chloroplast gene expression was that many of the identified factors appeared to be required for both mRNA stabilization and translation. This holds, e.g., for CRP1 and PPR10 from maize or Hcf107, CRR2, PGR3 from *A. thaliana* [15, 26, 30, 74, 86]. Recently, a model for the connection between RNA stabilization/processing and translation initiation has been proposed based on a detailed in vitro analysis of recombinant PPR10 protein from maize [77]. PPR10 recognizes a defined single-stranded RNA region comprising 17 nt in the intergenic region between *atpI* and *atpH* [74, 77]. This interaction serves as a barrier against exonucleolytic attack from both the 5' end as well as the 3' end of transcripts thereby defining the transcripts termini. In addition, PPR10 binding remodels the downstream RNA conformation releasing the *atpH* SD-sequence from an RNA duplex and thus makes it accessible to the small ribosomal subunit [77]. A similar mechanism has been proposed for the half-a-tetratricopeptide repeat protein HCF107 in enhancing PsbH synthesis [29].

This scenario of a dual function for RNA metabolism and translation in higher plants is reminiscent of the situation for *psbD* gene expression in *C. reinhardtii* which depends on the RNA stability factor NAC2 and the translational activator RBP40 (see above). In this case, the artificial stabilization of *psbD* transcripts in a mutant *nac2* background via the transgenic introduction of poly(G) tracts into the *psbD* 5' UTR led to accumulation of *psbD* mRNA but their translation was still hampered indicating that NAC2 is also required for D2 synthesis [67]. This function is—as mentioned above—exerted via its redox-controlled interaction with RBP40 which both together fulfil a PPR10-like concerted function for chloroplast gene expression. Similar insertions of poly(G) sequences into the 5'UTRs of *petD*, *psbB*, and *petA* transcripts all revealed the same picture, i.e., that chloroplast RNA stabilization factors are also required for translational control [20, 49, 112]. Furthermore apart from NAC2 and RBP40, complex formation between factors for RNA stabilization and translation has been described for MCA1 and TCA1 controlling *petA* gene expression in *C. reinhardtii* [12]. MCA1 represents the first algal PPR protein whose function has been to *petA* mRNA stabilization [49; see also Chap. 1]. MCA1 and TCA1 recognize adjacent targets on the first nucleotides of the *petA* message and act together to control *petA* gene expression with MCA1 serving also as a translational enhancer [12, 49, 80]. Consequently, the MCA1/TCA1 pair of trans-acting factors has been designated as single “*petA* gene expression system” underlining the fact that a single factor—or in the cases of NAC2/RBP40 or MCA1/TCA1 single

protein complexes—mediate dual functions, i.e., RNA stabilization and translation initiation [12, 91]. Interestingly, MCA1 was recently shown to be involved in the CES regulatory pathway, too, and thus connects processes of protein synthesis and assembly [12; see Chap. 13]. Taken together, the emerging picture on the posttranscriptional control of chloroplast gene expression in both algae and plants is that of a tight connection of RNA metabolism and protein synthesis which is manifested by factors/complexes of factors which mediate their functions mainly via the 5' UTRs of chloroplast transcripts.

## 2.5 Spatial Organization of Chloroplast Translation

One of the most fascinating new aspects on the regulation of chloroplast protein synthesis concerns the spatial organization of chloroplast translational events. Early electronmicroscopic studies as well as sucrose density gradient separations had demonstrated that polyribosomes associate with thylakoid membranes (TMs) [16, 43, 52]. This supported the idea that chloroplast-encoded integral membrane proteins are co-translationally inserted into TMs. This is also suggested by the fact that ca. 50% of these polysomes could only be released from TMs by the addition of puromycin catalysing a release of the nascent polypeptide chain from ribosomes (for a review see [121]). At least three different chloroplast subcompartments have been identified which contain the abovementioned regulatory activators, i.e., the chloroplast stroma, the TM system—especially non-appressed thylakoids—and a membrane subfraction named low density membranes (LDMs) which resembles the inner envelope membrane with regard to its acyl lipid composition but associates with thylakoids in a  $Mg^{2+}$  dependent manner [124].

