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## Chloroplast RNA-binding proteins

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**Abstract** Chloroplast gene expression is regulated by nucleus-encoded factors, which mainly act at the post-transcriptional level. Plastid RNA-binding proteins (RBPs) represent good candidates for mediating these functions. The picture emerging from recent analyses is that of a great number of differentially regulated RBPs, which are organized in distinct, spatially separated supramolecular complexes. This reflects the complexity of the regulatory network that underlies the intracellular communication system between the nucleus and the chloroplast.

**Keywords** Nucleus-to-chloroplast signaling · RNA binding · RNA metabolism · Translation

### Introduction

The transformation of a free-living cyanobacterium into an autonomy-lacking organelle, or more specifically into the chloroplast, represents one of the most important steps during the evolution of the eukaryotic plant cell. The key issue to be addressed during this process was the necessity to co-ordinate gene expression between the nuclear and plastid genetic systems. It is widely accepted that this coordination/communication is mediated by nucleus-encoded factors. These are imported by the organelle and regulate almost all steps of gene expression, which are principally of a prokaryotic nature due to the phylogenetic origin of the chloroplast.

Over the past years, the analysis of many photosynthetic mutants from green algae and vascular plants provided the basis for the application of genetic approaches; and some of the regulatory factors were successfully cloned (for reviews, see Barkan and Goldschmidt-Clermont 2000; Dent et al. 2001; Nickelsen 2003). To date, 16 nuclear loci have been isolated, encoding proteins involved in a variety of post-transcriptional steps of chloroplast gene expression. Although some of these factors have been shown to be part of high-molecular-weight complexes which contain their target RNAs, a direct interaction with plastid RNA probes has recently been documented for just one protein, called HCF152, from *Arabidopsis thaliana* (Meierhoff et al. 2003). HCF152 is involved in the processing of transcripts from the chloroplast *psbB* operon and contains a so-called pentatricopeptide repeat (PPR) motif, which has been hypothesized to form a RNA-binding domain (Lahmy et al. 2000; Small and Peeters 2000). Such PPR proteins appear to represent typical plant proteins belonging to a large family of more than 200 members in *A. thaliana* (Small and Peeters 2000). Nevertheless, the precise molecular working mode of these factors remains to be elucidated.

With the availability of the complete genome sequence of *A. thaliana*, bioinformatic searches became feasible for proteins containing additional, well characterized RNA-binding motifs, such as the RRM domain, which is also present in polypeptides from cyanobacteria (Sugita and Sugiura 1994), and the KH motif, which was first identified in the human (RNP) K protein. In total, 196 RRM and 26 KH domains containing proteins have been found in *A. thaliana*, which is more than in *Drosophila melanogaster* (117 domains) or in *Caenorhabditis elegans* (100 domains; Lorkovic and Barta 2002). However, these studies do not specify how many domains are chloroplast-localized. Independent estimations predict the existence of about 60 chloroplast RNA-binding proteins (cpRBPs) in *A. thaliana*, based on the computer-assisted analysis of putative chloroplast-targeting signals (Friedrich Ossenbühl, personal communication).

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An alternative and complementary biochemical approach for the identification of plastid factors controlling post-transcriptional processes, such as RNA stabilization, RNA maturation, or translation initiation, is the application of *in vitro* RNA-binding assays, which include gel-shift and UV-crosslinking techniques. Several chloroplast RNA-binding proteins have been detected by this strategy and the aim of this review is to give a brief overview of these factors by focusing on some well studied cases.

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### General RNA-binding proteins

