Processing, degradation, and polyadenylation of chloroplast transcripts

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Abstract

In this chapter, we describe the major enzymes and characteristics of transcript 5' and 3' end maturation, and polyadenylation-stimulated degradation. The picture which emerges is that maturation and degradation share many prokaryotic features, vestiges of the chloroplast endosymbiont ancestor. The major exoribonucleases are well-defined, being polynucleotide phosphorylase and RNase II/R. The endonucleases include CSP41, with largely informatic evidence for homologs of prokaryotic RNases E, J, and III. The polyadenylation-stimulated degradation pathway, which occurs in most living systems, is a major player in chloroplast RNA degradation. We discuss known or potential roles for polynucleotide phosphorylase and a prokaryotic-type poly(A) polymerase. Finally, we discuss nuclear mutations that affect RNA maturation and degradation, defining genes that are likely or known to encode regulatory factors. Major questions for future research include how the ribonucleases, which are inherently nonspecific, interact with these specificity factors, and whether newly-discovered noncoding RNAs in the chloroplast play any role in RNA metabolism.

1 Introduction

Chloroplasts originated from a cyanobacterial ancestor that entered a heterotrophically growing eukaryote some 1.5 billion years ago (Hoffmeister and Martin 2003). Ensuing gene transfer from the organelle to the nucleus has been extensive, resulting in a situation where the vast majority of the chloroplast proteome is encoded either by nuclear genes acquired from the endosymbiont, or by those that already existed in the nucleus of the mitochondriate host (Martin et al. 2002). Consequently, the chloroplast multisubunit complexes required for photosynthesis and gene expression contain both chloroplast- and nucleus-encoded components, necessitating coordinated gene expression in the two compartments. Plants and green algae have therefore evolved sophisticated intracellular communication systems that regulate chloroplast gene expression at multiple levels, many of which are reviewed in other chapters of this book.

This chapter concerns primarily posttranscription regulation of chloroplast gene expression, particularly RNA processing and degradation. RNA processing in

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chloroplasts is catalyzed by nucleus-encoded ribonucleases and includes 5' end maturation, which is catalyzed primarily by endoribonucleases and 3' end maturation, which is catalyzed by endonucleases and/or 3' to 5' exoribonucleases (Stern and Kindle 1993; Hayes et al. 1996). Like bacteria, chloroplasts often express genes from clusters or operons, leading to synthesis of polycistronic transcripts that are often cleaved intercistronically, requiring endoribonuclease activity and RNA-binding proteins (Barkan et al. 1994; Meierhoff et al. 2003). Although splicing and RNA editing are also important posttranscriptional processing events in the chloroplast, the reader is directed to the chapter by Christian Schmitz-Linneweber in this volume for a comprehensive discussion of these topics.

Although endo- and exoribonucleases feature prominently in RNA processing, these same activities are also important in catalyzing chloroplast RNA turnover. Chloroplast RNA accumulation increases significantly during leaf development and plastid differentiation. The accumulation of a specific transcript is controlled by the difference in its transcription and degradation rates, and can in principle be controlled at either one or both of these steps. Although global changes in plastid transcription are associated with leaf development and illumination (Deng and Gruissem 1987; Mullet and Klein 1987; Dhingra et al. 2006; Zoschke et al. 2007), chloroplast genes are rarely regulated individually at the transcriptional level, with the notable exception of *psbD*, which is regulated by a specialized promoter (Gamble and Mullet 1989; Kim et al. 1999; Thum et al. 2001). Instead, the significant differences in the accumulation of individual transcripts in various tissues and during leaf development and plastid differentiation are modulated in large part by transcript degradation rates, or at the level of RNA stability (Gruissem 1989; Monde et al. 2000b; Bollenbach et al. 2004). Chloroplast RNA stability is regulated primarily by its rate of degradation through a polyadenylation-stimulated turnover pathway, which is discussed in detail below. mRNA abundance for a handful of plant chloroplast genes has been shown to correlate with abundance of their respective proteins, consistent with the idea that regulation of mRNA accumulation is an important control point of chloroplast gene expression (Rapp et al. 1992; Mullet 1993). However, translation is also a key regulatory step, and *Chlamydomonas reinhardtii* chloroplasts maintain protein homeostasis even in the face of significant decreases in mRNA accumulation (Eberhard et al. 2002).

In this review, we describe the mechanisms of chloroplast RNA processing and degradation, including known and candidate endoribonucleases, exoribonucleases and regulatory proteins. The role of these nucleus-encoded factors, and the potential role of newly discovered chloroplast-encoded antisense RNAs in posttranscriptional regulation are discussed.

2 The enzymes of RNA degradation and maturation

2.1 Endoribonucleases

2.1.1 CSP41

CSP41a (Chloroplast Stem-loop binding Protein, 41 kDa) and CSP41b are widespread, highly conserved endoribonucleases, which are unique to photosynthetic organisms. The photosynthetic bacteria *Synechocystis* sp. PCC6803 and *Nostoc* sp. PCC7120 encode only a CSP41b homolog, whereas plant and algal nuclear genomes encode both CSP41a and CSP41b homologs (Yamaguchi et al. 2003). Phylogenetic and motif analyses have shown that CSP41a and CSP41b are paralogs of a cyanobacterial ancestor that diverged from a bacterial epimerase/dehydratase (Baker et al. 1998; Yamaguchi et al. 2003).

CSP41a and CSP41b are abundant proteins, and have been found in a number of chloroplast complexes by proteomics, including RNPs, chloroplast ribosomes, and the plastid-encoded RNA polymerase (Yang et al. 1996; Pfannschmidt et al. 2000; Yamaguchi et al. 2003; Suzuki et al. 2004; Peltier et al. 2006), although no primary function for these proteins in either transcription or translation has been demonstrated.

CSP41a was first purified from spinach chloroplasts as a *petD*-specific RNA-binding protein and a nonspecific endoribonuclease (Yang et al. 1996; Yang and Stern 1997). Spinach CSP41a was shown to cleave synthetic stem-loop-containing *petD*, *psbA*, and *rbcL* RNAs, and could cleave arbitrary single-stranded RNAs (Yang and Stern 1997), which suggested that it could initiate turnover of chloroplast transcripts by endonucleolytic cleavage, the first step in the poly(A)-stimulated turnover pathway (see Section 2). *In vitro* measurements of tobacco chloroplast mRNA degradation rates showed significant decreases in the rates of *rbcL*, *psbA*, and *petD* transcript turnover in CSP41a-deficient plants (Bollenbach et al. 2003), suggesting that CSP41a could participate broadly in chloroplast mRNA turnover.

Structure. A Hidden Markov model-based search of Genpept suggested that CSP41 proteins are homologous to sugar-nucleotide epimerases and hydroxysteroid reductases, and as such belong to the short-chain dehydrogenase/reductase (SDR) superfamily (Baker et al. 1998). This family comprises 1600 proteins, including more than 130 in *Arabidopsis* (Kallberg et al. 2002). Like other members of this family, CSP41 contains an N-terminal bidomain Rossman fold, including the βαβ-turn, which is responsible for binding the nucleotide portion of NAD(P)H in dehydrogenases. CSP41 homologs have, however, lost the conserved Gly-X-Gly-X_3-Gly NAD(P)H binding motif, and have therefore lost the ability to bind $NAD(P)^+$ or NAD(P)H (Baker et al. 1998; Bollenbach and Stern 2003a). Instead, deletion mutant analysis suggested that the N-terminal CSP41 Rossman fold is responsible for substrate (RNA) binding (Bollenbach and Stern 2003b).

Divalent metal requirement. Several SDR family proteins bind and cleave RNA, including glyceraldehyde phosphate dehydrogenase (GAPDH), and two endoribonucleases from the archaeon *Sulfolobus solfataricus*, but do not require di-

valent metal ions for activity (Evguenieva-Hackenberg et al. 2002). CSP41, a divalent metal-dependent ribonuclease, is therefore unique among RNA-cleaving SDR enzymes. CSP41a contains a single, broad specificity divalent metal binding site, but is optimally active in the presence of Mg^{2+}; the abundance of Mg^{2+} in the chloroplast suggests that this is the physiological activator of CSP41a (Bollenbach and Stern 2003a). Interestingly, the $K_{A,Mg^{2+}}$ for CSP41a is approximately 2 mM, a value that is within the physiological Mg^{2+} concentration range, which varies from 0.5 mM in etiolated leaves to 2-3 mM in young light-grown leaves and 10 mM in mature green leaves (Horlitz and Klaff 2000; Ishijima et al. 2003). Although CSP41b is known to catalyze a divalent metal-dependent reaction (Bollenbach and Stern, unpublished data), the biophysical parameters describing its interaction with Mg^{2+} remain to be tested.

