Cooperation of endo- and exoribonucleases in chloroplast mRNA turnover[1]

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Running title: Chloroplast mRNA turnover

[1]Abbreviations are: PAP, poly(A) polymerase I; PNPase, polynucleotide phosphorylase; UTR, untranslated region; GFP, green fluorescent protein; NTfr, nucleotidyltransferase; SDR, short chain dehydrogenase-reductase

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ABSTRACT

Chloroplasts were acquired by eukaryotic cells through endosymbiosis, and retain their own gene expression machinery. One hallmark of chloroplast gene regulation is the predominance of post-transcriptional control, which is exerted both at the gene-specific and global levels. This review focuses on how chloroplast mRNA stability is regulated, through an examination of poly(A)-dependent and independent pathways. The poly(A)-dependent pathway is catalyzed by polynucleotide phosphorylase (PNPase), which both adds and degrades destabilizing poly(A) tails, whereas RNase II and PNPase may both participate in the poly(A)-independent pathway. Each system is initiated through endonucleolytic cleavages which remove 3' stem-loop structures, which are catalyzed by the related proteins CSP41a and CSP41b, and possibly an RNase E-like enzyme. Overall, chloroplasts have retained the prokaryotic endonuclease-exonuclease RNA degradation system, despite evolution in the number and character of the enzymes involved. This reflects the presence of the chloroplast within a eukaryotic host, and the complex responses that occur to environmental and developmental cues.

I. INTRODUCTION

A. Evolutionary perspective

This chapter focuses on emerging models for chloroplast mRNA decay. With their evolutionary origin as photosynthetic prokaryotes, chloroplasts maintain many enzymes and pathways familiar from bacteria. On the other hand, some 1.5 billion years evolution since endosymbiosis [reviewed in 56] have resulted in substantial adjustments to the eukaryotic environment. Some estimates of the reshuffling of genes since endosymbiosis argue that in fact, the majority of the chloroplast proteome is encoded not by genes acquired by plant cells from cyanobacteria, but instead those which already existed in the nucleus of the mitochondriate host [92]. Some of the proteins involved in RNA metabolism are undoubtedly encoded by these "horizontally" transferred genes, for example PPR/TPR proteins implicated in various post-transcriptional regulatory steps [128].

Another evolutionary force acting to diversify the regulatory complement is gene duplication, which is widespread in ancient tetraploids such as maize [42] and *Arabidopsis* [138]. One example to be discussed below is the endoribonuclease CSP41, which is encoded by a single gene in *Synechocystis*, but which is duplicated in plants. In a similar vein, some genes can be shuffled, modified or lost. For example, *E. coli*-like RNAse E differs substantially from its putative plant homolog, apparently maintaining only the catalytic domain. Other examples include RNAse P, which may have lost its RNA component in chloroplasts [140], and poly(A) polymerase (PAP), which is present in *E. coli* but may be absent in chloroplasts (see below).

Detailed description of organellar ribonucleases in plants is complicated by the facts that first, apart from rice, *Arabidopsis* and *Chlamydomonas*, complete nuclear genome sequences are not available; and second, differentiating between mitochondrial and/or chloroplast targeting of a nucleus-encoded protein is often impossible without direct biochemical evidence. Finally, we note that in a few of the more gene-rich algal chloroplasts, genes encoding RNAse P RNA, RNAse G, and other "oddball" functions remain [127]. Whether these are pseudogenes or active contributors to RNA metabolism, remains unknown.

B. Chloroplast gene regulation

1. Transcriptional regulation

Unlike prokaryotes, chloroplast genes are rarely regulated individually at the transcriptional level. However, chloroplasts are polyploid, and changes in chloroplast genome copy number can result in overall transcriptional modulation [28, 99]. Furthermore, two highly expressed chloroplast genes are subject to transcriptional regulation. These are the evolutionarily related genes *psbA* and *psbD*, which encode the relatively unstable D1/D2 reaction center proteins of photosystem II. Because D1 and D2 are easily damaged under high light, illumination dramatically enhances their transcription rates [41, 73], which in the case of *psbD* appears to be mediated by specific DNA-binding proteins [71]. Chloroplast transcription also exhibits a circadian oscillation, at least in the unicellular green alga *Chlamydomonas reinhardtii* [58, 121].

Plastids also differ from their prokaryotic counterparts by virtue of possessing two RNA polymerase types [18]. One is the familiar eubacterial enzyme (α_2, β, β') which assembles with sigma factors, whereas the other resembles single-subunit phage T3/T7 RNA polymerase and assembles with unknown factors. In an evolutionary twist, all chloroplast sigma factors are nucleus-encoded, and have emerged as a gene family with six members, all expressed simultaneously [3]. One of these proteins, in maize, is also targeted to mitochondria, where its function remains to be discerned [11].

2. RNA processing

Although both chloroplasts and bacteria produce polycistronic transcripts, in chloroplasts nearly all of these are processed by endonucleases, and many are spliced [reviewed in 9, 98]. That processing is a prerequisite for efficient translation is best illustrated by nuclear mutants that affect processing of *Arabidopsis psbH* [38, 94] and maize *petD* [10] mRNAs, respectively. The data are consistent with models where intercistronic processing relieves secondary structures that otherwise might block ribosome assembly, perhaps the chloroplast counterpart to classical bacterial attenuation. Rather than encoding ribonucleases, the proteins encoded by the mutated loci are representatives of related protein families which possess multiple PPR or TPR domains [see above references for Arabidopsis and 40]. These penta/tetratricopeptide repeats are found throughout eukaryotes, but the PPR/TPR protein family has expanded dramatically in plants, where many of its members are predicted or known to be organelle localized [128].

3' end maturation in chloroplasts uses the prokaryotic pathway which follows rho-independent termination, resulting in mature termini flanking stem-loop structures. This process occurs for virtually all mRNAs because transcription termination is quite inefficient in chloroplasts, which lack T-rich sequences downstream of stem-loops [118, 130]. Available data suggest that at least in some cases, both endonuclease cleavage and exonuclease resection are involved [51, 131], which would facilitate 3' end formation in a situation where termination was stochastic and possibly far downstream of the eventual 3' end.

3. RNA stability

Because they are not covered in the main body of this review, it bears mentioning that chloroplast mRNAs require *trans*-acting factors in order to accumulate. First defined by *Chlamydomonas* nuclear mutants that failed to accumulate individual chloroplast transcripts [e.g. 77], it was later discovered that these nucleus-encoded factors protected mRNAs from net 5'→3' degradation [e.g. 31]. This mode of degradation was surprising, because prokaryotes do not encode 5'→3' exonucleases, and the possibility remains that the observed activity results from a

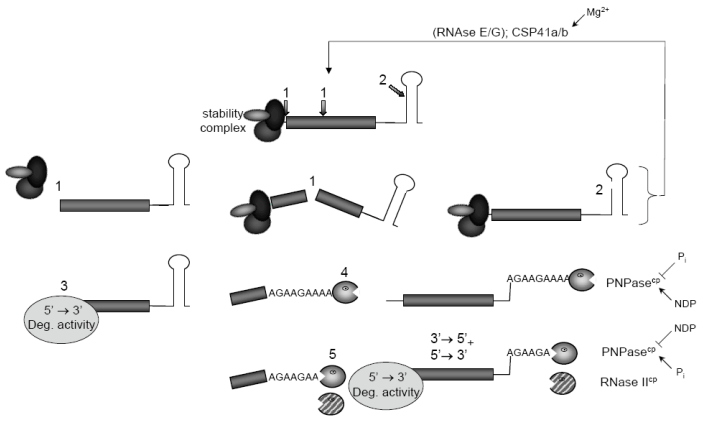

Figure 1. A working model for chloroplast mRNA degradation, illustrated for a typical stem-loop containing mRNA. Degradation is initiated by endonucleolytic cleavages within the coding region, the 5' UTR (1) or within the 3' UTR (2) by either CSP41a, CSP41b or RNase E/G. Cleavage at these positions yields distal products that may undergo further rounds of endonucleolytic cleavage, and proximal products that may be degraded by a 5'→3' activity (3), or may be polyadenylated by PNPase (gray) (4). The polyadenylated RNA is then degraded in the 3'→5' direction by either PNPase or by RNase II/R (diagonal stripes) (5), or may be simultaneously degraded in both the 5'→3' and the 3'→5' directions. Regulation of PNPase activity by NDP and Pi, and the regulation of CSP41a/b activities by Mg2+ are indicated. RNase E/G is labeled in parentheses since its participation in RNA turnover in land plant chloroplasts is hypothesized but has not been proven.

wave of endonuclease cleavages, such as those stimulated by the initial 3' processing step for the *Chlamydomonas atpB* mRNA [54]. Several genes encoding stability factors have been cloned, and most are of the PPR/TPR families mentioned above [14, 136]. These proteins are found in high molecular weight complexes *in vivo*, which contain their genetically defined RNA target. Whether disruption of these 5' stability complexes forms part of the cpRNA degradation pathway is not clear. If so, it would mirror the 5' cap removal which is characteristic of eukaryotic (cytosolic) mRNA decay [60].