Amongst the first cpRBPs to be identified was a group of proteins in the size range of 30 kDa. Cloning of the corresponding genes showed that they resembled human nuclear (hn)RNP proteins, which form the most abundant fraction of RNA-binding proteins in the nucleus of human cells. In tobacco, five different proteins, referred to as cp28, cp29A, cp29B, cp31 and cp33, were found, while a genome-wide computer-assisted search in *A. thaliana* revealed eight members of this group (Lorkovic and Barta 2002), which could be classified into subgroups I–III, based on phylogenetic comparisons (Ohta et al. 1995). A common feature of these polypeptides was that they contain two consensus sequence-type RNA-binding domains and an acidic N-terminal domain. *In vitro*, all cpRBPs exhibited a high affinity for poly-(G) and poly-(U) ribohomopolymers, but despite that, no distinct binding sites on chloroplast RNAs could be identified, suggesting that they represented general RBPs of the plastid compartment for which a physiological function was difficult to assign at that time. More recently, accumulating biochemical evidence provided some clues as to what role these proteins might play during chloroplast gene expression. Nakamura et al. (2001) demonstrated that the abundant cpRBPs form high-molecular-weight ribonucleoprotein complexes in the chloroplast stroma and function as stabilizing factors for ribosome-free mRNAs. In a detailed and elegantly conducted study, it was shown that the *psbA* mRNA encoding the D1 protein of photosystem II rapidly degraded in chloroplast extracts when these were immunodepleted of cpRBPs. Especially, cp33 appeared to be required for stable accumulation of chloroplast mRNAs. In addition, cpRBPs co-immunoprecipitated with chloroplast intron-containing precursor tRNAs, suggesting that at least some of them are involved in pre-tRNA splicing (Nakamura et al. 1999).

In spinach, a protein named 28RNP, with homology to cp28 and cp31 from tobacco, was analyzed in detail. 28RNP was shown to be essential for the 3' end formation of several chloroplast mRNAs *in vitro*. Since its affinity for RNA was substantially altered by phosphorylation, it was postulated to be directly involved in the regulation of plastid 3'UTR-mediated RNA stabilization (Lisitsky and Schuster 1995). Finally, tobacco cp31 was implicated in the editing process of *psbL* mRNA

(see next section). Hence, the emerging picture, as far as the physiological role of this group of small chloroplasts is concerned, is that of a relatively unspecific protein scaffold for plastid RNAs reminiscent of the situation found for hnRNP particles in the nucleus. Nevertheless, they may be involved in distinct steps of post-transcriptional gene expression by direct interaction with RNAs or, alternatively, by recruiting site-specific factors mediating RNA metabolism.

Interestingly, a recent *in vivo* UV-crosslinking approach led to the identification of PARBP33 and PARBP35 from avocado, which share a high identity with ATRBP33 from *A. thaliana* and other group II members of small RBPs (Daros and Flores 2002). PARBP33 was shown to bind to the RNA of the avocado sunblotch viroid (ASBVd). *In vitro*, recombinant PARBP33 was able to facilitate the hammerhead-mediated self-cleavage of dimeric ASBVd transcripts, which are normally formed during the rolling circle replication of the viroid within the chloroplast. This suggests that viroids can use host cpRBPs as RNA chaperones.

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### RBPs involved in splicing or editing of chloroplast transcripts

Chloroplast genes can be interrupted by introns, which are classified into the main groups I and II, based on primary and secondary structural features (Michel and Dujon 1983). Although some of these organellar introns have the capacity to self-splice *in vitro*, it is likely that these splicing processes are assisted by protein factors *in vivo* similar to the snRNP-mediated splicing events in the nuclear cell compartment.

The chloroplast *trnK* gene, for instance, is separated by a long intron, which encodes an open reading frame of 524 amino acids, called *matK*, which shares homologies with mitochondrial maturases. In mustard, recombinant *matK* gene product expressed in *Escherichia coli* was shown to selectively bind to precursor *trnK* and *trnG* transcripts *in vitro*, supporting the idea that MatK functions as a maturase during the excision of group II introns from these precursor RNAs (Liere and Link 1995).

Moreover, sedimentation analysis in transplastomic *Chlamydomonas reinhardtii* strains revealed native high-molecular-weight RNP complexes, which were formed with group II intron RNA and chloroplast proteins. Subsequent UV-crosslinking experiments then revealed the interaction of proteins, 31 kDa and 61 kDa in size, with distinct subdomains of group II intron RNAs (Bunse et al. 2001). As mentioned above, the small hnRNP-like cpRBPs were also found to co-precipitate with intron-containing precursor tRNAs in tobacco.