The physiological variation in stromal Mg^{2+} concentration suggested that light-dependent and developmental fluctuations in Mg^{2+} could regulate CSP41a activity *in vivo* (Yang et al. 1996). This hypothesis was verified by experiments in which the turnover of *rbcL* in lysed WT and CSP41a-deficient chloroplasts was measured as a function of free Mg^{2+}, which was varied from <1 mM to 12.5 mM (Bollenbach et al. 2003). Whereas the rate of *rbcL* turnover was invariant in chloroplasts from WT plants, its rate of turnover increased as a function of decreasing Mg^{2+} in chloroplasts from CSP41a-depleted plants. Together, these experiments suggested that CSP41a provides the primary route for transcript cleavage at high stromal Mg^{2+} concentrations but that it is bypassed, possibly by another endoribonuclease such as RNase E, RNase J, p54 or CSP41b (see Sections 1.1.2-1.1.4), at lower Mg^{2+} concentrations where CSP41a is only minimally active.

Substrate specificity. Most chloroplast open reading frames encode inverted repeat (IR) sequences in their 3' untranslated regions that can fold into stable stem-loop structures. Prior research has shown that these IRs act as processing determinants and protect upstream sequences against 3' to 5' exonucleolytic degradation (Stern and Gruissem 1987). CSP41 has no sequence specificity, but displays a substrate preference for stem-loop containing RNAs from *petD*, *psbA* and *rbcL in vitro* (Yang and Stern 1997). This property would potentially target CSP41 to mature RNAs for turnover (Bollenbach et al. 2003).

CSP41 activity was shown to be optimal with substrates containing fully base-paired stem-loops, whereas deletion of part or all of a stem-loop structure resulted in a 100-fold decrease in activity (Bollenbach and Stern 2003b). Mutations at the scissile bond, and mutations or deletions of the terminal loop structure had only minor effects on activity, whereas changes in stem torsion, either by intercalation of ethidium or though the introduction of single base bulges into either arm of the stem-loop, had more drastic effects. Together with *in vitro* measurements of several mRNA degradation rates in WT and CSP41a-deficient chloroplasts, this suggests that CSP41 has a broad substrate specificity, and that stem-loop structure is a major determinant of CSP41 cleavage rates, and therefore of transcript half-life.

2.1.2 RNase E/G

Ribonuclease E is generally believed to initiate RNA degradation in *E. coli* and also mediates the processing of certain rRNAs and tRNAs (Kushner 2002). *E. coli* and some other bacteria also encode a homolog, RNase G, which lacks the C-terminal domain (Fig. 1). RNase E, but not RNase G, is essential in *E. coli* and *Synechocystis* (Cohen and McDowall 1997; Rott et al. 2003).

Full-length or partial ESTs have been found for rice, *Arabidopsis*, tomato, barley, cocoa, grape, ice plant, sorghum, wheat, maize, soybean, and *Medicago truncatula*. Each of these RNase E/G homologs resembles the *E. coli* enzyme in the catalytic region, but lacks the C-terminal domain and contains an N-terminal extension.

In *E. coli* and several other related bacteria, RNase E is a component of the degradosome (Vanzo et al. 1998), a multiprotein complex that also contains PNPase, the DEAD-box RNA helicase RhlB, and the glycolytic enzyme enolase (Blum et al. 1999), which is believed to be important for mRNA degradation and processing (Symmons et al. 2002; Marcaida et al. 2006). Degradosome assembly is dependent on the RNase E C-terminal domain (Coburn et al. 1999). The absence of the C-terminal domain in plant RNase E/G homologs correlates with the absence of a degradosome in chloroplasts (Baginsky et al. 2001). The N-terminal extension is reminiscent of a chloroplast transit peptide (Fig. 1), and when the "plant-specific" extension of the *Arabidopsis* protein is analyzed for possible chloroplast targeting using bioinformatic tools, chloroplast localization is predicted (PCLR, 68%; TargetP, 69%; Predotar, 58%). A partial sequence of this protein was also reported in a Triton-insoluble pea chloroplast fraction (Phinney and Thelen 2005). Given this information, and the fact that RNase E has never been found in mitochondria, support the hypothesis that the nucleus-encoded RNase E homolog functions in the chloroplast and is responsible for an initial step in RNA degradation and/or for intercistronic processing (see Section 3.1.2). However, the function(s) of RNase E alone and/or within the context of other chloroplast endoribonucleases such as CSP41 remains speculative and awaits further analysis.

2.1.3 RNase J

Many organisms lack an RNase E homolog, suggesting that another endoribonuclease is responsible for endonucleolytic processing and turnover. Recently, the purification and identification of two novel *B. subtilis* endoribonucleases, RNases J1 and J2, was described (Even et al. 2005). These RNases, like the tRNA 3' processing endonuclease RNase Z, belong to β–CASP family of zinc-dependent metallo β–lactamases (de la Sierra-Gallay et al. 2005; Even et al. 2005) and *in vitro* assays suggest they are functionally homologous to RNase E, since they have the same substrate specificity, both in terms of cleavage site selection and in their preference for 5' monophosphorylated RNA substrates (Even et al. 2005).

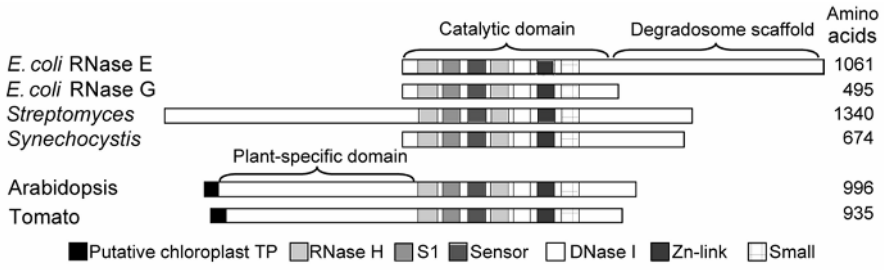

Fig. 1. Schematic amino acid alignment of RNase E homologs performed using MEME. Regions of significant homology are shown as textured boxes, with the catalytic subdomains named according to the recently solved structure (Callaghan et al. 2005). The catalytic and C-terminal degradosome scaffolding domains are highlighted by brackets at the top; the C-terminal domain is not conserved in any other protein shown. The "plant-specific" domains in *Arabidopsis* and tomato have no similarity to the *Streptomyces* N-terminal extension, and are preceded by putative plastid transit peptides (TP).

RNase J homologs are widespread in the eubacteria and archaea and although they appear to replace RNase E in many organisms, some encode both types of enzymes. The occurrence of both RNase E/G and RNase J in *Synechocystis* (Rott et al. 2003; Even et al. 2005) prompted us to search for RNase J homologs in the *Chlamydomonas* and *Arabidopsis* nuclear genomes. Each of these genomes contains a single *RNJ* gene (Positions 1136733-1144060, Scaffold 14 of the *Chlamydomonas* genome v3.0, and At5g63420, respectively), and the N-terminus of the *Arabidopsis* gene product targets GFP to chloroplasts in transient assays (Bollenbach and Stern, unpublished data). Any function of this enzyme in chloroplast RNA metabolism remains to be demonstrated, but it is essential for embryo development because plants heterozygous for a T-DNA insertion in the *RNJ* coding sequence produce siliques containing aborted embryos (www.seedgenes.org). This may be related to a function in 16S rRNA and/or ribosome assembly maturation, as was recently reported for the *B. subtilis* enzyme (Britton et al. 2007).

2.1.4 p54

RNase activities have been purified from chloroplasts for which no specific gene product has been associated (Nickelsen and Link 1989; Chen and Stern 1991). A well-characterized example is p54, a chloroplast RNA-binding protein and endoribonuclease originally identified by *in vitro* studies with mustard chloroplast protein extracts (Nickelsen and Link 1989, 1991). The interaction between p54 and RNA and its subsequent endonucleolytic cleavage were shown to be dependent on a heptamer motif located within the 3' non-coding regions of $tRNA^{Lys}$ and *rps16* mRNAs (Nickelsen and Link 1989). Therefore, p54 was hypothesized to be essential for $tRNA^{Lys}$ and *rps16* 3' processing, and *in vitro* cleavage sites correlated well with $tRNA^{Lys}$ and *rps16* mRNA 3' ends detected *in vivo* (Neuhaus et al. 1989; Nickelsen and Link 1991). Failure to bind $tRNA^{Gln}$, however, suggests that p54 is not a broadly specific in chloroplast tRNA 3' maturation (Nickelsen and

Link 1989); a role in tRNA 3' processing has recently ascribed to a chloroplast RNase Z homolog (Schiffer et al. 2002).

p54 is a divalent metal-independent ribonuclease and because its activity is not dependent on RNA secondary structure (Nickelsen and Link 1989, 1991) it has been suggested that it catalyzes RNA processing and/or turnover under conditions or on substrates where CSP41 is inactive (Bollenbach et al. 2003). Testing this hypothesis awaits identification of the p54 gene, and subsequent *in vivo* analysis. It cannot be ruled out, in fact, that p54 is none other than the Rubisco LS, which has recently been shown to have RNA-binding properties (Yosef et al. 2004), but which was not tested for endonuclease activity. Indeed, in our hands the two proteins co-purify (S. Preiss and D. Stern, unpublished results), and both p54 (Liere and Link 1997) and LS are redox-sensitive as RNA interactors.