C. A working model for cpRNA degradation

As in many systems, cpRNA lifetimes vary over a wide range, share few if any obvious sequence motifs in their untranslated regions (UTRs), and are regulated according to developmental and environmental cues. Any model for RNA decay, i.e. a molecular description of the apparatus that determines these lifetimes, must allow for these facts, and be consistent with extant biochemical and genetic data such as those outlined above. Roughly speaking, a working model should take into account *cis* elements in the mRNA and any proteins which bind to them, the resultant susceptibility of that RNA to the various ribonucleases present in the chloroplast, and how those binding proteins, RNA structures, or enzymes might be influenced by ionic strength, redox status, or other metabolic conditions.

Figure 1 presents such a model for cpRNA decay, including the data discussed above, and the nucleases discussed below. Initial steps might be removal of the 5' stability complex followed by 5'→3' exonucleolytic digestion or, more probably, by a wave of endonucleolytic cleavage in this direction followed by polyadenylation and 3'→5' exonuclease degradation. Alternatively, in a mechanism similar to that in *E. coli*, an endonuclease cleavage may destroy the 3' end stem-loop to initiate the degradation process. This endonuclease cleavage is followed by polyadenylation of the proximal cleavage product and 3'→5' exonucleolytic degradation. These steps could occur in cycles until the RNA molecule is degraded to short oligoribonucleotides or nucleotides. Given this model, two questions that arise are: what is the mechanism that first inactivates translatable mRNAs; and is the first endoribonucleolytic cleavage indeed the rate-limiting step? These questions should be considered in light of the discussion of each of the model's components, in the following sections.

II. POLYADENYLATION OF RNA IN THE CHLOROPLAST

A. Synopsis

The presence of relatively long poly(A) sequences in chloroplast and mitochondrial RNA preparations, and the presence of a RNA polyadenylating activity in chloroplast protein extracts, were first described 30 years ago [17, 49, 117]. Similar observations, and the first purification of a PAP, were reported for *E. coli*, but further characterization of this phenomenon was not immediately pursued [123], perhaps because a hypothesis explaining the biological role of polyadenylation in prokaryotes and organelles was lacking. Attention became refocused on polyadenylation, however, when it was discovered that *E. coli* strains harbouring deletions in genes encoding the exoribonucleases PNPase and RNase II accumulated significant levels of polyadenylated RNAs [21, 25, 27, 115, 123]. These results suggested that in contrast to the stability and translational competence imparted by polyadenylation of nuclear mRNA, polyadenylation of mRNA in *E. coli* targeted it for rapid degradation. This discovery prompted a re-examination of chloroplast polyadenylation and raised the question of whether polyadenylation is part of a degradation mechanism and if so, what this degradation pathway might be. Furthermore, if the chloroplast differed from *E. coli*, which might represents the ancient prokaryotic mechanism?

B. The biochemistry of chloroplast polyadenylation

Polyadenylation in spinach and *Chlamydomonas* chloroplasts was initially examined by PCR amplification of oligo dT-primed cDNAs [75, 78, 87]. These studies revealed that in contrast to the relatively short and homopolymeric poly(A) tails of *E. coli*, chloroplast tails could reach several hundred nucleotides in length and consisted in spinach of 70% adenosine, 25% guanosine, and 5% cytidine and uridine, but in *Chlamydomonas* were >95% adenosine. For the most part, the poly(A) or poly(A)-rich tails were added to sites within transcripts rather than to the mature 3' end. In fact, several polyadenylation sites within spinach *psbA* RNA matched endonucleolytic cleavage sites previously mapped by primer extension using the lysed chloroplast RNA degradation system [87], and a polyadenylation site in spinach *petD* RNA was found to coincide with the major *in vitro* cleavage site of a partially purified endoribonuclease [78]. These results implied that most, if not all of the polyadenylation sites are produced by the endonucleolytic cleavage of mature RNAs and not from truncated molecules resulting from premature transcription termination [reviewed in 124].

The fact that polyadenylation occurs at least 50 times less frequently at mature 3' ends than at internal sites suggests that mature 3' ends are poor substrates for the polyadenylating enzyme. Taking into account that most chloroplast mRNAs are processed at their 3' ends and that these ends are often coincident with the 3’ ends of stem-loop structures, it seems likely that in addition to protecting mRNA 3’ ends from exonuclease attack, the stem-loop also protects the RNA by sterically

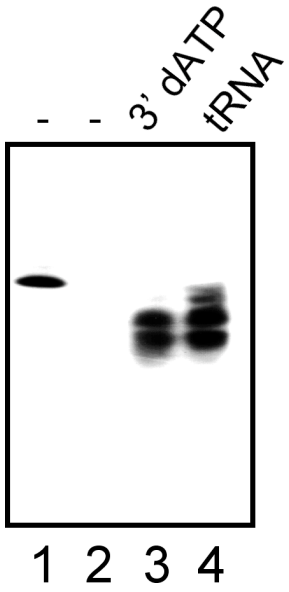

Figure 2. The inhibition of polyadenylation results in the inhibition of exonucleolytic degradation of RNA in lysed chloroplasts. Lysed chloroplasts were either harvested at time 0 (lane 1) or incubated for 60 min without (lane 2) or with (lane 3) the polyadenylation inhibitor 3’-dATP, or with excess yeast tRNA (which inhibits exoribonuclease activity; lane 4). After 60 min at 25°C, RNA was purified and analyzed for the presence of the *psbA* transcript by RNA gel blot hybridization.

blocking polyadenylation. In turn, this forces most mRNA degradation into an endonuclease-dependent pathway. Indeed, RNA molecules with a 3' stem-loop structure are poorly polyadenylated *in vitro* in chloroplast protein extracts [87].

C. Polyadenylation is part of the cpRNA degradation mechanism

That polyadenylation targets endonucleolytic cleavage products for rapid exonucleolytic degradation was confirmed by experiments using the lysed chloroplast system and the polyadenylation inhibitor cordycepin (3'-dATP). When lysed chloroplasts were treated with cordycepin, it was found that full-length *psbA* transcripts were endonucleolytically cleaved but that the cleavage products accumulated **(Figure 2)**. Furthermore, polyadenylated cleavage products accumulated when lysed chloroplasts were incubated with yeast tRNA, a non-specific exonuclease inhibitor [88]. These results implied that as in *E. coli*, polyadenylation targets chloroplast mRNAs for rapid exonucleolytic degradation following endonucleolytic cleavage.

