Expression of Plastid Genes: Organelle-Specific Elaborations on a Prokaryotic Scaffold[1]

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Chloroplasts and their nonphotosynthetic relatives in the plastid organelle family evolved from a cyanobacterial endosymbiont (for review, see Timmis et al., 2004). The subsequent coevolution of the chloroplast and nuclear genomes produced an organelle that is eubacterial at its core but with extensive chloroplast-specific embellishments. Of the thousands of genes in the cyanobacterial ancestor, only approximately 100 are retained in chloroplast genomes. These genes fall into three categories: those encoding (1) components of the chloroplast gene expression machinery (RNA polymerase, ribosomal proteins, tRNAs, and rRNAs), (2) subunits of photosynthetic enzymes (Rubisco, PSII, the cytochrome b_6f complex, PSI, the ATP synthase, and the NADH dehydrogenase), and (3) proteins involved in other metabolic processes (e.g. ClpP, AccD, Ycf1, and Ycf2). The chloroplast proteome has a complexity of several thousand proteins and is dominated by nuclear gene products that are synthesized in the cytosol and imported into the organelle. Many of these are encoded by genes of cyanobacterial ancestry that were transferred to the nucleus and that have retained their ancestral functions. As a result, the chloroplast gene expression and photosynthesis machineries consist of proteins that are derived from two physically separate genetic systems. Detailed knowledge of chloroplast gene expression and the nucleus-encoded proteins that influence it are prerequisites for understanding nuclear-organellar cross talk and chloroplast evolution, and will aid in optimizing transgene expression in the plastid compartment.

The use of genetic and biochemical approaches, together with the ability to manipulate the chloroplast genome in several species, have brought most aspects of chloroplast gene expression out of the "black box" and into the realm of concrete, mechanistic hypotheses. The intent of this contribution is to highlight new perspectives that have resulted from recent observations and instances in which current data warrant the revision of previous paradigms. For more comprehensive information, I refer the reader to recent reviews of chloroplast RNA metabolism (Stern et al., 2010), transcription (Liere and Börner, 2007; Lerbs-Mache, 2010), and translation (Peled-Zehavi and Danon, 2007). Mechanisms of chloroplast gene expression have been studied primarily in land plants and in the green alga *Chlamydomonas reinhardtii*. Here, I emphasize findings with land plants, as detailed reviews of chloroplast gene expression in *Chlamydomonas* have been published in a recent volume (Stern and Harris, 2009).

THE CORE GENE EXPRESSION MACHINERIES IN CHLOROPLASTS RETAIN STRONG RESEMBLANCE TO THOSE IN BACTERIA BUT HAVE ORGANELLE-SPECIFIC EMBELLISHMENTS

The bacterial ancestry of chloroplasts is readily apparent in the organization of chloroplast genomes and in the machineries for chloroplast transcription, translation, and RNA turnover. Polycistronic transcription units that resemble bacterial operons predominate in land plant chloroplasts (Bock, 2007). Chloroplast ribosomes are similar in protein content and antibiotic sensitivities to bacterial ribosomes (Peled-Zehavi and Danon, 2007). A bacterial-type RNA polymerase contributes to chloroplast transcription (Liere and Börner, 2007; Lerbs-Mache, 2010), and chloroplast RNA turnover employs ribonucleases that are derived from those in bacteria (Stern et al., 2010). Superimposed on this bacterial infrastructure are features that were acquired only after the chloroplast became incorporated into a eukaryotic cell. Examples include a plethora of introns, a phage-type RNA polymerase, the modification of mRNA sequences by RNA editing, and the processing of polycistronic primary transcripts to generate complex transcript populations.

CHLOROPLAST GENE EXPRESSION EMPLOYS UNUSUAL RNA-BINDING PROTEINS THAT EMERGED IN THE CONTEXT OF NUCLEAR-ORGANELLAR COEVOLUTION

The analysis of nonphotosynthetic mutants in maize (*Zea mays*), Arabidopsis (*Arabidopsis thaliana*), and *Chlamydomonas* has revealed numerous nucleus-encoded RNA-binding proteins that participate in the expression of chloroplast genes. Two major themes emerged from this large body of work: (1) the repertoire of nucleus-encoded chloroplast RNA-binding

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proteins is remarkably complex given the small coding capacity of the chloroplast genome; and (2) most such proteins belong to protein families that function almost exclusively in organellar gene expression. Both of these points are exemplified by the pentatricopeptide repeat (PPR) family, whose members are defined by degenerate 35-amino acid helical repeats (for review, see Schmitz-Linneweber and Small, 2008). PPR proteins are not represented in bacteria, and PPR proteins in eukaryotes function almost exclusively in organellar gene expression. The PPR family consists of over 400 members in angiosperms, approximately half of which are predicted to localize to chloroplasts and half to mitochondria. Several other predicted helical repeat protein classes have also been implicated in chloroplast RNA metabolism (Stern et al., 2010); it is likely that these share mechanistic similarities with PPR proteins, so these and PPR proteins are referred to together below as "PPR-like" proteins.

Current data support the view that PPR tracts are sequence-specific RNA-binding motifs that bind single-stranded RNA along a surface formed by stacked, helical repeating units (Schmitz-Linneweber and Small, 2008; Williams-Carrier et al., 2008; Prikryl et al., 2011). Genetic data have implicated PPR proteins in many aspects of organellar RNA metabolism, and it is often suggested that they mediate their multifarious effects by recruiting different effector proteins to specific RNA sites. Indeed, there is good evidence that an appended domain found in a subset of PPR proteins functions in this manner during the process of plant organellar RNA editing (see below). However, recent data also support an alternative view: that many of the functions attributed to PPR proteins may result directly from the unusual nature of the PPR-RNA interface, which sequesters an extended RNA segment such that it cannot interact with other proteins or RNAs (Prikryl et al., 2011; Fig. 1).