2.2 Exoribonucleases

2.2.1 PNPase (polynucleotide phosphorylase)

PNPase (EC 2.7.7.8) was discovered during studies of biological phosphorylation in *Azotobacter vinelandii* (Grunberg-Manago and Ochoa 1955), and was later characterized in the context of its role in *E. coli* RNA synthesis (Littauer and Soreq 1982). In fact, PNPase was the first enzyme shown to catalyze the formation of polynucleotides from ribonucleotides; unlike RNA polymerases, PNPase catalyzes this reaction in a template-independent manner.

As a phosphorylase, PNPase catalyzes both processive 3' to 5' degradation and RNA polymerization, and in bacteria and organelles, participates in the degradation, processing and polyadenylation of RNA (Hayes et al. 1996; Grunberg-Manago 1999; Littauer and Grunberg-Manago 1999; Jarrige et al. 2002; Bollenbach et al. 2004; Slomovic et al. 2006a). PNPase was also reported to be a global regulator of virulence and persistency in *Salmonella enterica* (Clements et al. 2002), and its activity in some way regulates both chloroplast isoprenoid metabolism (Sauret-Gueto et al. 2006) and in *Chlamydomonas*, its ability to survive phosphate starvation (Yehudai-Resheff et al. 2007). Human PNPase was recently shown to be localized to the mitochondrial inter-membrane space (Chen et al. 2006; French et al. 2006; Rainey et al. 2006), and was identified in an overlapping-pathway screen to discover genes displaying coordinated expression as a consequence of terminal differentiation and senescence of melanoma cells (Leszczyniecka et al. 2002; Sarkar et al. 2003). Genes encoding PNPase homologs have been identified in almost all prokaryotes and eukaryotes with the exception of the *Mycoplasma*, trypanosomes and yeast (Slomovic et al. 2006a). In addition, there is no PNPase in archaea, though the hyperthermophiles and some methanogenic archaea contain an exosome that is very similar to the PNPase (Fig. 2) (Lorentzen et al. 2005; Portnoy et al. 2005; Slomovic et al. 2006a).

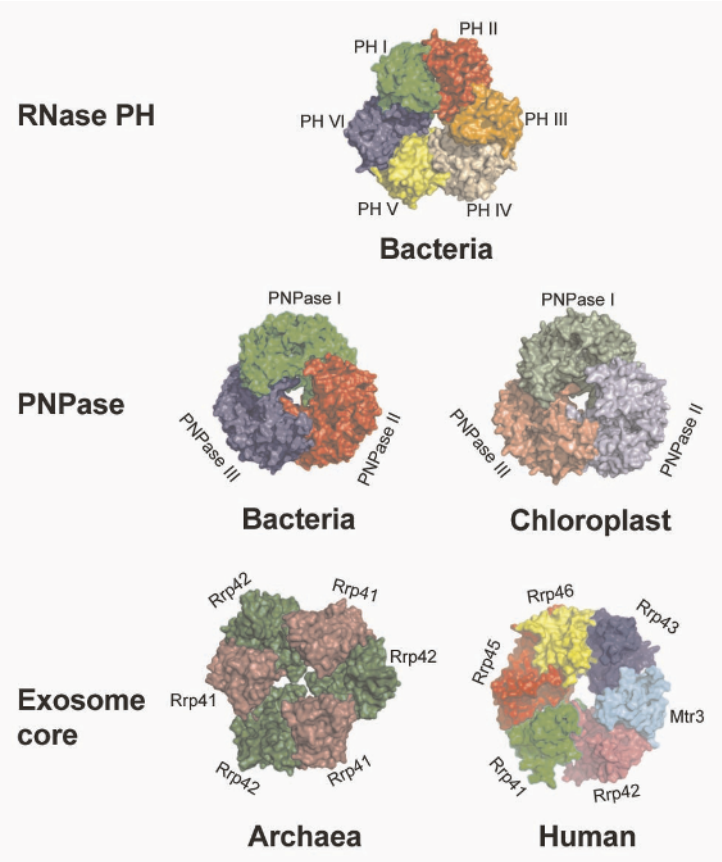

Fig. 2. Similarities in the structures of RNase PH, bacterial and chloroplast PNPase and the archaeal and human exosome cores. The bacterial RNase PH structure (Ishii et al. 2003; Harlow et al. 2004) and bacterial PNPase (Symmons et al. 2000a), archaeal (Buttner et al. 2005; Lorentzen et al. 2005) and human (Liu et al. 2006) exosomes, as well as the predicted structure of the chloroplast PNPase (Yehudai-Resheff et al. 2003), are shown in order to compare the ring shapes. The molecular surfaces are represented such that each protein subunit is differently colored. The structures were generated using PyMOL.

The primary structures of PNPases from bacteria and the nuclear genomes of plants and mammals comprise five domains, which are two N-terminal core domains homologous to the *E. coli* phosphorylase RNase PH, which are separated by an α-helical domain, and two C-terminal RNA-binding domains (KH and S1) (Symmons et al. 2000b, 2002; Zuo and Deutscher 2001; Raijmakers et al. 2002; Yehudai-Resheff et al. 2003). X-ray crystallographic analysis was used to reveal the three-dimensional structure of the PNPase from the bacterium *Streptomyces antibioticus*. The enzyme is arranged in a homotrimeric complex forming a circle

(doughnut), which surrounds a central channel that can accommodate a single-stranded RNA molecule (Fig. 2) (Symmons et al. 2000b, 2002).

The domains of spinach chloroplast PNPase were analyzed in detail using a series of recombinant proteins (Yehudai-Resheff et al. 2003). It was found that the first core domain, which was predicted to be inactive in bacterial enzymes, was active in RNA degradation but not in polymerization. Surprisingly, the second core domain was found to be active only in degrading polyadenylated RNA, suggesting that non-polyadenylated molecules can be degraded by this domain only if tails are added, apparently by the same protein (see Section 2.4.2). The high-affinity poly(A) binding site was localized to the S1 domain.

Recent observations suggest the unexpected conclusion that bacterial and chloroplast PNPases are evolutionary related to the archaeal and eukaryotic exosomes. The exosome functions in 3' to 5' RNA degradation, processing, and quality control of gene expression in the cytoplasm and nucleus of eukaryotic cells (Houseley et al. 2006), and is comprised of 10-11 proteins including six related to the phosphorylase RNase PH and two to the S1 and KH RNA-binding domains. Overall, the exosome is structurally similar to trimeric PNPase (Fig. 2)(Aloy et al. 2002; Raijmakers et al. 2002; Yehudai-Resheff et al. 2003; Hernandez et al. 2006; Liu et al. 2006). Therefore, the PNPase/archaeal exosome/eukaryotic exosome represent a functionally and evolutionary conserved machine for 3' to 5' exonucleolytic degradation.

2.2.2 RNase II/R

The RNR exoribonuclease family, which is typified by *E. coli* RNase II and RNase R, are hydrolytic processive 3' to 5' exoribonucleases that release 5' monophosphates. These enzymes are widely distributed among eukaryotes, eubacteria, mycoplasma and archaea. While most eukaryotic nuclear genomes encode at least three RNR homologs, some encode only a single RNR-like enzyme, and exceptional ones such as *Mycoplasma* encode a single RNR homolog as the only exoribonuclease (Zuo and Deutscher 2001). The halophilic archaea also contain an RNR homolog, while hyperthermophiles and several methanogens contain the archaeal exosome, which is similar to PNPase (Portnoy et al. 2005; Portnoy and Schuster 2006). Interestingly, no homolog of could be detected in methanogens that do not contain the archael exosome (Ng et al. 2000; Portnoy and Schuster 2006). The *Arabidopsis* nuclear genome encodes three homologs including RNR1, which is both plastid and mitochondria-localized, and RNR2 and RNR3, which based on GFP fusions are localized to the nucleus and cytosol, respectively, and are therefore putative exosome subunits (Perrin et al. 2004; Bollenbach et al. 2005).

In *E. coli*, the RNR family enzymes differ in their ability to remain processive through secondary structures. For example, RNase II becomes distributive near stem-loops and is eventually inhibited by them, while RNase R can melt secondary structures (Cheng and Deutscher 2002). Therefore, although in *E. coli* both enzymes are nonspecific exonucleases, RNase II is more active on single-stranded

homopolymeric transcripts such as poly(A), and RNase R has a preference for rRNAs (Cheng and Deutscher 2002).

An RNase II crystal structure has recently shed light on the catalytic activity and substrate specificity of RNR enzymes (Frazao et al. 2006; Zuo et al. 2006). RNase II folds into four domains comprising two N-terminal RNA-binding moieties, a central catalytic domain, and a C-terminal S1-like RNA binding region (Frazao et al. 2006; Zuo et al. 2006). The N- and C-terminal domains form a clamp atop the catalytic domain, which funnels the ssRNA substrate into a narrow channel that houses the active site. Although domain structure and sequence motifs are highly conserved among RNR family members, it is thought that differences in the clamp arrangement and thus RNA binding properties play an important role in regulating the activity on transcripts containing secondary structures.