To date, five nuclear genes required for chloroplast splicing have been isolated by genetic means. These include *Raa1*, *Raa2*, *Raa3* from *C. reinhardtii*, which are involved in a complex two-step *trans*-splicing mechanism leading to mature chloroplast *psaA* mRNA. In addition,

Crs1 from maize represents a splicing factor which mediates intron removal from the *atpF* precursor RNA. In the *crs2* mutant, the splicing of a whole subgroup of class IIB introns is defective (Vogel et al. 1999). For most of these factors, it has been shown that they are part of high-molecular-weight complexes, which contain their target RNAs (Jenkins and Barkan 2001; Rivier et al. 2001; Till et al. 2001). However, to date, no direct contact between these and the respective transcripts has been reported. Thus, the precise *cis*-acting RNA elements involved and, accordingly, the precise molecular working mode of these proteins remains to be elucidated.

The only exception is represented by the above-mentioned HCF152 factor from *A. thaliana*, which is involved in both the cleavage of polycistronic precursor RNAs between the *psbH* and *petB* genes and the splicing of *petB* intron sequences. UV-crosslinking assays with various RNA probes from the *psbB-psbT-psbH-petB-petD* operon revealed a high affinity binding of recombinant HCF152 to distinct RNA regions covering either the *psbH-petB* intergenic region or the exon-intron boundaries of *petB* transcripts. These data suggest that HCF152 is directly involved in the maturation of *petB* mRNA (Meierhoff et al. 2003).

RNA-editing represents another RNA maturation process which alters internal nucleotides of plastid transcript-coding regions, mainly by C-to-U conversions at specific sites. In algae, no editing can be observed, indicating phylogenetically a later development of this gene expression mechanism. While the essential *cis*-acting elements on chloroplast RNAs have been narrowed down by analyzing site-directed plastid mutants (Hermann and Bock 1999), relatively little is known about the interacting *trans*-acting factors, for some of which genetic data indicate that they may be nucleus-encoded (Bock and Koop 1997; Schmitz-Linneweber et al. 2001). Recently, in vitro RNA-editing systems for both tobacco and pea chloroplasts were developed, which now enable a rapid identification of both the involved *cis*-elements and, furthermore, the proteins recognizing these RNA regions by UV-crosslinking. In these editing extracts, four different RBPs were detected that bound to different RNA editing elements. A 25-kDa protein specifically recognized the *psbL*-editing site and the above-mentioned general cpRBP cp31 was required for editing both *psbL* and *ndhB* mRNAs, as demonstrated by immunodepletion of this factor from the extract (Hirose and Sugiura 2001). In tobacco, proteins of 56 kDa and 70 kDa were shown to bind to the *psbE* and *petB* sites, respectively. Interestingly, pea chloroplasts have no editing capacity for the *psbE* site and lack the 56-kDa protein, suggesting a co-evolution of editing sites and their cognate editing factors (Miyamoto et al. 2002).

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### **RBPs involved in 3' end formation of chloroplast RNAs**

Apart from the internal regions of chloroplast RNAs, the 5'UTRs and 3'UTRs have attracted special attention

with regard to their potential role in the regulation of post-transcriptional gene expression steps. 3'UTRs often contain stem-loop structures, which function as general transcript-stabilizing determinants and, in addition, serve as processing signals for correct 3' end formation. This 3' end formation is a two-step process, which involves an initial endonucleolytic cut downstream of the stem-loop structure and a subsequent exonucleolytic trimming in the 3'-to-5' direction. Removal of the stem-loop structure, for instance by internal endonucleolytic cuts, results in rapid degradation of the investigated RNAs, both in vitro and in vivo. Interestingly, similar to the situation in bacteria, polyadenylation of the resulting cleavage products at their 3' ends targets them for degradation (Hayes et al. 1999; Schuster et al. 1999). In *E. coli*, polyadenylation of RNAs and their subsequent degradation are performed by a poly(A)polymerase and the exoribonuclease polyphosphorylase (PNPase), respectively. Also in chloroplasts, a PNPase has been detected which can be UV-crosslinked to many chloroplast RNA probes. In contrast to the *E. coli* enzyme, chloroplast PNPase apparently does not form a degradosome-like structure containing both endonuclease and helicase activities (Carpousis et al. 1999), but forms a homo-oligomeric complex of about 600 kDa, as judged by biochemical and mass-spectrometric analyses (Baginsky et al. 2001). Recently, a reverse genetic approach revealed a more complex picture of the function of PNPase in the chloroplasts of *A. thaliana*. In co-suppressed plants exhibiting a drastically reduced amount of PNPase, correct 3' end formation of *rbcL* and *psbA* mRNAs was significantly reduced. However, their total transcript accumulation remained unaffected by the absence of PNPase and no obvious phenotype of co-suppressed plants was observed. These data indicate that PNPase represents a critical determinant for the 3' processing of plastid mRNAs but not for their half-lives. Furthermore, the processing of rRNAs and the decay of tRNAs appear to be affected by PNPase (Walter et al. 2002). Another aspect of PNPase function concerns its relation to processes of polyadenylation of chloroplast RNA fragments. Biochemical data suggest that PNPase itself acts as a poly(A)polymerase in both higher plants and cyanobacteria (Yehudai-Resheff et al. 2001; Rott et al. 2003). However, analysis of the mentioned co-suppressed PNPase lines of *A. thaliana* reveals that polyadenylation is enhanced in the absence of PNPase, suggesting that a poly(A)polymerase distinct from PNPase is responsible for RNA modification similar to the situation in *E. coli* (Walter et al. 2002). Future work will have to clarify these contradictory results.