A role for polyadenylation was recently confirmed *in vivo* using the green alga *Chlamydomonas reinhardtii* [74]. A chimeric, polyadenylated green fluorescent protein (*gfp*) gene was introduced into the *Chlamydomonas* chloroplast genome such that a 3' poly(A) tail would be exposed after RNase P cleavage upstream of an ectopic *trnE*. As shown in **(Figure 3)**, no GFP fluorescence could be detected in the this strain, whereas in a control strain transformed with a construct lacking a poly(A) tail or with a heteropolymeric [A + U] tail, the amount of GFP fluorescence was relatively high. Similar results were obtained by modification of the endogenous *atpB* gene, whose product is required for photosynthesis. Since engineered polyadenylation conferred transcript instability, the strain became an obligate heterotroph, allowing for the selection of autotrophic suppressors [74]. This *in vivo* approach corroborated results obtained using *in vitro* and lysed chloroplast assays, and opened up the possibility of regulating RNA stability of chloroplast transgenes by genetic engineering.

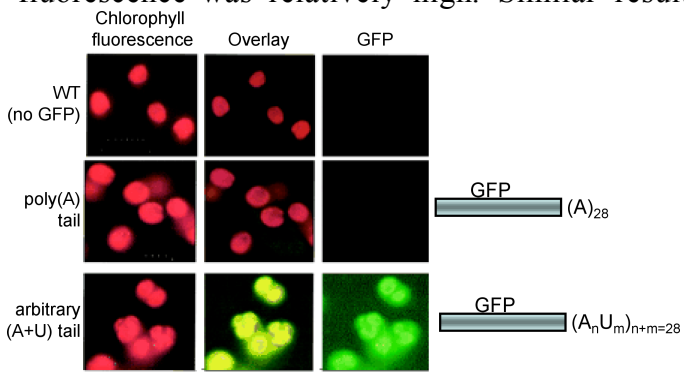

Figure 3. Utilization of GFP to show instability conferred by a poly(A) tail *in vivo*. From top to bottom wild-type cells, a chloroplast transformant possessing a GFP gene engineered to terminate in an A_{28} tail, and a similar transformant where an arbitrary A+U sequence was used in place of the poly(A) tail. Reproduced by permission from Komine et al. [74].

In a soluble chloroplast protein extract, polyadenylated and non-polyadenylated RNAs are degraded at similar rates. When the two types of RNA were mixed together, however, the non-polyadenylated RNAs were more stable than the polyadenylated molecules. This result implied that polyadenylated RNAs were preferentially degraded by exonucleases present in the extract [78, 89]. The same results were obtained when these competition experiments were performed using only chloroplast PNPase, either purified from spinach or expressed as a recombinant protein in *E. coli* [89, 146]. The preferential degradation of polyadenylated RNA by PNPase was subsequently explained by the presence of a high affinity poly(A) RNA binding site within its S1 domain (see below). Therefore, it is possible that as a processive enzyme, most of the PNPase population is bound to RNA in the chloroplast and its high affinity for polyadenylated RNA sequesters it from degrading non-polyadenylated molecules.

Taken together, these results describe an mRNA degradation pathway initiated by endonucleolytic cleavage(s), followed by the addition of a poly(A)-rich tail to the proximal cleavage product. This poly(A)-rich tail targets the cleavage products for rapid degradation by an exonuclease(s), which preferentially degrades polyadenylated RNAs [32, 50, 98, 124].

D. What is the polyadenylation enzyme in the chloroplast?

In *E. coli*, the major enzyme responsible for polyadenylation is PAP, which is encoded by the *pcnB* gene [97]. The amino acid sequence of PAP displays a very high homology to the nucleotidyltransferase (NTfr) family [114]. In fact, the amino acid sequence identity among these family members is so high that assignment of catalytic activities as either PAP or NTfr must be verified biochemically. This indicates that PAP was relatively recently derived from an NTfr by gene duplication and indeed, both PAP and NTfr enzymes have the ability to specifically add a C or A to RNA without using a DNA template [83, 147].

Initial attempts at isolating and purifying chloroplast homologs of *E. coli* PAP met with limited success [e.g. 84]. Subsequently, it was discovered that poly(A) tails in *pcnB* deletion strains of *E. coli* were long and heterogeneous, very similar to those characterized in spinach chloroplasts [87, 97]. This suggested that PNPase, which until that time was believed to be active in bacteria and chloroplasts only as an exonuclease, could also carry out polyadenylation, at least in bacteria [97]. This led to the finding that purification of PAP activity from spinach chloroplasts yielded only PNPase, which was efficient at polyadenylating unstructured RNA *in vitro*, but inefficient at polyadenylating substrates which terminated in stem-loops, mirroring the specificity of polyadenylation activity *in vivo* and in chloroplast protein extracts [51, 87, 145, 146]. Taken together, these results suggested strongly that PNPase was responsible for both polyadenylation and degradation of RNA in spinach chloroplasts [145].

How could one enzyme perform the opposing activities of polyadenylation and degradation? Biochemical analyses have shown that chloroplast PNPase is neither post-translationally modified nor bound to other proteins, both of which could potentially influence its activity [7]. However, *E. coli* PNPase catalyzes a nearly freely reversible reaction with an equilibrium constant that varies from approximately 0.25 to 4 depending on free Mg^{2+} concentration [86]. In addition, PNPase directionality is directly influenced by the P_i/NDP ratio [91], suggesting that it could be shifted toward net exonucleolytic or polymerization activities as the concentrations of its substrates change, something that was recently shown to be true of chloroplast PNPase *in vitro* [145, 146]. This model is summarized in **Figure 4**.

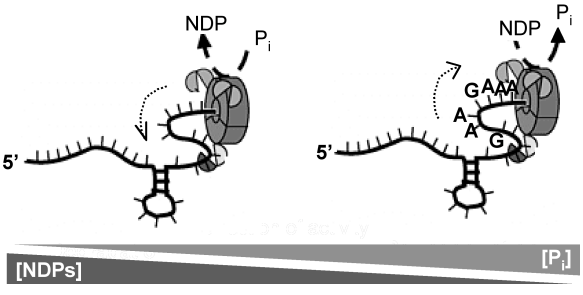

Figure 4. A model for possible modulation of PNPase activity as an exoribonuclease or a polymerase. The PNPase is modeled as a homotrimer according to the crystal structure, as discussed in the text. When degrading RNA, PNPase consumes P_i and produces nucleotides, thereby increasing the local concentration of nucleotides (left side of Figure). However, when polymerizing RNA, it consumes nucleotides and generates P_i (right side of Figure). Therefore, while working in one direction, and if no other mechanism controls the activity, it could reach a situation whereby the local concentrations of nucleotides and Pi would cause the enzyme to reverse its activity.

Although a plant PNPase null mutant has not been studied, an *Arabidopsis* line in which the expression of PNPase was reduced to about 1% of WT by cosuppression was recently described

[139]. These plants had no observable phenotype, but were impaired in the 3' end processing of *psbA*, *rbcL* and 23S RNAs. Interestingly, a DNA gel blot of oligo(dT)-primed cDNA showed a pronounced increase in the amount of polyadenylated RNA in the cosuppressed plants. This suggested that an enzyme other than PNPase could polyadenylate RNA in these plants, arguing that PNPase is not the only enzyme capable of polyadenylation in the chloroplast, at least in this mutant background. Alternatively, the residual PNPase in these plants may be somehow shifted to toward the polyadenylation reaction. Distinguishing these possibilities, and ultimately resolving whether a PAP-like enzyme exists in the chloroplast, will require analysis of chloroplast PNPase null mutants, and/or the discovery of a *bona fide* chloroplast-targeted PAP.

E. Cyanobacterial mechanisms resemble those of chloroplasts

The discovery that PNPase was likely to be the major polyadenylating enzyme in the chloroplast raised the question of what the ancestral state of polyadenylation-linked RNA degradation might be. It was shown recently that PNPase is responsible for polyadenylation in several Gram positive bacteria and in cyanobacteria, believed to be related to the chloroplast ancestor [114, 119]. Interestingly, mRNA, rRNA, tRNA and the single intron located within $tRNA^{fmet}$ were all found to be polyadenylated in *Synechocystis* [119], mirroring the polyadenylation of mRNA, tRNA and rRNA reported both in *E. coli* [85] and *Chlamydomonas* [75]. It may be hypothesized, therefore, that *E. coli* and perhaps other proteobacteria acquired PAP relatively late in evolution through the interconversion of a CCA-adding enzyme, as has been suggested by Yue and coworkers [147]. Therefore, RNA polyadenylation in the chloroplast may represent a more ancient evolutionary state of the system present in *E. coli*, the opposite of what might be expected given the numerous adaptations organelles have made in deference to the eukaryotic environment. If this hypothesis stands the test of time, elucidating the functional implications of PAP vs. PNP-based polyadenylation will require analogous investigations in other prokaryotes and organelles.