Chloroplast RNR1 is inhibited by secondary structures when assayed *in vitro* (Perrin et al. 2004; Bollenbach et al. 2005). Therefore, it could participate in the processing of precursor RNAs, in particular 3' ends. Since mature transcripts often contain terminal stem-loops any degradative action of RNR1 would require prior endonucleolytic cleavage and polyadenylation, or recruitment of an RNA helicase. The latter tactic is employed by yeast mitochondrial Dss1, an RNase R homolog that digests secondary structures by complexing with a helicase. It should be noted that there is no PNPase in yeast mitochondria, thus Dss1 is the only exonuclease so far identified in that organelle (Dziembowski et al. 1998).

RNase II, RNase R, and PNPase, which represent the major exoribonuclease activities in *E. coli*, have significantly different substrate specificities and catalytic properties *in vitro* but share overlapping functions *in vivo*. In *Synechocystis*, there is a single RNase II/R homolog. In addition, PNPase functions as the only polyadenylation enzyme (in addition to its function in degradation). Accordingly, deletion of *Synechocystis* PNPase- or RNase II/R-encoding genes, unlike the situation in *E. coli* (Donovan and Kushner 1986), leads to inviability (Rott et al. 2003). Similarly, since there is no PNPase in yeast mitochondria, deletion of the RNase II/R homolog *DSS1* leads to mitochondrial dysfunction and eventually to loss of its genome (Dziembowski et al. 1998, 2003).

Plant chloroplast PNPase and RNR1 catalyze distinguishable reactions *in vivo*, but may functionally overlap. Repression of the *pnp1* gene, for example, leads to defects in mRNA and 23S rRNA 3' processing, but plants retaining only minimal amounts of chloroplast PNPase are viable and grow on soil (Walter et al. 2002). In contrast, *rnr1* null mutants are defective in rRNA 3' processing but not in mRNA 3' processing (Kishine et al. 2004; Bollenbach et al. 2005). RNR1 mutants are inviable on soil, owing to a dependence on RNR1 for chloroplast development in cotyledons, and perhaps an effect on mitochondrial mRNA metabolism (Perrin et al. 2004). On the other hand, *pnp1*/*rnr1* double null mutants have an embryo lethal phenotype (Bollenbach, Gutierrez, and Stern, unpublished data), suggesting either that these enzymes are redundant or additive for an essential processing or regulatory step(s).

2.2.3 Evidence for a 5' to 3' pathway

A major player in eukaryotic RNA decay is a 5' to 3' pathway catalyzed by the exonuclease Xrn1/Rat1. First described in *S. cerevisiae* and subsequently in animals (Newbury et al. 2006), Xrn1 is encoded by a small gene family in plants (Kastenmayer and Green 2000), with at least one member involved in miRNA metabolism (Souret et al. 2004). None of the family members, however, are known or suspected to be organelle-targeted.

It is therefore surprising that chloroplasts possess a 5' to 3' RNA degradation activity, which was revealed through the phenotypes of nuclear mutants affecting the stabilities of individual chloroplast transcripts (see Section 3.1.2). This suggests several possibilities: (1) one of the Xrn1-like proteins may be organelle-localized or dual targeted; (2) an organellar protein with Xrn1-like activity may exist but have little sequence homology; and/or (3) the apparent 5' to 3' RNA degradation maybe be a net activity, in fact catalyzed by a processive endonuclease.

Current literature best supports the concept of a net 5' to 3' pathway. Evidence for this comes from studies of endonuclease cleavage sites in the 3' UTRs of the *Chlamydomonas rbcL* and *atpB* mRNAs. When cleavage occurs, presumably as part of 3' end maturation (see Section 3.3), the downstream moiety is rapidly degraded (Stern and Kindle 1993). Subsequent studies showed that the degradation cannot be blocked using polyguanosine [poly(G)] or a stem-loop structure, which prevent exonuclease attack (Hicks et al. 2002). On the other hand, the 5' to 3' degradation found in RNA stability mutants can be blocked by poly(G), leaving open the possibility that chloroplasts have multiple 5' to 3' activities (Drager et al. 1998, 1999; Nickelsen et al. 1999).

If a vectorial endonuclease exists in chloroplasts, the best candidate would be an RNase E-like enzyme (Mackie 1998). As discussed in Section 1.1.2, however, its function in chloroplasts is still speculative. Furthermore, there is no evidence as yet that 5' to 3' pathway(s) occur in higher plant chloroplasts. Indeed, none of the plant nuclear mutants affecting cpRNA metabolism appear to mimic the RNA stability mutants of *Chlamydomonas* (see Section 4). Whether this is an artifact of the small number of mutants characterized to date or an evolutionary difference, remains to be established.

3 Polyadenylation

3.1 Historical perspective on polyadenylation

Polyadenylation is an important posttranscriptional modification of prokaryotic, eukaryotic and organellar RNA. In the cytoplasm and nucleus, the molecular mechanism of the addition of stable poly(A) tails to the 3' ends of most mRNAs and the importance of this process for translation initiation have been well established (Wickens et al. 1997; Dreyfus and Regnier 2002a; Edmonds 2002). In addition, transient polyadenylation was recently described for the yeast nucleus as part of an exosome-dependent RNA quality control mechanism (Lacava et al. 2005;

Vanacova et al. 2005; Wyers et al. 2005; Houseley et al. 2006). In bacteria, the major proteins involved in the polyadenylation-stimulated pathway have been identified and the relationship between polyadenylation and RNA decay has been characterized (Coburn and Mackie 1999). Polyadenylated RNA was first detected in the chloroplast more than 30 years ago (Haff and Bogorad 1976). Using hybridization experiments with cpDNA and ^{125}I-labeled RNA from maize seedlings, it was determined that about 6% of the poly(A)-containing RNA hybridized to cpDNA, and that the chloroplast poly(A) tracts averaged about 45 nucleotides in length.

Since polyadenylation is a phenomenon observed in almost all organisms, a major point is the assumption that a basal mechanism of polyadenylation-stimulated degradation of RNA was present in the last universal common ancestor of the three domains of life. During evolution, this basal mechanism was subjected to many modifications and variations that can be observed today in different organisms and organelles (Table 1) (Slomovic et al. 2006a). Moreover, different and perhaps conflicting biological functions for polyadenylation were acquired in several cases, such as transcript stabilization and translation initiation in the case of eukaryotic mRNA, and stimulation of turnover in the case of bacteria and organelles (Dreyfus and Regnier 2002a; Slomovic et al. 2006b).

The addition of a stable poly(A) tail to most nucleus-encoded mRNAs was first observed many years ago, and shown to occur following endonucleolytic cleavage in the 3' UTR, by a complex of several proteins providing enzymatic, RNA-binding and regulatory functions (Weiner 2005). Therefore, even though the first PAP was identified in *E. coli*, polyadenylation has long been considered a unique feature of eukaryotic cells and one of the major differences between eukaryotes and prokaryotes.

3.2 The polyadenylation-stimulated degradation pathway in bacteria

As mentioned above, even though the purification of *E. coli* PAP was reported many years ago, polyadenylation in bacteria was not studied extensively, perhaps because no biological role had been conceived (Sarkar 1997; Deutscher and Li 2001; Kushner 2004). However, attention was refocused on polyadenylation when it was discovered that mutations in *pcnB* (encoding PAP) resulted in a tenfold increase in accumulation of RNA I, which represses plasmid replication. These results suggested that polyadenylation targets RNA I for rapid degradation, in contrast to the stability and translational competence imparted by the stable poly(A) tails at the 3' ends of nuclear mRNA.

Considerable progress has subsequently been made in understanding bacterial RNA polyadenylation and degradation, mostly by analyzing *E. coli* (Deutscher 2006). The first step in RNA degradation is endonucleolytic cleavage, which is believed to be carried out mainly by RNase E or RNases J1 and J2 (see Section 1.1.3). In chloroplasts, CSP41a was also shown to be a key enzyme in endonucleolytic cleavage (see Section 1.1.1), thus chloroplasts may have two or even three endonucleases in the polyadenylation pathway.

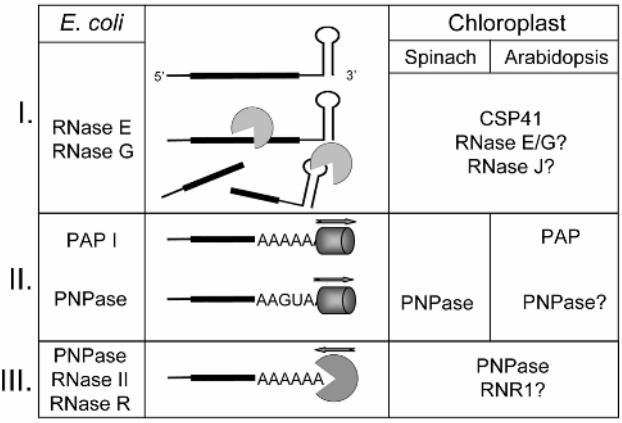

Fig. 3. A comparison of polyadenylation-stimulated RNA turnover pathways in *E. coli* and chloroplasts. The three stages of polyadenylation-stimulated RNA turnover are highlighted at left: endonucleolytic cleavage (I), polyadenylation (II), and exonucleolytic turnover (III).