In addition to PNPase, several other proteins have been shown to interact with various chloroplast RNA 3'UTRs. Among these, CSP41 from spinach was analyzed in detail. It belongs to the short-chain dehydrogenase/reductase superfamily and exhibits both RNA-binding and endonuclease activity in vitro (Yang et al. 1996). A thorough investigation of various

different RNA substrates showed that recombinant CSP41 especially recognizes and cleaves fully base-paired stem-loop structures, suggesting that it plays a broad role in the degradation of stem-loop-containing plastid RNAs (Bollenbach and Stern 2003). Interestingly, this protein appears to be associated with the plastid transcription machinery, pointing to a tight connection between transcriptional and post-transcriptional processes (Pfannschmidt et al. 2000). Another RBP from mustard chloroplast, named p54, also shows endonucleolytic activity and is involved in the 3' end formation of the stem-loop lacking 3'UTRs from *trnK* and *rps16* transcripts (Nickelsen and Link 1993). Both the redox state and phosphorylation of p54 regulate its activity in vitro (Liere and Link 1997).

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### RBPs interacting with 5'UTRs

5'UTRs of chloroplast transcripts harbor the *cis*-acting determinants for the stabilization of plastid transcripts and, moreover, represent the platform on which translation initiation takes place. For instance, analysis of chloroplast reporter gene constructs revealed that, in both *C. reinhardtii* and tobacco, *rbcL* transcripts are stabilized via elements within their 5' regions in vivo (Salvador 1993; Shiina et al. 1998). The same applies to 5'UTRs of *psbD*, *petD* and *psbB* transcripts in *C. reinhardtii*, which were shown to contain the target sites for nucleus-encoded factors involved in the gene-specific stabilization of the corresponding mRNAs (Nickelsen et al. 1994; Drager et al. 1998; Vaistij et al. 2000a). For *psbD* and *petD* RNAs, site-directed mutagenesis enabled the precise localization of distinct RNA elements mediating RNA stabilization (Higgs et al. 1999; Nickelsen et al. 1999).

Similarly, analysis of mutants and reporter genes confirmed that 5'UTRs also contain essential translation elements, some of which represent the target sites for nucleus-encoded functions regulating the basic bacteria-like translation apparatus (for reviews, see Bruick and Mayfield 1999; Zerges 2000). To date, three nuclear genes have been cloned from *C. reinhardtii* which encode proteins mediating their function via chloroplast 5'UTRs. These include *Nac2* and *Mbb1*, controlling the stabilization of *psbD* and *psbB* transcripts, respectively, and *Tbc2*, regulating translation initiation on the *psbC* mRNA (Boudreau et al. 2000; Vaistij et al. 2000b; Auchincloss et al. 2002). Similar to the above-mentioned genetically defined factors involved in chloroplast-splicing, all three factors have been detected in high-molecular-weight complexes which also contain RNA; but it remains to be clarified whether or not they directly interact with their cognate transcripts.