It should be noted here that another major difference between the RNA degradation systems in *E. coli*, cyanobacteria and the chloroplast is the presence of the high molecular weight complex, the “degradosome” [reviewed in 20]. In *E. coli*, the degradosome is comprised of RNase E (but not RNase G), part of the PNPase population, an RNA helicase and the glycolytic enzyme enolase. The nature of its components led to the presumption that the degradosome was a major player in the RNA degradation process. However, later it was found that the degradosome is not essential for viability in *E. coli*, nor does it exist in the chloroplast or in cyanobacteria [7, 119]. Therefore, the degradosome may have been a relatively late acquisition by *E. coli*, or perhaps it was lost in cyanobacteria prior to endosymbiosis. Different complexes of ribonucleases, helicases and additional proteins have been described for other bacteria [61] and yeast mitochondria [35]. Even taking into account possible impurities, it appears that ribonucleases in prokaryotic-like systems come in many macromolecular forms.

F. Is RNA degradation in the chloroplast always polyadenylation-dependent?

Polyadenylation-mediated RNA turnover has been established as a general mechanism in bacteria, chloroplasts and plant mitochondria. However, although important, the polyadenylation-mediated pathway may not be the only route to RNA degradation in the chloroplast. If there are two pathways, one polyadenylation-dependent and the other polyadenylation-independent, it would be important to define the differences between them, to determine what regulates flux through each, and to determine which enzymes are involved.

To begin to address these questions, it would be instructive to consider polyadenylation-independent RNA degradation pathways from other prokaryotic-like systems. Because PNPase is a key player in all polyadenylation-dependent degradation pathways described to date, one can look to genomes which appear not to encode a PNPase, such as those of *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and the *Halobacterium* (Archaeal) lineage [76]. Indeed, analysis of yeast mitochondria and *Halobacterium volcanii* revealed no evidence for polyadenylated species (Portnoy, Zipor, Oliel and Schuster, unpublished results) and in the case of yeast, earlier reports were conflicting [45, 53], whereas a recent publication also argues against it [35]. In these systems, we speculate that the exonuclease RNase II/R plays a central role in degradation. It remains to be seen whether RNase II/R is part of a polyadenylation-dependent degradation pathway in most prokaryotes and organelles, where both PNPase and RNase II/R exist, or if this enzyme operates independently of PNPase. The fact that *E. coli* easily tolerates the absence of PNPase, but not both RNAse II and PNPase [30], points to at least a partial functional redundancy.

III. CHLOROPLAST ENDORIBONUCLEASES

A. RNase E/G

RNA turnover in bacteria is thought to begin with endonucleolytic cleavage by RNase E, encoded in *E. coli* by the *ams* gene [80, 105]. RNase E is essential for cell growth and its inactivation results in an increase in the half life of bulk RNA in *E. coli*, and inhibition of rRNA and tRNA processing [43, 44, 113]. *E. coli* RNase E can be divided into two major domains, the N-terminal catalytic domain and the C-terminal domain, which is thought to serve as a scaffold for degradosome assembly and to participate in autoregulation of *ams* expression *in vivo* [26, 63].

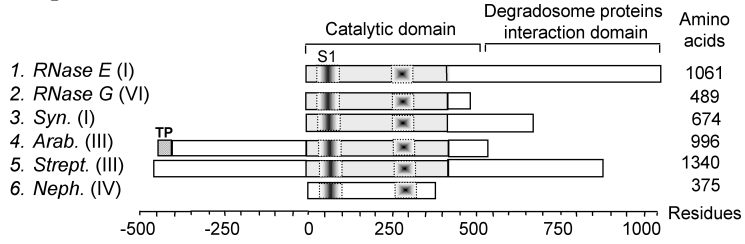

Figure 5. Homology-based alignment of several RNase E-like proteins
The proteins are: (1) *E. coli* RNase E; (2) *E. coli* RNase G; and RNAse E/G-like proteins from (3) *Synechocystis* PCC6803 (Cyanobacteria); (4) *Arabidopsis thaliana* (higher plant, nuclear encoded); (5) *Streptomyces coelicolor* (Actinobacteria) and (6) *Nephroselmis olivacea* chloroplast (Chlorophyta, chloroplast encoded). The proteins were aligned for maximum homology using the BLAST and ClustalW programs. Regions scoring >200 to *E. coli* RNase E, using BLAST, are shown in gray. The S1 domain and another conserved domain are highlighted. The N-terminal catalytic domain and the C-terminal scaffold for degradosome assembly are shown by brackets at the top; the C-terminal domain is not conserved in any other protein shown. The putative transit peptide (TP) at the N-terminus of the *Arabidopsis* protein may direct chloroplast import. The roman numerals in parentheses indicate the group number of this enzyme as designated by Lee and Cohen [81].

Figure 5 compares the primary structures of RNase E-like proteins from several bacterial and plant genomes. The *E. coli cafA* gene encodes an RNase E homolog called CafA or RNase G [93]. RNase G shares homology with the catalytic domain of RNase E, but lacks the C-terminal domain. A *Synechocystis* RNase E/G homolog has also been characterized, and both it and the *E. coli* protein exhibit a preference for 5' monophosphorylated RNA substrates. *Synechocystis* RNase E/G can substitute for RNase E in *ams* mutants, although it cannot regulate *ams* expression [63], nor is it incorporated into a degradosome-like complex [119]. Disruption of the *Synechocystis* RNase E/G gene is lethal, suggesting that it is essential, and although it is assumed to play a

role in initiating RNA turnover, this has not been experimentally verified [119].

An RNase E/G homolog is encoded in cpDNA of the red alga *Porphyra* and the green alga *Nephroselmis olivacea* [116, 135]. This observation suggests that a nucleus-encoded RNase E/G exists in the chloroplasts of organisms where the gene is absent in cpDNA. Indeed, the *Arabidopsis* nuclear genome encodes a single gene annotated as an RNase E/G-like protein (At2g04270), for which alternatively spliced cDNAs, and therefore putative protein variants exist. A portion near the C-terminus has 30% identity (99/331 residues) to *Porphyra* RNase E/G, but the remainder has no similarity. The identity of the 5' end of the major accumulating mRNA is not clear at present, due to the existence of multiple cDNA species, making targeting predictions difficult. Other plant nuclear genomes – but not that of *Chlamydomonas* (D. Stern, unpublished results) – appear to encode similar proteins, but whether any are targeted to the chloroplast awaits experimental confirmation.

B. CSP41a

CSP41a (41 kDa Chloroplast Stem-loop binding Protein) is an abundant, highly conserved nucleus-encoded endoribonuclease, first purified from spinach chloroplast protein extracts as a sequence specific *petD* 3' UTR RNA binding protein, and as a nonspecific endoribonuclease [143]. CSP41a cleaves efficiently within the 3' stem-loop structures of synthetic *petD*, *psbA* and *rbcL* RNAs *in vitro*, and also cleaves arbitrary double-stranded RNA substrates, albeit at a low rate [144]. Given the importance of 3' stem-loops in mRNA stability, its substrate specificity implied that CSP41a might play a role in initiating mRNA turnover *in vivo*, a hypothesis that was recently verified in tobacco using an antisense approach [12]. In this study, RNA degradation rates were measured using an assay [124] in which intact chloroplasts from WT and antisense plants were lysed and analyzed for residual full-length mRNAs as a function of time. In chloroplasts from antisense plants, degradation of full length *psbA*, *rbcL* and *petD* RNAs decreased by 7-, 2- and 5-fold, respectively, as compared to the WT control. Because turnover of chloroplast RNAs probably begins with a rate-limiting endonucleolytic cleavage, these data suggested that CSP41a could initiate and regulate turnover of these transcripts *in vivo*.