In the second step, the cleavage product is polyadenylated and thus targeted for rapid exonucleolytic degradation (Fig. 3). In *E. coli*, polyadenylation is carried out mainly by a nucleotidyltransferase-type PAP (Ntr-PAP) producing homopolymeric poly(A) tails and to a certain extent by PNPase, which produces heteropolymeric poly(A)-rich tails containing all four nucleotides (Mohanty and Kushner 2000b). The protein Hfq, which resembles the eukaryotic Sm-like protein, was recently found to be involved in the modulation of polyadenylation activity between Ntr-PAP and PNPase (Mohanty et al. 2004; Folichon et al. 2005). The final step in the polyadenylation pathway is exonucleolytic degradation, which is performed by PNPase, RNase II, and RNase R in *E. coli* (Cheng and Deutscher 2005).

These findings along the way stimulated related research in other prokaryotes and in organelles. Indeed, evidence for the evolution and adaptation of the basic ancient polyadenylation-stimulated degradation process and the proteins involved have been revealed (Table 1) (Slomovic et al. 2006b), and the reader is referred to several recent reviews (Coburn and Mackie 1999; Grunberg-Manago 1999; Marujo et al. 2000; Deutscher and Li 2001; Dreyfus and Regnier 2002b; Kushner 2002, 2004; Condon 2003; Deutscher 2006).

3.3 PNPase as the major polyadenylating enzyme: variations from *E. coli*

Only limited studies have been carried out on Gram-positive bacteria. When *Streptomyces coelicolor* and *B. subtilis* transcripts were analyzed, heteropolymeric tails containing all four nucleotides were found, suggesting that PNPase and not Ntr-PAP is the major polyadenylating enzyme (Bralley and Jones 2002; Campos-Guillen et al. 2005). Accordingly, the sole Ntr proteins encoded by both these

Table 1. Similarities and differences between RNA polyadenylation systems among prokaryotes, chloroplasts, and eukaryotes.

	Prokaryotes			Chloroplast		Eukaryotes
	G-	G+	Cyano-bacteria	Plants	algae	Nucleus +Cytoplasm
	E. coli	*S. coe.* *B. sub.*	*Syn.*	Spinach, *Arabidopsis*	*Chlamydomonas*	Yeast Human
Endo.	E G	E J	E J	E CSP41 J	? J	?
Polyad-enylation	PAP I PNP	PNP PAP?	PNP	PNP PAP	PNP? PAP?	PAP TRAMP Exo.?
Exo.	PNP II R	PNP R	PNP R	PNP R	PNP R	3' → 5' Exo 5' → 3'
Poly(A)	Hom.	Het.	Het.	Het. Hom.	Hom.	Hom. Het.
Poly(A) RNA	Unstable					Stable +Unstable

Note: Within the bacteria, *E. coli* represents the Gram-negative (G-) and *Streptomyces coelicolor* (*S. coe.*) and *Bacillus subtilis* (*B. sub*.) the Gram positive (G+). Cyanobacteria are represented by *Synechocystis* (*Syn.*). Land plants are represented by spinach and *Arabidopsis* while algal data are from *Chlamydomonas*.
Symbols and abbreviations are: E, proteins homologous to RNase E or RNase G of *E. coli*; G, RNase G; PAP, poly(A) polymerase; PNP, polynucleotide phosphorylase; II and R, proteins homologous to RNase II and RNase R of *E. coli*; (?), Unknown or only based on prediction from genomic sequences. Hom., homopolymeric poly(A); Het., heteropolymeric poly(A)-rich. A gray background marks systems where both stable and unstable poly(A) tails are present.

organisms were active as Ntrs and not PAPs *in vitro* (Raynal et al. 1998; Sohlberg et al. 2003). Nevertheless, the analysis of PNPase-deficient *B. subtilis* revealed pronounced polyadenylation with homopolymeric poly(A) tails. This result suggested that *B. subtilis* has both PNPase and PAP-like activities, although the enzyme encoding the PAP-like activity has not been identified.

Cyanobacteria are related to the evolutionary ancestor of the chloroplast (Dyall et al. 2004), suggesting that an analysis of cyanobacterial RNA turnover could shed light on the ancient evolutionary form of the polyadenylation-stimulated pathway. Studies of *Synechocystis* revealed that mRNA, rRNA, tRNA and the single intron located at the $tRNA^{fMet}$ undergo polyadenylation (Rott et al. 2003), mirroring results for the same RNA classes in *E. coli* (Li et al. 1998), *Chlamydomonas* (Komine et al. 2000) and human mitochondria (Slomovic et al. 2005). The nature of the tails, which were poly(A)-rich and not homopolymeric, indicated that the polyadenylating enzyme is PNPase and not an Ntr. Therefore, PNPase is the major polyadenylating enzyme in cyanobacteria, spinach chloroplasts, and *Strep-*

tomyces. These results support the hypothesis that *E. coli,* other proteobacteria and *Arabidopsis* chloroplasts (see Section 2.4.2) acquired PAP relatively late in evolution through the conversion of a CCA-adding Ntr (Yue et al. 1996). Therefore, the RNA polyadenylation mechanism in cyanobacteria may represent a more ancient evolutionary state of the version found in *E. coli*.

3.4 Polyadenylation in the chloroplast

3.4.1 Discovery of heteropolymeric tails and relationship to degradation

Assuming that RNA metabolic pathways in the chloroplast were retained from its prokaryotic ancestor and following elucidation of the polyadenylation-degradation pathway in *E. coli*, the way was paved for dissecting this process in the chloroplast. RT-PCR analysis of oligo(dT)-primed cDNAs revealed polyadenylation in spinach chloroplasts (Kudla et al. 1996; Lisitsky et al. 1996). These studies revealed heteropolymeric, poly(A)-rich tails, the first observation of such tails in any organism. In addition, at the time of this discovery, there was still no explanation of how the heteropolymeric tails were formed. Nevertheless, heteropolymeric tails were later discovered in bacteria, archaea and human cells, as discussed above.

Several polyadenylation sites within the spinach *psbA* RNA matched endonucleolytic cleavage sites mapped by primer extension (Lisitsky et al. 1996). In addition, a polyadenylation site identified by RT-PCR in the spinach *petD* RNA was found to coincide with the cleavage site of a partially purified endoribonuclease when incubated with RNA resembling the *petD* transcript (Kudla et al. 1996). These results implied that the polyadenylation sites are produced by endonucleolytic cleavage of mature RNA and do not arise from polyadenylation of truncated molecules resulting from premature transcription termination (reviewed in Hayes et al. 1999; Schuster et al. 1999).

That polyadenylation stimulates degradation was observed by several biochemical and molecular approaches, as well as by experiments using the green alga *Chlamydomonas reinhardtii*. A DNA construct was engineered to express GFP mRNA and protein in *Chlamydomonas* chloroplasts such that the 3' end poly(A) tail would be exposed after RNase P cleavage upstream of an ectopic *trnE* (Komine et al. 2002). Indeed, no GFP protein or polyadenylated *gfp* transcript could be detected in this strain. In contrast, the expression of GFP was relatively high in strains where the *gfp* mRNA either lacked a poly(A) tail or contained an arbitrary (A+U) tail (Komine et al. 2002). This result, together with those obtained using *in vitro* and lysed chloroplast assays demonstrated that polyadenylation-stimulated degradation in chloroplasts and bacteria were similar. Therefore, the next step was to identify the proteins responsible for initial endonucleolytic cleavage, polyadenylation and exoribonucleolytic degradation.

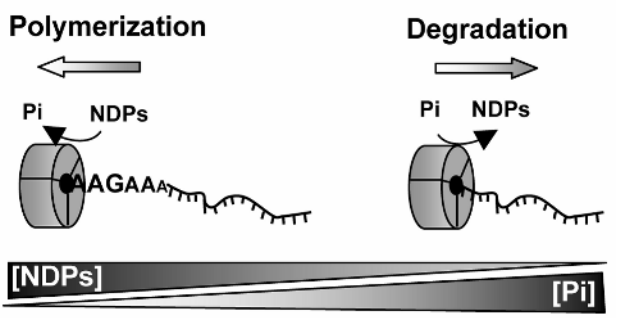

Fig. 4. PNPase acts as both a polymerase and a 3' to 5' exoribonuclease. PNPase is presented schematically as a homotrimer. When polymerizing RNA (left side), PNPase consumes nucleotide diphosphates (NDPs) and produces inorganic phosphate (P_i). When PNPase is an exoribonuclease and catalyzes RNA degradation (right side), it consumes P_i and produces NDPs. Because the equilibrium of this reaction lies close to unity, PNPase is exquisitely sensitive to P_i and NDP concentrations (grey wedges). Therefore, the reaction catalyzed by PNPase can theoretically be dictated by local concentrations of each substrate.