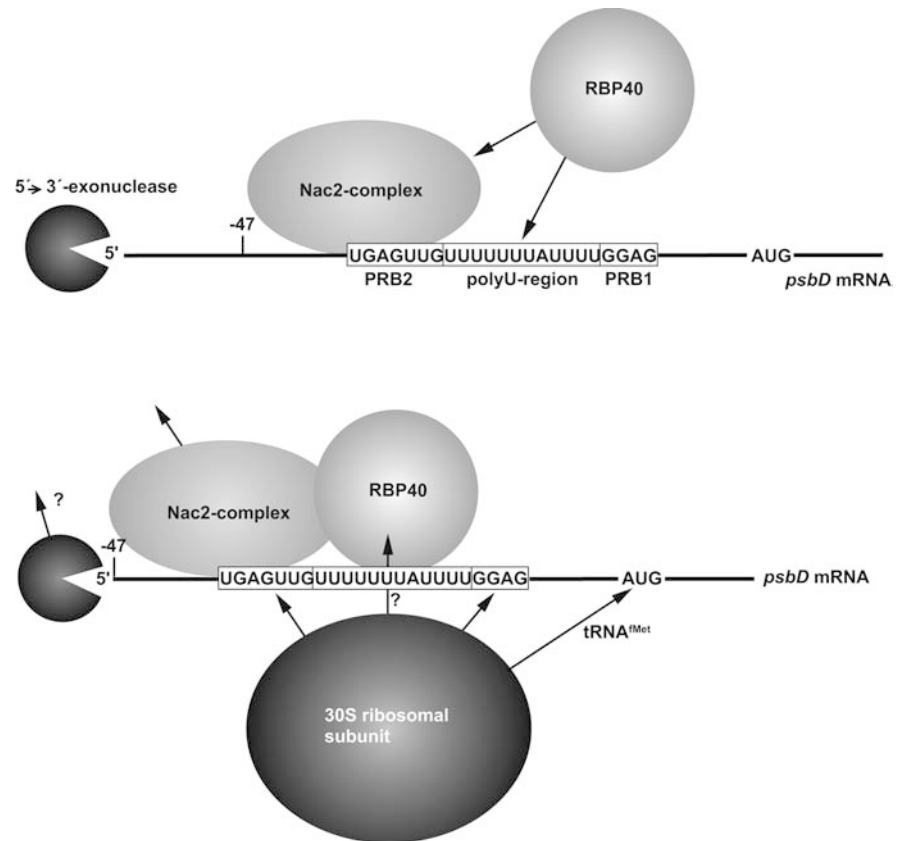
To identify factors regulating plastid translation initiation at 5'UTRs, which is considered to be the rate-limiting step for polypeptide synthesis, mainly in vitro analyses have been applied in the past. During extensive RNA-binding experiments using a variety of different

systems, a large number of RBPs were detected, which interact with chloroplast leader regions (Danon and Mayfield 1991; Nickelsen et al. 1994; Hauser et al. 1996; Alexander et al. 1998; Zerges and Rochaix 1998; Hotchkiss and Hollingsworth 1999; McCormac et al. 2001). Moreover, a promising in vitro system has been developed, based on a tobacco chloroplast protein extract, which is capable of accurately translating exogenously added mRNAs (Hirose and Sugiura 1996). Gel-shift experiments with this extract confirmed an RNA-protein complex formation with the AU-rich element of the *psbA* 5'UTR, which is required for protein synthesis. Also, in spinach, the ribosomal protein S1 was shown to interact with the *psbA* leader in addition to several other RNAs (Alexander et al. 1998; Shteyman-Kotler and Schuster 2000).

Currently, the most comprehensive analysis of *psbA* gene expression has been performed in *C. reinhardtii*. Using RNA affinity chromatography, a *psbA* 5'UTR-interacting protein complex was isolated, consisting of four subunits referred to as RB47, RB60, RB55 and RB38. Among these, only RB47 directly contacts the RNA, as was determined by UV-crosslinking assays. The genes for both RB47 and RB60 were cloned and shown to encode a poly(A)-binding protein (cPABP) and a protein disulfide isomerase (cPDI), respectively (Kim and Mayfield 1997; Yohn et al. 1998). The binding activity of this complex was decreased in nuclear mutants, which affected D1 synthesis, underlining its function for translation initiation. In vitro, both phosphorylation and redox reactions could modulate protein-binding to the *psbA* 5'UTR through RB60 and/or RB47 (Fong et al. 2000; Trebitsh et al. 2000). Accordingly, a model for the regulation of D1 synthesis was proposed, which predicts a direct coupling of translation initiation with photosynthetic activity via NADPH and ATP (Bruick and Mayfield 1999). Consistent with this, redox signals originating from both photosystem I and photosystem II were found to influence D1 synthesis in isolated *C. reinhardtii* chloroplasts. However, since the synthesis of other chloroplast proteins was regulated in the same manner, it remains to be clarified whether this reflects a *psbA*-specific or a more general effect on the translation machinery (Trebitsh and Danon 2001). A related redox-regulation of RNA-protein complex formation on the *psbA* 5'UTR has also been reported for *A. thaliana* (Shen et al. 2001).