1. CSP41a Structure

The spinach *CSP41A* gene encodes a 416 amino acid protein, which includes the mature 331 amino acid enzyme and an 85 amino acid chloroplast transit peptide. Despite its robust endoribonuclease activity, CSP41a is homologous to nucleotide-sugar epimerases and hydroxysteroid dehydrogenases and is therefore a member of the short chain dehydrogenase/reductase (SDR) superfamily, a widespread family of more than 1,600 members [**Figure 6**; 65]. SDR proteins contain a characteristic "Rossman" dinucleotide binding fold [16], which is found near the N-terminus in CSP41a.

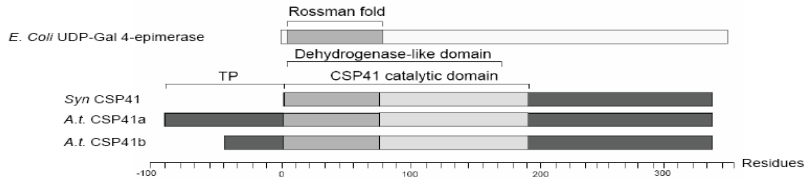

Figure 6. Domain structure of CSP41. Schematic representations of *E. coli* UDP-galactose 4-epimerase, *Synechocystis* CSP41 and *Arabidopsis* CSP41a and CSP41b are shown. Numbering at the bottom is relative to the mature plant CSP41 polypeptides. The position of the Rossman-type fold and the predicted CSP41 dehydrogenase-like domain are indicated, as previously designated [8]. The CSP41 catalytic domain (residues 1-191 of the mature protein) is indicated, as are the transit peptides (TP) of *Arabidopsis* CSP41a and CSP41b.

CSP41a, however, has lost the conserved Gly-X-Gly-X_3-Gly consensus nucleotide binding motif and no longer interacts with NADH or NADPH *in vitro* [8, 13].

The Rossman fold is a known RNA binding domain in several dehydrogenases [100] and was recently shown to be important for the endoribonuclease activity of glyceraldehyde-3-phosphate dehydrogenase and two aldehyde dehydrogenases from the archaeon *Sulfolobus solfataricus* [36]. A 74 amino acid CSP41a C-terminal deletion which retains the Rossman fold (between amino acids 11 and 41 of the mature protein) is inactive, suggesting that the CSP41a Rossman fold alone is insufficient for endonuclease activity [13, 144]. Competition experiments, however, showed that the mutant protein could compete with the full-length enzyme for its RNA substrate, implicating the CSP41a Rossman fold in substrate recognition rather than in bond cleavage [13]. The function of the C-terminal 140 amino acids of CSP41a, which are dispensable for its endonuclease activity, is not known.

2. Divalent metal requirement

Like RNase E, CSP41a has an absolute requirement for Mg^{2+}, which likely provides transition state stabilization and supplies a nucleophile for phosphodiester bond cleavage. Like other divalent metal-requiring endonucleases, CSP41a cleaves on the 5' side of the phosphodiester bond [13] and generates 3' hydroxy terminated products, which is essential for their entry into the polyadenylation-dependent degradation pathway. The divalent metal binding site on CSP41a, formed by the side chains of Asn71, Asp89 and Asp103, is relatively nonspecific and binds Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+} and Zn^{2+}. However, only Mg^{2+} activates CSP41a significantly and it is probably the physiological divalent metal, based on its abundance in the chloroplast stroma. Mg^{2+} activates CSP41a with a K_A of 2.1 mM, which is on the same order of magnitude as stromal free Mg^{2+} concentrations, which fluctuate between 0.5 mM in dark-adapted leaves and 10 mM in mature, light-grown leaves [13, 57, 59]. This suggests a mechanism whereby the chloroplast could fine-tune CSP41a activity, and therefore the rate of transcript turnover, upon transition of plants to darkness and during leaf development, a time during which CSP41a abundance does not vary significantly [143].

Using lysed chloroplast extracts from WT and *CSP41A* antisense plants [12], it was discovered that full-length *rbcL* RNA degraded with half lives of 72 and 10 min in *csp41a* antisense plants at 12.5 mM Mg^{2+} and at < 1 mM Mg^{2+}, respectively, whereas the degradation rate was invariant in WT extracts, with a half-life of approximately 10 min over the same range of Mg^{2+} concentrations. These data shed new light on several important facets of cpRNA turnover, namely that CSP41a provides the primary route to *rbcL* turnover at Mg^{2+} concentrations found in mature, light-grown leaves, and that an alternative route to *rbcL* turnover must exist at < 1 mM Mg^{2+}, conditions under which CSP41a turns over at less than 20% of its maximal rate.

3. Substrate specificity

Chloroplast RNAs share few sequence motifs in their untranslated regions (UTRs) that might act in *cis* as endonuclease targeting elements. Instead, it has been proposed that it is the structure of 3' stem-loops which modulates chloroplast mRNA turnover rates [46]. CSP41a has no sequence specificity, but has a strong preference for cleaving stem-loop containing RNAs, and is sensitive to the size and to the tertiary structure of the stem below the scissile bond [144]. When analyzing the activity on different molecules related to *petD* transcripts, CSP41a was found to optimally cleave the mature *petD* RNA 3' UTR, whereas deletion of 8 nucleotides on the distal

arm of the stem below the primary cleavage site decreased substrate binding and therefore cleavage rate by 100-fold. This suggests that CSP41a has a requirement for a minimal stem length for optimal substrate recognition, similar to the yeast RNase III homolog, Rnt1p [2]. Furthermore, disruption of the *petD* stem-loop either by intercalation of ethidium or by introduction of a bulge into the stem, decreased the cleavage rate by 4- to 5-fold. Because the activity of CSP41a is modulated by substrate structure, and given the natural variation in size and the presence of helix-disrupting internal loops and bulges in chloroplast mRNA stem-loops, the structures of the stem-loops themselves may act as *cis* elements that regulate CSP41a cleavage rate, and therefore turnover rate of a particular transcript *in vivo*. Although model substrates used to test CSP41a activity *in vitro* are derived from cpRNA 3' UTRs, the lack of sequence specificity of CSP41a suggests that it could also cleave RNAs within their coding regions, where many poly(A) addition sites have been observed [72]. This point remains to be addressed experimentally.

C. CSP41b

A probable ortholog of CSP41a, named CSP41b in plants and RAP38 in *Chlamydomonas*, was recently discovered [12, 142]. While homologues of CSP41a have been identified in green algae and plants, only CSP41b is present in the cyanobacterium *Synechocystis* [8]. *Arabidopsis* CSP41a and CSP41b share 35% amino acid identity, and a phylogenetic analysis suggests that these genes are paralogs of a common cyanobacterial ancestor, which was derived from a bacterial epimerase/dehydratase [142]. A preliminary *in vitro* analysis with purified, overexpressed *Arabidopsis* CSP41b has shown that it has *petD* stem-loop RNA cleavage activity (Bollenbach and Stern, unpublished results).

To date, the role of CSP41b in RNA metabolism is unknown. It was found to be a stochiometric component of the *Chlamydomonas* chloroplast 70S ribosome, which may suggest that it is somehow involved in a process involving RNA-cleavage and translation [142]. CSP41a, on the other hand, was reported to copurify with the chloroplast prokaryotic-like RNA polymerase [107]. It should be noted that CSP41a (and probably CSP41b) are relatively abundant in the chloroplast, which could result in co-purification as a contaminant with ribosomes or transcription complexes. However, possible involvement of RNA cleavage processes in translation and transcription in the chloroplast should not be excluded.