3.4.2 Different enzymes perform polyadenylation in spinach and Arabidopsis chloroplasts

Interestingly, species differences for polyadenylation enzymes were found in chloroplasts as they were in bacteria. In 2000, it was discovered that poly(A) tails in *pcnB* deletion strains of *E. coli* were heteropolymeric, very similar to those characterized before in spinach chloroplasts, and that these heteropolymeric tails were produced by PNPase (Mohanty and Kushner 2000a). This meant that PNPase was likely responsible for polyadenylation in spinach chloroplasts and indeed, purification of PAP activity from spinach chloroplasts yielded only PNPase, whose activity was the same as the stromal extracts from which it was isolated (Yehudai-Resheff et al. 2001).

How can one enzyme perform the opposing activities of polyadenylation and degradation? Biochemical and molecular analyses revealed that the directionality of the nearly freely reversible reaction that chloroplast PNPase catalyzes is directly influenced by the P_i/NDP ratio (Yehudai-Resheff et al. 2001, 2003; Bollenbach et al. 2004). This suggests that PNPase activity may be shifted towards net exonucleolytic or polymerization activities by shifting concentrations of its substrates (Fig. 4).

A different situation exists in *Arabidopsis* chloroplasts where as in *E. coli*, an Ntr-like PAP may be responsible for polyadenylation (Fig. 3). This is because the tails identified so far in *Arabidopsis* chloroplasts are virtually homopolymeric (our unpublished results). Moreover, several putative chloroplast- and mitochondrially-targeted PAPs were identified bioinformatically in the *Arabidopsis* genome (Martin and Keller 2004). If one or more of these PAPs can be confirmed experimentally to be chloroplast-localized and to act as a PAP rather than an Ntr, this would suggest that the conversion of Ntr to PAP occurred independently in the

evolution of *E. coli* and *Arabidopsis* chloroplasts. The third observation suggestive of PAP activity in *Arabidopsis* chloroplasts came from the analysis of a transgenic line in which the amount of PNPase was significantly reduced, but chloroplast polyadenylation appeared to be undiminished or even increase (Walter et al. 2002).

Together, these observations show that while PNPase performs polyadenylation in spinach chloroplasts and in *Synechocystis*, PAP seems to be responsible for this process in *Arabidopsis* chloroplasts. This suggests that chloroplast lineages containing PAP vs. Ntr may have split relatively recently in evolutionary terms.

4 RNA maturation

4.1 5' end maturation

4.1.1 5' ends can be processed or primary transcripts

Chloroplast mRNAs are not capped but instead accumulate as unprocessed primary transcripts or processed transcripts, which are characterized by a 5' di- or triphosphate, or by a 5' hydroxyl group, respectively. 5' phosphorylated RNAs are cappable by GDP and guanylyltransferase, whereas hydroxylated 5' ends are not. In angiosperm chloroplasts, many RNAs accumulate both in primary and processed forms, whereas no cappable chloroplast RNAs have been detected in *Chlamydomonas*, suggesting that all transcripts are 5' processed. Although 5' processing sites and the mode of processing have been identified for a number of chloroplast RNAs, the enzymes that catalyze these reactions have not.

Chloroplast RNA 5' processing can result in differential translation efficiencies, as exemplified by tobacco *atpB*, *atpH*, *psbB*, and *rbcL*, which are processed within their 5' UTRs and accumulate in multiple forms (Tanaka et al. 1987; Orozco et al. 1990; Kapoor et al. 1997; Miyagi et al. 1998; Serino and Maliga 1998). *In vitro* assays suggested that translation efficiencies of unprocessed and processed tobacco *rbcL* and *atpH* 5' UTRs were comparable, while processing of *atpB* and *psbB* 5' UTRs resulted in enhanced translation efficiencies (Yukawa et al. 2006). In an extreme case, one of five spinach *atpB* transcripts, whose 5' end mapped to the start of the coding region, was associated with crude polysomes (Bennett et al. 1990). This variation is likely to reflect species differences, in particular *cis* elements in the 5' UTRs.

RNA processing in *Chlamydomonas* chloroplasts is also linked to translation when two different 5' ends are present. For example, mutagenesis experiments with *psbA* and *psbD* have suggested that only the shorter of the two transcripts that accumulate for each gene is competent for translation (Bruick and Mayfield 1998; Nickelsen et al. 1999). In at least one case, the differences in translation efficiency have been correlated with the presence of sequence elements in the 5' UTR that are present in the longer transcript, but not in the shorter one (Bruick and Mayfield 1998), while the causal relationship between processing and translation in other cases is not as clear-cut (Yukawa et al. 2006). This type of processing-dependent

regulation is also true for 5' ends generated by intercistronic processing, as described below.

4.2 Intercistronic processing

Plastid-encoded genes are often clustered into transcription units, reflecting their post-endosymbiotic assembly from different cyanobacterial genes and operons (Douglas 1998, 1999). Typical transcript patterns from these regions are complex, the result of extensive posttranscriptional processing including 5' and 3' maturation, intercistronic cleavages, and splicing, which are catalyzed by nucleus-encoded enzymes and are regulated by nucleus-encoded proteins (Barkan and Goldschmidt-Clermont 2000; Nickelsen 2003).

4.2.1 Clusters encoding mRNAs

The *psbB* gene cluster has long been a paradigm for studying the processing of plastid transcription units (Barkan 1988; Westhoff and Herrmann 1988). This cluster encodes five thylakoid membrane proteins, three of which are PSII components (*psbB*, *psbT*, *psbH*) and two of which are components of the cytochrome b_6/f complex (*petB*, *petD*).

Significant evidence suggests that intercistronic processing of the *psbB* gene cluster is required for efficient translation. *hcf107* is an *Arabidopsis* mutant impaired in *psbH* 5' processing, which results in a decrease in accumulation of monocistronic *psbH* and therefore in a decrease in the PsbH protein (Felder et al. 2001). This is thought to arise because the cleavage at position -45 of the *psbH* 5' UTR is required to alleviate inhibition by an intramolecular base pairing interaction that obscures the ribosome binding site. Similarly, the maize *crp1* mutant is impaired in cytochrome b_6/f complex accumulation, which is thought to result from the masking of the *petD* ribosome binding site, which requires endonucleolytic cleavage and formation of a monocistronic *petD* RNA to alleviate an intramolecular base pairing interaction (Barkan et al. 1994). On the other hand, tobacco and *Arabidopsis* chloroplasts do not accumulate monocistronic *petD* RNA and therefore do not require this same type of processing for translation, even though the *petB-petD* intergenic spacer contains elements important for translation (Monde et al. 2000a).

Although not affected in translation initiation, a third mutant of note that affects *psbB* operon processing is *Arabidopsis hcf152*, which is defective in *petB* intron splicing and therefore in cytochrome b_6/f accumulation (Meierhoff et al. 2003). Although the endoribonucleases responsible for intercistronic cleavage and splicing have not been identified, *HCF107*, *CRP1*, and *HCF152* each encode TPR/PPR family proteins (see Section 4.3), suggesting that this abundant class of proteins plays an important role in regulating the processing of polycistronic RNAs in the chloroplast. Further supporting this conclusion is a recent report showing that a *Physcomitrella* PPR protein is required both for intercistronic cleavage between *clpP* and 5'-*rps12*, and for *clpP* splicing (Hattori et al. 2007).

A highly regulated chloroplast gene cluster is the *ndhH-D* operon. This operon encodes, in order, *ndhH*, *ndhA*, *ndhI*, *ndhG*, *ndhE*, *psaC*, and *ndhD*. The *ndh* genes encode components of the low abundance NADH dehydrogenase complex, and *psaC* encodes subunit VII of photosystem I. Despite being co-transcribed, the *psaC* message accumulates two orders of magnitude higher than the *ndh* messages (Meurer et al. 1996). In leek and barley, the *psaC-ndhD* dicistronic intermediate is cleaved within the *ndhD* coding sequence, which provides monocistronic *psaC* with a stabilizing 3' UTR and yields a non-translatable monocistronic *ndhD* (del Campo et al. 2002, 2006). Alternative *psaC-ndhD* intergenic cleavages produce translationally competent *ndhD* at low levels, but only from dicistronic messages in which C to U editing has restored the *ndhD* start codon (Hirose and Sugiura 1997; del Campo et al. 2002). *In vitro* evidence from tobacco translation extracts suggested that the *psaC-ndhD* dicistronic RNA is not translationally competent, and that production of monocistronic RNAs is required to alleviate a base pairing interaction between the *ndhD* 3' UTR and an 8 nt element contained within the *psaC* coding region, thus allowing translation to occur (Hirose and Sugiura 1997). Mutations of the negative control element destabilized this base pairing and resulted in the translation of *ndhD* from the dicistronic RNA. This highly regulated system apparently ensures that processing and accumulation of *ndhD* does not exceed that of other Ndh complex subunits, while still allowing the *psaC* message, and PSI subunit VII, to accumulate to high levels.