One particularly intriguing, but not entirely resolved, aspect of chloroplast gene expression is the mode of subcompartmentalizing different gene expression steps within the organelle. In addition to the stroma or the membrane phases of thylakoids and the inner envelope membrane, another membranous subcompartment, the so-called "low density membrane" (LDM), has been described (Zerges and Rochaix 1998). LDMs resemble the inner envelope with regard to their lipid composition and associate with thylakoids in a magnesium-dependent manner. It is hypothesized that they represent intermediate states during thylakoid membrane biogenesis,

**Fig. 1** Model of *psbD* gene expression in *Chlamydomonas reinhardtii*. See text for explanation



which might originate from the inner chloroplast envelope via vesicle transport (Zerges 2000; Kroll et al. 2001). Interestingly, several light-activated RNA-binding activities in *C. reinhardtii* were shown to be significantly enriched in the LDM fraction (Zerges and Rochaix 1998; Zerges et al. 2002), suggesting that the envelope/LDMs represent the site of thylakoid membrane protein synthesis. Consistent with this, the *psbA*-specific translational regulator RB47 was localized to LDMs and RB60 was found to be partitioned between the soluble and membrane phase during chloroplast fractionation experiments (Zerges and Rochaix 1998; Trebitsh et al. 2001). Recently, a novel *psbA*-specific RBP, named RBP63, was identified in *C. reinhardtii*, which recognizes a translational A-rich element within the *psbA* leader, but, in contrast to RB47, is exclusively associated with stromal thylakoid membranes (Ossenbühl et al. 2002). This is the place where photo-damaged D1 is co-translationally exchanged during photosystem II repair (Adir et al. 1990; Zhang and Aro 2002). Thus, two different, spatially separated processes of photosystem II generation may have to be considered: One is the de novo assembly of PSII in developing chloroplasts, which takes place at the LDM system, and the other is its thylakoid-located maintenance in mature chloroplasts. Whilst *psbA* mRNA translation at LDMs is likely to be regulated by the complex containing RB47, RB60, RB55 and RB38, D1-repair synthesis might be targeted to stromal thylakoid membranes via a molecular tether represented by RBP63. Future work will show whether two different

pathways actually do exist for *psbA* gene expression in chloroplasts.

Many of the molecular details we are aware of today regarding site-specific RNA-protein interactions are mainly based on in vitro investigations. Research on *C. reinhardtii*, which lends itself to the application of both genetic and biochemical approaches, married in vitro work on RBPs with genetic approaches. Possibly, one of the most comprehensive analyses of this kind was performed for the chloroplast *psbD* gene, encoding the D2 protein of the PSII reaction center. Analysis of the photosynthetic mutant *nac2* revealed that the stability of the *psbD* mRNA depends on a nucleus-encoded tetratricopeptide repeats protein, which is part of a high-molecular-weight complex mediating its function via the *psbD* 5'UTR (Boudreau et al. 2000). However, a thorough site-directed mutagenesis of the *psbD* 5' region after biolistic transformation of chloroplasts from wild-type cells identified several essential *cis*-acting elements required for either stabilization or translation of the *psbD* message (Nickelsen et al. 1999). Concomitant in vitro RNA binding assays with the mutant *psbD* 5' versions then demonstrated that the deletion of an U-rich element leading to a defect in *psbD* mRNA translation in vivo resulted in the inability of binding a protein of 40 kDa (RBP40) to the 5'UTR in vitro. Furthermore, a suppressor screen led to the isolation of a photosynthetic revertant, which contained a 5-bp duplication within the mutated U-rich element. This *cis*-acting suppressor mutation was then shown to be