IV. EXONUCLEASES

A. Polynucleotide phosphorylase (PNPase)

PNPase catalyzes both processive 3'→5' phosphorolysis and polymerization of RNA [90]. In *E. coli*, PNPase is mostly active in 3'→5' phosphorolysis during RNA degradation and 3' end processing [47, 62]. PNPase was also reported to be a global regulator of virulence and persistency in *Salmonella enterica* [24]. Recently, the human PNPase was identified in an screen to discover genes displaying coordinated expression as a consequence of terminal differentiation and senescence of melanoma cells [82, 122]. Its expression was found to be induced by interferon β and the protein kinase C inhibitor mezerein [82]. While this group reported the protein to be located in the cytoplasm, it was found to be located exclusively in the mitochondria of HeLa cells by others [109].

As noted above, a small proportion of bacterial PNPase is a constituent of the degradosome, but what was originally postulated to be the chloroplast degradosome was later found to be homotrimeric PNPase complexes [7, 51] and to date, no interactions with other proteins have

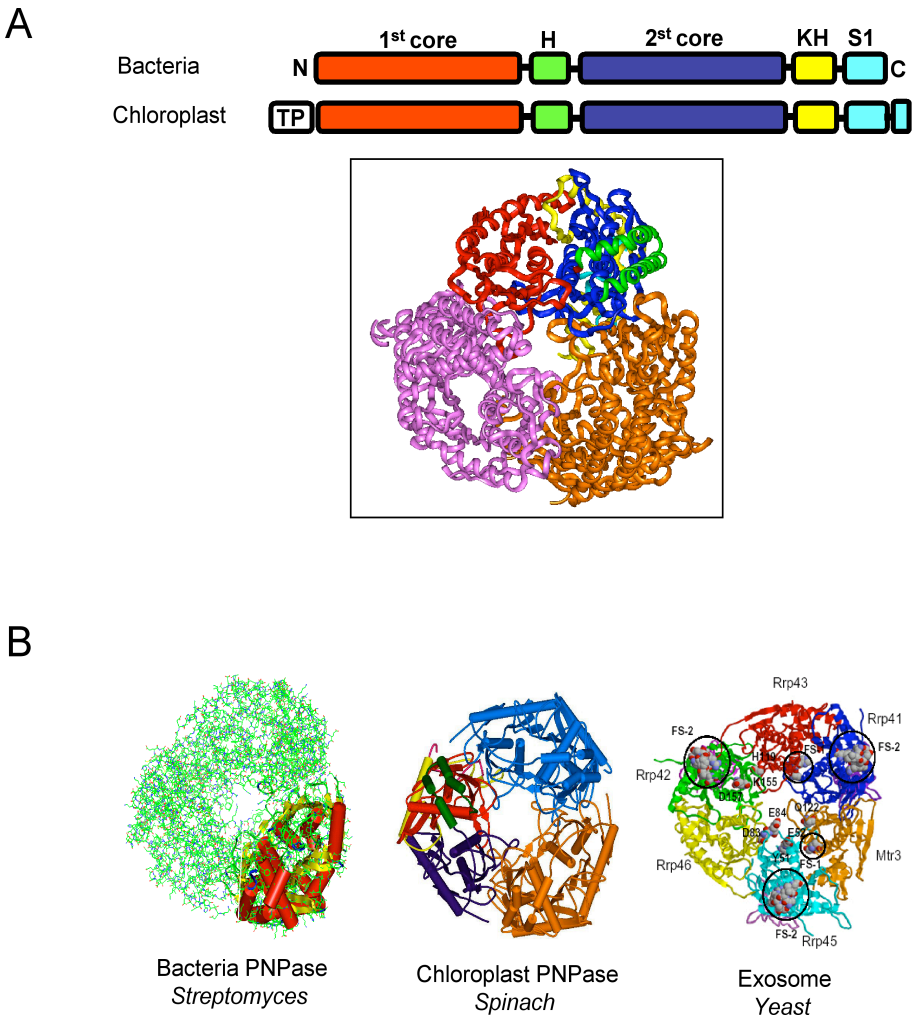

Figure 7. Structural aspects of PNPase. (A) The domain organization (top) or proposed tertiary structure of the PNPase from spinach chloroplasts, as reported by Yehudai-Resheff *et al.* [146]. (B) Structural similarity of PNPase and the exosome, a 3'→5' RNA degradation machine. The structure of bacterial PNPase was resolved by X-ray crystallography [133], and its coordinates were used to predict the spinach chloroplast structure. The yeast exosome structure was reported by Aloy et al. [4].

been confirmed. The amino acid sequences of PNPases from bacteria, as well as from the nuclear genomes of plants, yeast and mammals, display a high level of homology and feature similar structures comprised of five motifs [**Figure 7A**; 111, 133, 134, 148]. These motifs include two core domains having different degrees of homology to the *E. coli* phosphorylase RNase PH, an α-helical domain separating them, and two adjacent RNA-binding domains (KH and S1), which are also found in other RNA-binding proteins. 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 donut shape, surrounding a central channel that could accommodate a single-stranded RNA molecule [**Figure 7A**; 133, 134]. The domains of spinach chloroplast PNPase were recently analyzed in detail [146]. It was found that the first core domain, which was predicted to be inactive in the bacterial enzymes, was active in RNA degradation but not in polymerization. Surprisingly, the second core domain was found to be active in degrading polyadenylated RNA only, suggesting that non-polyadenylated molecules can be degraded only if tails are added, apparently by the same protein. The high affinity poly(A) binding site was localized to the S1 domain.

Recently, it was observed that the exosome, which is composed of 10-11 exoribonucleases and RNA-binding proteins, is structurally similar to trimeric PNPase [4, 111, 146], including a similar structure and homology of the two core domains (**Figure 7B**). The exosome functions in 3'→5' RNA degradation in the cytoplasm and nucleus of eukaryotic cells and from these

observations, we may speculate that the PNPase trimer in bacteria and chloroplasts is the evolutionary ancestor of the eukaryotic exosome.

B. Ribonuclease II/R

Ribonuclease II (RNase II) is a non-specific processive 3'→5' exoribonuclease yielding 5'-phosphomononucleotide products [19], a 67 kDa monomer, and requires Mg^{2+} and K^{+} for activity. As a member of the RNR superfamily of ribonucleases, RNase II displays high homology to proteins of bacteria, plants, *Drosophila*, *C. elegans* and mammals [148]. However, no homologues have being detected in any Archaea except the *Halobacterium* NRC-1 [102]. Interestingly, this is the only archaeon lacking members of the phosphorylase exoribonuclease family [76]. As mentioned above, no polyadenylation could be detected in the related archaeon *Halobacterium volcanii*, which implicates RNase II/R as a major player in its presumably poly(A)-independent RNA degradation. Some RNR superfamily proteins were shown to be RNA hydrolases and also to be subunits of the exosome, whose ribonucleases are mostly phosphorylases [96, 111]. *E. coli* contains another ribonuclease termed RNase R, which is very similar to RNase II and is widespread in eubacteria, even more so than RNase II. In fact, this is apparently the only exoribonuclease present in *Mycoplasma*, according to its genome sequence [23]. While γ-*Proteobacteria* contain both RNase II and RNase R, most of the other bacterial lineages appear to contain only one member, which is more similar to RNase R. Eukaryotes, including plants, encode several members of the RNR family [148]. One member present in the *Arabidopsis* genome contains a typical chloroplast transit peptide and its N-terminal was indeed found to target YFP to this organelle (R. Gutierrez, T. Bollenbach and D. Stern, unpublished results).

E. coli deletion strains lacking either RNase II or RNase R are viable, probably because they have a significant level of functional overlap with the remaining exoribonucleases [67, 79, 97]. However, this was found not to be the case in *Synechocystis*, which contains only one gene encoding an RNR family member [119]. Similarly, inactivation of the *DSS1* gene, which encodes the hydrolytic exoribonuclease of yeast mitochondria, resulted in respiratory incompetence, inhibition of mitochondrial translation and loss of the mitochondrial genome [34, 35]. Is RNase II/R involved in the polyadenylation degradation pathway? Experiments in *E. coli* have shown that the inactivation of RNase II and PNPase resulted in significant stabilization of polyadenylated RNAs [27, 123]. Because the deletion of PNPase alone was insufficient for the accumulation of polyadenylated RNA, it could be concluded that RNase II is involved in the rapid degradation of polyadenylated RNAs. However, whether the protein displays high affinity for poly(A), as does PNPase, remains an open question.