4.2.2 The chloroplast rrn operon

Chloroplast rRNA genes resemble those of bacteria, in that their coding sequences are conserved and that they are co-transcribed as part of an operon with the gene order 16S-23S-4.5S-5S. The operon also encodes two tRNAs within the 16S-23S spacer, and is flanked by tRNA genes. Chloroplast ribosome biogenesis requires considerable processing and maturation of rRNAs, which requires both endo- and exoribonuclease steps. The primary *rrn* transcript is cleaved endonucleolytically by an unidentified enzyme(s), which releases pre-tRNAs and pre-rRNAs. Pre-tRNAs are matured by chloroplast homologs of RNase P and RNase Z at their 5' and 3' ends, respectively (Wang et al. 1988; Schiffer et al. 2002). The pre-16S and 5S RNAs differ considerably from their bacterial counterparts in that they are not processed close to their mature termini and therefore accumulate long 3' tails, which require 3' to 5' exonucleolytic processing by RNR1 and/or PNPase (Yamamoto et al. 2000; Walter et al. 2002; Bollenbach et al. 2005).

The 23S rRNA in plants appears to co-migrate with the *E. coli* 23S rRNA under non-denaturing conditions, but migrates as smaller RNAs under denaturing conditions due to cleavage at the so-called "hidden breaks" (Leaver 1973). The 4.5S RNA, which is unique to angiosperms, is homologous to the bacterial 23S rRNA 3' end, and is separated from the remainder of the 23S sequence by a 100 nt internal transcribed spacer (ITS). The 23S-4.5S processing intermediate undergoes 3' maturation prior to cleavage at the 4.5S 5' end, in a series of steps that requires prior assembly into pre-50S ribosomal subunits, as evidenced by the accumulation of this transcript in mutants defective in both rRNA 3' processing and ribosome

assembly (Bellaoui et al. 2003; Bisanz et al. 2003; Bellaoui and Gruissem 2004; Bollenbach et al. 2005). 23S rRNA then undergoes a two-step 3' maturation that in *Arabidopsis* requires both PNPase and RNR1 (Walter et al. 2002; Bollenbach et al. 2005), but appears to be PNPase-independent in *Chlamydomonas* (Yehudai-Resheff et al. 2007). The translational consequences of a failure to remove the 23S ITS in plants is unknown and may be phenotypically silent as it is in bacteria (Kordes et al. 1994; Gregory et al. 1996; Mattatall and Sanderson 1998). On the other hand, the *Chlamydomonas ac20* mutant, which accumulates unspliced 23S rRNA and fewer mature ribosomes, fails to grow photoautotropically (Holloway and Herrin 1998).

4.3 3' end maturation

The 3' IRs of bacterial mRNAs promote transcript stability and can act as rho-independent transcription terminators. In chloroplasts, transcription termination is not influenced by 3' IRs and is probably stochastic (Stern and Gruissem 1987, 1989). Therefore, chloroplast mRNAs require 3' processing for maturation by processive 3' to 5' exoribonucleases (Stern and Gruissem 1987; Rott et al. 1996). 3' end maturation and 3' IR function has been studied in detail in *Chlamydomonas* using the *atpB* mRNA as a model. Termination of *atpB* transcription by its 3' IR is less than 50% efficient (Rott et al. 1996) and the resultant heterogeneous pre-mRNAs undergo two-step processing that begins with cleavage at a specific endonucleolytic cleavage site (ECS), and is completed by 3' to 5' exonucleolytic trimming (Stern and Kindle 1993), and may involve polyadenylation (Komine et al. 2000). Recent analysis of the *atpB* and *rbcL* 3' IR and ECS, together referred to as the 3' processing determinant (PD), suggested that these elements contain a significant amount of redundancy, since deletion of one or the other *cis*-element did not cause changes in *atpB* maturation (Rymarquis et al. 2006b). Redundancy in 3' PDs may be fairly common. For example, the *Chlamydomonas* chloroplast *petA* gene has at least ten possible mature 3' termini (Jiao et al. 2004).

Genetic screens have identified at least two nuclear genes important to chloroplast 3' RNA processing, *CRP3* and *MCD4*. The *crp3* mutant was isolated as a suppressor of a chloroplast *atpB* 3' IR deletion mutant, and was later found to affect the 3' maturation of several chloroplast-encoded RNAs (Levy et al. 1997, 1999). The *mcd4* mutant, which has numerous chloroplast 3' processing defects, is described in Section 4.2. The genes encoding CRP3 and MCD4 have not been cloned, but evidence suggests that they either represent endoribonucleases or RNA-binding proteins that guide ribonucleases to the ECS.

PNPase has been shown to be important for mRNA processing in *Arabidopsis*, since plants in which PNPase expression was inhibited by co-suppression were defective in *rbcL* and *psbA* 3' maturation, and accumulated RNAs with multiple 3' ends. Unlike the case with *Chlamydomonas atpB*, however, these transcripts were not differentially polysome associated versus their processed counterparts (Walter et al. 2002). *Chlamydomonas* cells nearly lacking PNPase due to RNAi suppression, however, accumulated apparently normal chloroplast mRNAs, suggesting a redundancy in this organism (Yehudai-Resheff et al. 2007).

4.4 Non-coding RNAs

Antisense RNA (asRNA)-mediated gene regulation occurs widely in prokaryotes and eukaryotes and bacteria express both *cis*- and *trans*-encoded antisense transcripts (reviewed in Gottesman 2004; Storz et al. 2005). For example, accumulation of *Synechocystis isiA* was shown to correlate inversely with the *cis*-encoded asRNA *isiR*, and it was suggested that the *isiA*-*isiR* duplex could be targeted for turnover by a dsRNA-specific RNase, such as RNase III (Duhring et al. 2006). The *Arabidopsis* nuclear genome encodes two RNase III homologs with putative chloroplast transit peptides at the loci At4g37510 and At3g20420.

Because posttranscriptional regulation is important in chloroplasts, it stands to reason that antisense-mediated mechanisms may operate in these organelles, although a role for noncoding RNAs (ncRNAs) remains to be clearly established. The tobacco chloroplast-encoded *sprA* gene (Vera and Sugiura 1994) encodes a *trans*-encoded RNA that was hypothesized to control 16S rRNA 5' maturation, but this function could not be confirmed by further experimentation with transgenic plants (Sugita et al. 1997). More recently, a search for chloroplast-encoded ncRNAs in tobacco identified several short sequences including two *cis*-asRNAs, Ntr-5 and Ntr-7, which are complementary to *atpE* and the *rps16* intron, respectively (Lung et al. 2006). Thus chloroplasts, like their prokaryotic ancestors, may encode functional asRNAs.

Evidence that asRNAs can regulate their targets in chloroplasts is currently restricted to transgenic contexts. For example, fortuitous expression of a synthetic asRNA following a chloroplast genome rearrangement in *Chlamydomonas* resulted in the stabilization of an otherwise unstable polyadenylated *atpB* transcript (Nishimura et al. 2004). In another case, expression of asRNAs decreased the efficiency of sense RNA editing in tobacco chloroplasts (Hegeman et al. 2005). Thus, chloroplasts have the potential to utilize natural asRNAs for gene regulation.

5 Regulatory factors

Generalized screens have led to identification of cpRNA mutants. In *Chlamydomonas*, mutants were obtained by isolating colonies unable to grow on minimal medium (acetate-requiring). These nonphotosynthetic mutants affect all stages of gene expression, as well as metabolic functions (Harris 1989; Rochaix 1995). The analogous screens in higher plants are seedling lethality in maize and a sucrose requirement in *Arabidopsis* (Barkan 1998; Stern et al. 2004). These plants display chlorotic or ivory phenotypes and if blocked in photosynthetic electron transport, high chlorophyll fluorescence (hcf). The hcf screen has also been used in *Chlamydomonas*, simplified by a video imaging approach (Bennoun and Béal 1997). While some of these mutants have turned out to affect ribonucleases, as discussed above, most remain uncloned or encode regulatory proteins. In this section, we discuss mutant characteristics and the PPR/TPR protein families, which are emerging as key regulators of organellar RNA metabolism.

5.1 Mutations affecting single chloroplast loci

A mutant class essentially unique to *Chlamydomonas* is gene-specific RNA stability mutants. These recessive mutants lack factors that stabilize certain transcripts, generally against 5' to 3' degradation. Known targets include *petA*, *psbB-psbT*, *petD*, *psbC*, *atpB*, and *psbD* (Barkan and Goldschmidt-Clermont 2000). The specificity of such mutants is somewhat presumptive, since in only one case was each chloroplast transcript checked in the mutant background; a microarray analysis of the *petD* mutant *mcd1* confirmed its specificity (Erickson et al. 2005).