sufficient to restore both D2 synthesis and binding of RBP40 to the *psbD* leader, indicating a strong correlation of these two processes. Interestingly, the binding of RBP40 was also dependent on the presence of the RNA stabilization factor, Nac2. This suggests that a tight connection between 5'UTR-mediated RNA stabilization and translation initiation exists in the chloroplast. Based on the data collected so far, the following model for *psbD* gene expression has been proposed, which is depicted in Fig. 1. Indirect evidence strongly suggests that, after transcription, the Nac2 complex interacts with a RNA element within the *psbD* 5'UTR, called PRB2 (Nickelsen et al. 1999). This leads to the protection of the RNA against exonucleolytic degradation from the 5' end of the message. Furthermore, Nac2 guides RBP40 to its U-rich binding site immediately downstream of PRB2 (Ossenbühl and Nickelsen 2000). Once RBP40-*psbD* mRNA complex formation has been established, Nac2 is released from the RNA. This transient interaction of the Nac2 complex with the *psbD* 5'UTR is supported by the finding that Nac2 cannot be identified at polysomes from *C. reinhardtii* chloroplasts (Boudreau et al. 2000). Afterwards, RBP40-mediated ribosomal assembly takes place and translation is initiated.

Similar experiments based on the analysis of various *cis*-acting suppressor strains in combination with *in vitro* RNA-binding assays were carried out for the 5'UTR of the chloroplast *rps7* gene in *C. reinhardtii*. A 20-kDa protein, which was shown to be the S7 protein of the small ribosomal subunit encoded by *rps7* itself, was detected in UV-crosslinking experiments with the wild-type *rps7* 5'UTR probe. It failed to bind to mutant versions, which affected translation of a reporter gene when introduced into the chloroplast genome. However, 5'UTRs derived from isolated *cis*-acting suppressor strains bound to S7, highlighting the *in vivo* significance of this interaction. The above-mentioned RB47 factor implicated in the regulation of *psbA* mRNA translation was also found to recognize the *rps7* 5'UTR during these experiments. However, in contrast to S7, no correlation between the *in vitro* and the *in vivo* data could be observed, indicating that the function of RB47 during *rps7* gene expression is not essential. Overall, these data suggest a positive auto-regulation of the *rps7* gene by its own product and possibly also the regulation of other genes (Fargo et al. 2001).

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## Conclusions and perspectives

The biogenesis of chloroplasts is controlled by numerous nucleus-encoded factors, which mediate the crosstalk between the plastid and nuclear genomes by regulating organellar gene expression, mainly at the post-transcriptional level. In addition to genetic approaches, biochemical analyses of RNA protein complex formation within chloroplasts have proven to be powerful tools for the characterization of RBPs and the identification of their corresponding genes.

In conclusion, data available to date indicate the existence of a high number of different plastid RBPs, which appear to possess differentially regulated RNA-binding activities. Some of these exhibit sequence-specific and, thus, gene-specific binding affinities, while others represent more general RBPs that might establish a protein scaffold for chloroplast transcripts enhancing RNA stability and/or RNA-folding. Another outcome of more recent research is that most RBPs form part of high-molecular-weight complexes, which are likely to represent the regulatory units controlling gene expression. Their chloroplast sublocalization and their supra-molecular organization in distinct complexes now provide the basis for the selective enrichment of RBPs in plastid subfractions. Further biochemical processing of these fractions by affinity chromatography in combination with recently improved proteomic techniques will facilitate the identification of even low-abundance RBPs and constituents of the RBP-containing complexes, thereby enabling the cloning of their respective genes. The parallel development of chloroplast *in vitro* systems for editing and translation and the site-directed mutation of plastid genes in transplastomic transformants is expected to reveal precise molecular details of the working mode of the regulatory network that underlies intracellular communication between cell organelles. With the understanding of this communication system, we might be able to answer a fundamental question of cell biology, i.e., how the photoautotrophic cell which we see today has developed by starting from two independent organisms, a photoautotrophic prokaryote and a heterotrophic eucaryotic host which became fused. Especially, the harmonization of the two genetic systems involved via this communication system provides the molecular basis for organelle biogenesis and, thus, reflects a milestone in the development of higher organisms.

Furthermore, the identification of the molecular machinery mediating gene regulation in chloroplasts, together with established techniques for both nuclear and chloroplast transformation, will accelerate the development of high-efficiency systems for the expression of foreign genes within plastids, which recently became of outstanding interest in terms of biotechnical applications (Bock 2001; Maliga 2003).

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