Intriguingly, the precise in situ localization of mRNAs as well as ribosomes and/or translational activators in the chloroplast of *C. reinhardtii* by FISH analyses using confocal microscopy revealed the existence of distinct so-called T-zones (for translation zones) which are located close to the periphery of the pyrenoid. Pyrenoids represent algae-specific structures which mainly consist of concentrated Rubisco enzyme and, thus, are centers where plastid  $CO_2$  fixation takes place. At these suborganellar structures, especially the synthesis and assembly of PSII subunits is proceeding while *psaA* mRNAs did not co-localize to them indicating that PSI biogenesis is separated from T-zones [109]. A further specialization of T-zones is that they are dedicated only to the translation during de novo synthesis of PSII but D1 repair synthesis is distributed all along thylakoid membranes and thus spatially separated from T-zones [109]. This provides further compelling evidence for the idea that two completely different systems for *psbA* mRNA translation probably co-exist in chloroplasts (see above). How *psbA* mRNA is sorted between these two systems remains to be shown, nevertheless, one candidate for a membrane targeting factor for *psbA* mRNA is represented by the abovementioned DLA2 subunit of the pyruvate dehydrogenase which is required for proper localization of the *psbA* mRNA [9].



The translation of *rbcL* mRNA in *C. reinhardtii* is also highly localized, i.e., it is selectively enriched at the outer perimeter of the pyrenoid but not organized in T-zones [111]. Since *rbcL* mRNA depleted of ribosomes via lincomycin treatment did not localize to the pyrenoid a translation dependent targeting mechanism, presumably via the nascent RbcL polypeptide chain was suggested [111].

In addition to the observed light-dependent localizations of *psbA/psbC* and *rbcL* mRNAs, at two distinct regions at the periphery of the pyrenoid, another mRNA localization process was observed in cells which were exposed to high light or oxidative stress. Under these conditions, chloroplast stress granules (cpSGs) are formed in the internal perimeter of the pyrenoid where—embedded in pockets of stroma—plastid mRNAs are stored in a translationally repressed form [110]. One of the essential cpSGs-forming components has been hypothesized to be the RbcL subunit itself which has—as mentioned above—intrinsic RNA binding activity [111]. Furthermore, a flux of mRNAs between cpSGs and polysomes was observed suggesting a very dynamic mRNA trafficking in chloroplasts [111]. Additional genetic support for the existence of storage particles for chloroplast mRNA was obtained when the OPR protein Tda1 was analysed. Tda1 is specifically required for the translation of the *atpA* mRNA in *C. reinhardtii* and has been shown to act via its 5' UTR [24]. Interestingly, the OPR domain of Tda1 is involved in translational activation while the distinct N-terminus recognizes the 5' UTR and mediates a fine-tuning of *atpA* mRNA partition between polysomes and a high molecular weight, non-polysomic, storage particle. These storage particles might be identical or related to cpSGs as speculated by the authors [24]. Clearly, more work is required to obtain a more comprehensive picture on the spatial organization of chloroplast gene expression. Especially, in chloroplasts of higher plants a pyrenoid is lacking raising questions on the organizing structural element in these organelles.

## 2.6 Conclusions and Future Perspectives

Translation represents a key step during chloroplast gene expression and, accordingly, is highly regulated for instance by light. With the recent identification and molecular characterization of the involved regulatory factors, we are now starting to gain first insights on the regulatory principles which underlie a concerted synthesis of components for photosynthetic complexes. The overall picture emerging reveals helical repeat proteins playing an essential role for the organization of regulatory units which often physically and functionally connect RNA metabolism and translation initiation. These units also appear to represent the targets for signal transduction pathways affecting chloroplast protein synthesis and, as a consequence, chloroplast biogenesis. For the future, the interconnection between these pathways, which utilize redox signals or other metabolic semaphores, and chloroplast translation will represent an important issue of research efforts which might then dismantle novel unexpected crosstalks between different plastid biosynthetic pathways. A second focus of chloroplast cell biology will be the visualization

of the three-dimensional spatial network which ensures chloroplast protein synthesis to take place at those sites where e.g. photosynthetic complexes are assembled. Maybe one of the most intriguing suggestions of recent RNA localization studies is that thylakoid membrane ultrastructure does not only determine photosynthetic performance but also affects gene expression.

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