Several *Chlamydomonas* RNA stability factors have been cloned. *MCA1*, which stabilizes *petA* mRNA, encodes a pentatricopeptide repeat (PPR) protein (Lown et al. 2001), a motif which is discussed below. Some nomenclature confusion exists because *MCA1* was previously attributed to mitochondrial carbonic anhydrase (Eriksson et al. 1998). The *psbB/T* and *psbD* stability factors are encoded by *MBB1* and *MBD1/NAC2*, respectively, which both feature tetratricopeptide (TPR) repeats (Boudreau et al. 2000; Vaistij et al. 2000), another motif that is discussed below. The *petD* stability factor MCD1, however, possesses neither of these motifs nor any recognizable domains (Murakami et al. 2005). From just this small sample, it appears that even within *Chlamydomonas* various solutions have arisen to protect transcripts, and possibly to promote their translation.

While no higher plant mutants are fully analogous to the *Chlamydomonas* RNA stability mutants, in at least one case an orthologous gene has been found. The *Arabidopsis* mutant *hcf107* (Felder et al. 2001) has defects in the processing of *psbH* mRNA (see Section 3.2.1). The Hcf107 protein is homologous to Mbb1 (Sane et al. 2005), and the slightly different phenotypic consequence of its absence can be ascribed to the different gene arrangements in the respective chloroplast genomes. Other homologous pairs of genes have been identified for chloroplast biogenesis, such as *TAB2* (Dauvillee et al. 2003; Barneche et al. 2006), which functions gene-specifically in translation initiation in *Chlamydomonas* but appears to have multiple targets in *Arabidopsis*. This may suggest that evolution of these proteins has been more closely constrained by the RNA target, rather than interaction with cellular machinery such as ribosomes or nucleases. Otherwise, one might anticipate common motifs accompanied by a "gene specificity domain."

Several higher plant mutants, like *hcf107,* appear to have a single primary target. For example, *Arabidopsis HCF152* encodes a PPR protein that also affects *psbH* maturation (Meierhoff et al. 2003; Nakamura et al. 2003). Hcf152 has been reported to have structural similarity to Crp1, a maize protein whose primary target is cleavage between *petB* and *petD*, with a concomitant or secondary effect on PetA translation (Barkan et al. 1994). Because *psbH* and *petB-D* are in the same gene cluster, the functions of Crp1 and Hcf152 are in a sense related. In turn, cloning of Crp1 revealed sequence similarity to at least two fungal regulators of mitochondrial translation (Fisk et al. 1999), which is most related to its maize function for PetA. Crp1 was also reported to share homology with p67, an RNA-binding PPR protein from radish chloroplasts (Lahmy et al. 2000). The *Arabidopsis* homolog of p67 (At4g16390) and Hcf152 (At3g09650), however, are minimally re-

lated, making the situation somewhat ambiguous, and pointing to the difficulty of assigning correct homologies in large, degenerate gene families.

One nearly universal feature of the regulatory factors described above is that they are found in high molecular weight complexes. These have most often been revealed by gel filtration, and tend to show broad peaks in the 350 kDa – 600 kDa range, such as for Nac2, Crp1, and Mbb1 (cited above). A major unanswered question is the composition of these complexes, apart from the presence of the cognizant RNA, which has been detected in some cases (e.g. *psbD* mRNA in the Nac2 complex). One difficulty is their low abundance, which is a consequence of their single or dual-gene specificity. However, affinity methods are likely to lead to purification in the near future. The reader is also directed to the chapter by Schmitz-Linneweber and Barkan for a somewhat better-developed knowledge of chloroplast splicing complexes.

A final point regarding gene-specific regulators is the implication of co-evolution of the regulatory factor and the gene sequence. Evidence for this includes the lack of conservation between 5' UTRs of different chloroplast mRNAs, the targets of the vast majority of the regulators. Furthermore, small sequence motifs, when mutated, phenocopy the cognizant nuclear mutations. For example, 4-nt changes in the 5' UTR of the *Chlamydomonas petD* mRNA destabilize the transcript, phenocopying the *mcd1* mutation (Higgs et al. 1999); similar results were obtained for *psbD* (Nickelsen et al. 1999). Interestingly, the *petD* regulatory motifs tend to be highly conserved among *Chlamydomonas* species whose cpDNAs are otherwise highly divergent (Kramzar et al. 2006). This argues in favor of constraints on the *cis* elements in a given gene, most likely because of their interactions with specific motifs in the regulatory proteins.

Because transcript destabilization for these genes leads to a loss of photosynthetic capability, genetic screens can be carried out for restoration of photosynthetic growth. In the case of *psbD*, three unlinked nuclear suppressors were obtained which restored *psbD* expression, but did not affect *psbA* expression (Nickelsen 2000). For *petD* three suppressors were also obtained, again in unlinked nuclear loci. Most surprisingly, the restoration of *petD* expression was accompanied by pleiotropic effects on other chloroplast mRNAs (Rymarquis et al. 2006a), which are described in more detail in the next section. Direct screens for suppressors of the mutated nuclear factors have been less successful. A suppressor of an *mcd1* mutant was isolated and found to be allele-specific and semi-dominant, however it was revealed to encode a suppressor tRNA, rather than a new effector of *petD* expression (Murakami et al. 2005). In summary, studies of genetic interactions with gene-specific regulators is scattered, and understanding the basis of the specific interactions awaits knowledge of complex components and suitable *in vitro* systems.

5.2 Pleiotropic mutations

In principle, mutation of general RNA regulators should cause pleiotropic phenotypes, much as the maize nuclear mutant *crs1*, which is affected in the splicing of

many chloroplast introns (Jenkins et al. 1997). Indeed, the *Arabidopsis rnr1* and PNP- lines have pleiotropic defects (Walter et al. 2002; Bollenbach et al. 2005). Another class of pleiotropic mutations affects mRNAs, and is exemplified by *mcd3*, *mcd4* and *mcd5*, which were isolated as suppressors of *petD* 5' UTR mutations as described above (Rymarquis et al. 2006a). Most pleiotropic were *mcd3* and *mcd4*, which accumulated numerous transcripts with extended 3' ends, particularly in gene clusters. This implicates the genes in 3' end formation, which is counterintuitive since they were isolated as suppressors of 5' UTR mutations causing RNA instability. Some resolution of this dilemma may be offered by the fact that 5' ends of chloroplast transcripts are often generated by endonucleolytic processing, which also occurs at the 3' end, as exemplified by *Chlamydomonas atpB* and *rbcL*, among others (Blowers et al. 1993; Stern and Kindle 1993).

5.3 The PPR/TPR protein superfamilies

As noted above, at least some of the RNA regulators are members of the PPR and TPR protein classes. While the *Chlamydomonas* nuclear only encodes about two dozen TPRs and less than ten PPR family members, the protein class has been highly amplified in flowering plants. Indeed, *Arabidopsis* was found to encode 441 PPR proteins, many of which appear to encode essential functions in mitochondria and chloroplasts (Lurin et al. 2004). Why the families would be expanded in plants vs. *Chlamydomonas* is not yet known, however it may related to the lack of RNA editing in *Chlamydomonas*, and also to the extreme simplicity of its mitochondrial genome relative to that of the flowering plants. As components of multiprotein RNA processing complexes, TPR/PPR proteins are very likely to interact with catalytically active complex members. It could be that chloroplast RNA processing complexes, or processosomes, will be analogous to the bacterial degradosome, which contains both ribonucleases and scaffolding factors, in particular the C-terminal part of ribonuclease E (Vanzo et al. 1998). The degradosome, however, is not gene-specific, so the analogy is likely to be imperfect.

6 Conclusions

The last few years has seen a number of advances in the understanding of cpRNA processing and turnover, including a broader knowledge of how chloroplast polyadenylation has evolved and its extant diversity in the broader organismal context, the identification of new enzymatic and regulatory components of RNA metabolizing pathways, and the identification of chloroplast-encoded ncRNAs.

Much of the understanding of the polyadenylation pathway has been underpinned by comparative genomics, which permitted correlations between polyadenylation mechanisms and its enzymatic machinery (Slomovic et al. 2006a). Candidate gene approaches have been key to establishing the basic enzymatic framework of the cpRNA processing and turnover, setting the stage for a phase in

which regulation of their activities and specificities will be investigated. Whether these enzymes turn out to be regulated by metabolites, as in the case of CSP41a and PNPase (Yehudai-Resheff et al. 2001; Bollenbach and Stern 2003a; Bollenbach et al. 2003), or by plant-specific proteins such as members of the PPR family(Lurin et al. 2004), remains to be seen. Answering these questions will likely take a multidisciplinary strategy, combining forward and reverse genetics, biochemistry and enzymology.

Finally, we note the timely identification of antisense RNA-mediated gene regulation in *Synechocystis* (Duhring et al. 2006), and the recent identification of small, chloroplast-encoded ncRNAs (Lung et al. 2006). Whether these small RNAs turn out to be regulatory transcripts remains to be determined, as we move from cataloging them to determining the mechanisms by which they might regulate chloroplast gene expression.

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