C. 5'→3' exonuclease

Data obtained so far uniquely from *Chlamydomonas* have shown that unlike all prokaryotic systems explored to date, the chloroplast contains a 5'→3' degradation pathway, defined as a pathway which can be inhibited by the structure formed by a polyguanosine tract [31]. 5'→3' degradation has been well characterized in the yeast cytoplasm and nucleus, where the related exonucleases Xrn1p and Rat1p, respectively, harbor these functions [64]. However, in the chloroplast it is unknown whether 5'→3' degradation is mediated by Xrn1/Rat1 type proteins, or is in fact a net 5'→3' degradation pathway catalyzed by a processive endonuclease. Data in favor of the former include a variety of studies demonstrating that in nuclear mutants unable to accumulate individual chloroplast mRNAs, those RNAs are degraded in a 5'→3' direction, but

that decay can be blocked by insertion of a poly(G) tract. On the other hand, rapid 5'→3' degradation of the chloroplast *atpB* distal cleavage product created during 3' end maturation was not inhibited by poly(G), suggesting that an endonuclease was responsible [54]. While the processive endonuclease activity which degrades the *atpB* cleavage product was observed *in vitro*, no Xrn1/Rat1-like activity has been reported in chloroplast extracts. Still, given the existing data, a conservative conclusion at the moment is that both pathways exist.

A search for genes encoding organellar Xrn1/Rat1 proteins reveals that in both *Arabidopsis* and *Chlamydomonas*, small nuclear gene families exist. In *Arabidopsis* there are three members, AtXRN2-4, and in two cases fusions to GFP result in nuclear localization [66]. An AtXRN4-GFP fusion, however, resulted in cytoplasmic localization, either in a diffuse pattern, or in a punctate pattern more reminiscent of mitochondrial targeting. In the absence of more biochemical data, this result remains somewhat ambiguous. Based on the available *Chlamydomonas* nuclear sequence, this organism also encodes 3, or possibly 4, Xrn1-like proteins. One of these, *CrXrn1*, has three corresponding mRNA 5' ends, including a minor one which encodes a protein with a predicted chloroplast localization, although this remains to be experimentally verified (D. Higgs, S. Murakami and D. Stern, unpublished data). In the absence of Xrn1/Rat1-like proteins in the chloroplast, a candidate for a processive 5'→3' endonuclease would be RNase E, which is known to act in this manner [reviewed in 25]. However, as noted above, evidence for this enzyme in chloroplasts is lacking, and so far no corresponding gene has been annotated in the *Chlamydomonas* nuclear genome.

D. Ribonuclease summary

In **Table 1**, we compare and contrast the known enzymes of RNA metabolism between the major groups of bacteria and plant organelles. This Table illustrates conservation on one hand, and diversity on the other, resulting in a gradual evolution of RNA decay pathways, presumably reflecting evolutionary forces. The Table also illustrates both the limits of our knowledge and thus areas for future study.

V. Regulation of RNA Decay

A. Phosphate regulation of cpRNA decay

Phosphate is a component of many crucial molecules including DNA, RNA, phospholipids, storage molecules, and (ribo)nucleotides. Because of this, phosphate limitation requires short-term responses by organisms, and in the longer term can impact ecosystem health and makeup [39, 132]. One short-term response to P limitation is induction of scavenging mechanisms, for example through secretion of phosphatases, mobilization of internal phosphate pools, or in plants, changes in root architecture and associations with symbiotic mycorrhizal fungi [1, 110]. Another way in which organisms mobilize P is by inducing the expression of ribonucleases. Although in plants several cytosolic and vacuolar ribonucleases are known to be induced under P limitation [reviewed in 1], how P limitation impacts the metabolism of cpRNA in higher plants is currently unknown.

One can hypothesize, that one or more chloroplast ribonucleases is affected by P limitation. An obvious candidate is PNPase, since *in vitro* data suggest that PNPase activity may be regulated dynamically by the P_i:NDP ratio [145], although gene expression changes in the nucleus could also result in altered RNase levels in the chloroplast. If plant cells limited for P also become depleted for P in the chloroplast, which is believed to be the case at least in the stroma [reviewed in 112], then one might predict a tendency of PNPase to be in a polymerizing

vs. degradative mode. In turn, this might result in longer, or at least longer-lived poly(A) tails, and thus enhanced cpRNA stability. Metabolically, one could argue that this "preservation" of cpRNA would reduce the need for new transcription, thus conserving NTPs for other uses. On the other hand, increased polymerization by PNPase could deplete ADP (and to some extent GDP), but this would eventually liberate enough P_i to disfavor polymerization and promote degradation. Fluctuations in the P_i:NDP ratio must occur in chloroplasts under circumstances other than P limitation, however actual measurements are technically challenging. Should such measurements become possible, it will be interesting to relate cpRNA metabolism to the flux of these potential regulators.

B. Mg^{2+} regulation of chloroplast RNA decay

Magnesium is the most abundant divalent cation in living cells and, in the chloroplast, is an important component of chlorophyll and an essential cofactor for several enzymes of the Calvin cycle [reviewed in 141]. Stromal Mg^{2+} levels are dynamic and are modulated by leaf development and by light, which causes Mg^{2+} release from thylakoid membranes into the stroma via a membrane cation channel [55, 57, 59]. It has been proposed that the fluctuations in stromal Mg^{2+} concentration play an important regulatory role in CO_2 fixation and, more recently, that Mg^{2+} fluctuations play a role in regulating chloroplast RNA stability [12, 57, 125]. A striking example of this is the approximately 3- to 4-fold increase in the stability of *psbA* and *rbcL* transcripts over the physiological range of stromal Mg^{2+} concentrations. Magnesium influences both the rate of degradation and endonucleolytic cleavage site selection within *psbA*, and can do so through direct interactions with the transcript, by regulating the activity of divalent metal-dependent ribonucleases, and/or by mediating the assembly of ribosomes and RNP complexes [12, 57, 72]. The regulation of cpRNA by Mg^{2+} is, therefore expected to be multifaceted.

Mg^{2+} can bind directly to high affinity sites on highly structured RNAs and is essential for their proper folding, stability and biological activity. For example, Mg^{2+} is an essential cofactor for the catalytic activities of the self-splicing *Tetrahymena* group I intron and the M1 RNA of RNase P [68, 108], for the stabilization of certain yeast and *E. coli* tRNAs, 5S rRNA [reviewed in 95] and for chloroplast *trnH* [57]. The observed increase in *psbA* and *rbcL* half-lives as a function of Mg^{2+} are probably mediated through interactions of these transcripts with ribosomes and in RNP complexes [57, 72, 101]. The Mg^{2+}-dependent differential binding of chloroplast RNA binding proteins to form RNPs has been demonstrated by UV crosslinking of chloroplast proteins to the *rbcL* 5' UTR, which is an important *cis*-acting RNA stability determinant in this transcript both in plants and in *Chlamydomonas* [57, 120, 126].

In addition to activating CSP41a, Mg^{2+} is an essential cofactor for CSP41b, PNPase and RNase II/R. Therefore, it is conceivable that these enzymes are either activated or inhibited by light-induced and developmental fluctuations in stromal Mg^{2+} concentration. The chloroplast also contains at least two divalent metal-independent endoribonucleases whose activity would be insensitive to changes in stromal Mg^{2+}. A 54 kDa endoribonuclease, p54, was purified from mustard chloroplasts and was shown to participate in processing the 3' ends of *trnK* and *rps16* RNAs *in vitro* [103]. However, a role for this enzyme in chloroplast RNA turnover has not been demonstrated. Spinach chloroplasts contain a divalent metal-independent endonuclease, EndoC2, which cleaves *petD* RNA at the translation termination codon *in vitro* [22], but which has an unknown function *in vivo*. If either p54 or EndoC2 did participate in chloroplast RNA turnover, they would most likely be part of the poly(A)-independent pathway since, as divalent metal independent endonucleases, they would generate phosphoryl-terminated products, which are not

substrates for PNPase. These products could, however, be digested by RNase II/R, which has no 3' end preference [23]. The interplay between potentially constitutive divalent metal-independent endoribonucleases and regulated divalent metal-dependent endoribonucleases during chloroplast development and as a response to environmental cues remains to be elucidated.

Mg^{2+} has a destabilizing effect on *psbD* 5' UTR RNA in *Chlamydomonas* chloroplast protein extracts, suggesting that it is a cofactor for an endonuclease [104]. In spinach and tobacco extracts, the opposite has been observed, namely that Mg^{2+} stabilizes transcripts [12, 57]. Our results with *CSP41A* antisense plants have shown that while the stability of *rbcL* is highest at >10 mM Mg^{2+}, CSP41a is also most active under these conditions. This somewhat paradoxical finding again suggests that the regulation of chloroplast mRNA decay by Mg^{2+} is controlled not only by rate-limiting endonucleolytic cleavages, but also through the concerted effects of RNA-Mg^{2+} complexes, the formation of Mg^{2+}-dependent RNP complexes and the assembly of polysomes.

C. Regulation of chloroplast mRNA stability by light

Light impacts chloroplast transcription, RNA processing, and translation. Gene expression early in seedling development is transiently regulated at the level of transcription and is later controlled at the level of RNA stability in pea seedlings [33]. In barley, increases in mRNA stability during chloroplast development has been shown to be largely light-independent with the exception of *rbcL*, which is 2- to 10-fold less stable in the light than in the dark, and *psbA*, which accumulates to high levels in illuminated developing pea chloroplasts by virtue of its high stability [33, 70, 126].

Dark-adapted spinach leaves accumulate less *petD* RNA than light grown leaves, but accumulate more polyadenylated *petD* transcripts [78]. Subsequent analysis of *petD* RNA degradation in spinach chloroplast protein extracts *in vitro* suggested two routes for RNA turnover, one in the light and one in the dark [6]. Both routes begin with endonucleolytic cleavage followed by polyadenylation-dependent 3'→5' degradation or, in the case of *petD* RNA, by an increase in polyadenylation of the mature 3' end. In light grown plants, CSP41a plays a major role in initiating turnover of *psbA*, *petD* and *rbcL* transcripts, but does not participate in RNA degradation in dark-adapted plants (our unpublished observations), probably because the dark adapted chloroplasts have been depleted of Mg^{2+}. In undifferentiated plastids or dark-adapted chloroplasts, the endonucleolytic cleavage enzyme is unknown, however candidates include RNase E/G, EndoC2 and p54.

PNPase has been implicated in polyadenylation and turnover of endonucleolytic cleavage products in both light and dark [6]. An increase in the level of polyadenylated RNA in the dark was suggested to signal an increase in overall rate of *petD* and *psbA* turnover [78, 87]. However, an increase in the steady-state pool of polyadenylated transcripts suggests that either the rate of endonucleolytic cleavage has increased, that the rate of polyadenylation of endonucleolytic cleavage products has increased, and/or that the rate of 3'→5' degradation of polyadenylated RNAs has decreased. These possibilities have not been formally addressed. To what extent this increase in degradation activity is linked directly to light, and therefore, to redox poise of the cell remains to be seen since fluctuations in Mg^{2+} concentration, which influences degradation rates, are also controlled by light. To date, only one chloroplast ribonuclease, p54, has been shown to be under the control of redox conditions [103].

VI. CONCLUSIONS AND PERSPECTIVE

As in bacteria [29], chloroplast ribonucleases can be described as an "alphabet soup". Their overlapping functions probably reflect a mix of evolutionary remnants, requirements for adaptation to environmental and developmental signals, and redundancy to assure continuity of this vital function. The genomics era has provided tools for the dissection of ribonucleases, in particular genome sequences, mutant collections, and high-throughput methods. One strategy that is likely to bear fruit in the near future is increasing application of microarray technology to RNA degradation. Genome-wide approaches have already been taken to study the function of the *DST* RNA stability locus [106], and for general characterization of unstable mRNAs [48], in *Arabidopsis*, to assess the relative contributions of 5'→3' vs. 3'→5' exonuclease pathways in yeast [52], and to characterize RNA stability patterns in a variety of other organisms [reviewed in 69]. The relevance of such investigations to phenotyping ribonuclease mutants is clear.

The work cited here also highlights the power of comparative genomics to understand the origins of complex RNA degradation pathways. Based on our current knowledge of enteric and photosynthetic bacteria, Archaea, yeasts, plants and animals, the regulation of cellular components through modulation of mRNA levels is achieved in a variety of ways. Broader investigations will reveal different combinations of enzymes, which in turn will help establish the role of each one in a given organism.

Finally, we note that the regulation of ribonucleases by small molecules such as Mg^{2+} and P_i is well known based on *in vitro* biochemistry. How or if such regulation occurs *in vivo* is an important frontier in chloroplast biology. Utilization of genetic resources, metabolic manipulation (e.g. phosphate depletion), and perhaps even direct manipulation of the chloroplast contents [137], may allow direct tests of some of the hypotheses proposed above.

VII. ACKNOWLEDGEMENTS

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Table 1. Similarities and differences between RNA degradation/polyadenylation systems among prokaryotes and organelles.

	Prokaryotes[a]			**Archaea**[b]	**Organelles**				**Eukaryotes**
	Bacteria G -	G+	**Cyano-bacteria**	**Halobacteria**	**Chloroplast**	**Mitochondria**			**Nucleus +Cytoplasm**
	E. coli	***S. coe.***	***Syn.***	***H. volcanii***	**Plants/ algae**[c]	**Plant**	**Yeast**	**Human**	**Human**
Endonuclease[d]	E/G	E/G?	E/G	E/G	E/G?* CSP41a CSP41b	?	?	?	-
Polyadenylation	PAP I	PNP	PNP	-	PNP	PNP?	-	PNP? PAP?	PAP
Exonuclease	PNP II R	PNP II/R	PNP II/R	II/R	PNP II/R	PNP II/R	R II/R	PNP II/R	3'→5' 5'→3'
Protein complex	Degradosome	Yes	No	?	No	No	RII helicase	?	Exosome
Poly(A) tails	Hom.	Het.	Het.	-	Het./Hom.*	Hom.	-	Hom.	Hom.
	Non-stable poly(A) tails			No poly(A)	Non-stable poly(A) tails		No poly(A)	Stable poly(A) tails	

[a]Within the bacteria, *E. coli* represents the Gram negatives (G-) and *Streptomyces coelicolor* the Gram positives (G+). Cyanobacteria are represented by *Synechocystis*.

[b]In the domain of Archaea only a halophyte is presented, for the purpose of exemplifying a system in which polyadenylation apparently does not occur. Genes encoding PNPase or phosphorylases are present in Archaea such as methanogens and thermophiles, suggesting that polyadenylation occurs in these species.

[c]Plants indicates land plants, whereas most algal data are from *Chlamydomonas*. The asterisks mark instances where plants and *Chlamydomonas* may differ, namely that no RNase E/G gene has been found in *Chlamydomonas* nuclear DNA, and that poly(A) tails found in the chloroplast are nearly homopolymeric, in contrast to those in plants.

[d]Symbols and abbreviations are: (-) or "No", not present; E/G, proteins homologous to RNase E, RNase G of *E. coli*; E/ PAP I, poly(A) polymerase I of *E. coli*; PAP, poly(A) polymerase; PNP, polynucleotide phosphorylase; II, R and II/R, proteins homologous to RNase II and RNase R of *E. coli*; Hom, homopolymeric; Het, heteropolymeric; ? indicates unknown or only based on predictions from genomic sequence, or that there is conflicting experimental evidence. References not already cited in the text are [5, 15, 37, 81, 129].

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