Three additional classes of organellar RNA-binding proteins illustrate a similar theme, albeit on a smaller scale. The CRM (Barkan et al., 2007), PORR (Kroeger et al., 2009), and APO (Amann et al., 2004; Watkins et al., 2011) domains are represented in small plant-specific gene families. All members of these families are predicted to localize to chloroplasts or mitochondria, and all that have been studied bind RNA and promote intron splicing in plant organelles. That PPR, CRM, PORR, and APO proteins are dedicated to promoting organelle-specific steps in RNA metabolism implies a coevolutionary process through which these protein families were spawned in concert with the molecular processes they engender.

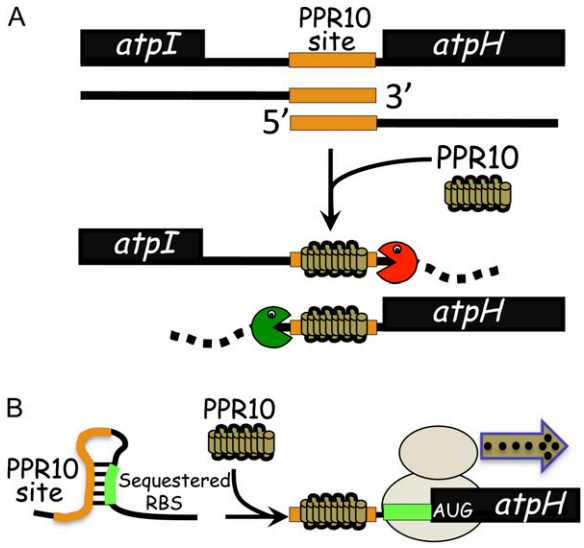

Figure 1. Sequestration of a segment of single-stranded RNA by a long PPR tract may account for many functions attributed to PPR proteins. This is exemplified by PPR10, which stabilizes specific processed RNA termini (A) and enhances translation (B) by sequestering the same RNA segment (Pfalz et al., 2009; Prikryl et al., 2011). Analogous interactions could influence RNA processing, stability, and translation in other ways. A, Site-specific barrier activity of PPR10 defines processed RNA termini. The processed RNA termini derived from polycistronic transcripts spanning *atpI* and *atpH* are shown at the top. PPR10 binds this intergenic region and promotes the accumulation of processed RNAs by blocking exoribonucleases intruding from either direction. The processed *atpI* and *atpH* transcripts derive from different precursor molecules. B, Site-specific RNA-remodeling activity of PPR10 enhances translation. The *atpH* ribosome-binding site (RBS) is sequestered in a duplex with a portion of the PPR10-binding site. PPR10 binding frees the *atpH* ribosome-binding site for translation (Pfalz et al., 2009; Prikryl et al., 2011).

CHLOROPLAST GENE EXPRESSION IS REGULATED AT MANY STEPS

The balance of chloroplast-encoded proteins changes in response to developmental and environmental inputs. The developmental component is particularly important in multicellular plants, where members of the plastid organelle family adopt different forms in different cell types. For example, the amyloplasts in potato (*Solanum tuberosum*) tubers and the chromoplasts in tomato (*Solanum lycopersicum*) fruit lack chloroplast gene products involved in photosynthesis but maintain the expression of chloroplast genes involved in other aspects of cellular metabolism (Kahlau and Bock, 2008; Valkov et al., 2009). The effects of light and hormones are superimposed on developmental programs to further influence the synthesis of chloroplast gene products.

A key difference between chloroplasts and bacteria concerns the point at which gene expression is controlled: whereas transcription initiation is the most common point of regulation in bacteria, in chloroplasts, the regulation of posttranscriptional steps features prominently (Eberhard et al., 2002). Rapid progress in the identification of nuclear genes required for various steps in chloroplast gene expression has provided many candidates for regulatory factors. Examples of gene regulation and potential regulatory

mechanisms are discussed in the context of each step of gene expression below.

The precise regulation of gene expression may be less important in land plant chloroplasts than in bacteria, where the efficient use of resources is likely to have a stronger impact on organismal fitness. In fact, the stoichiometric accumulation of subunits within each photosynthetic enzyme complex is mediated, in part, by proteolysis of unassembled subunits (Adam, 2007). The relative contributions of coordinated protein synthesis versus posttranslational proteolysis has been most thoroughly explored in *Chlamydomonas*, where a set of mechanisms known as control by epistasy of synthesis (CES) coordinate the synthesis of subunits of each photosynthetic enzyme complex via negative feedback loops that are triggered by specific unassembled subunits (Choquet and Wollman, 2009). The degree to which CES-type mechanisms exist in land plants is unclear. Several unassembled proteins that trigger CES in *Chlamydomonas* have been shown not to do so in land plants (McCormac and Barkan, 1999; Monde et al., 2000). On the other hand, a CES-like mechanism coordinates the synthesis of the nucleus- and plastid-encoded subunits of Rubisco in tobacco (*Nicotiana tabacum*; Rodermel et al., 1996; Wostrikoff and Stern, 2007). An alternative strategy for coordinating the expression of multiple chloroplast genes could employ nucleus-encoded proteins that promote the expression of subsets of chloroplast genes (e.g. RNA-binding proteins or σ-factors), as many such proteins have been described (Schmitz-Linneweber et al., 2005; Pfalz et al., 2009; Tillich et al., 2009; Lerbs-Mache, 2010).

CHLOROPLAST TRANSCRIPTION

Large-scale changes in chloroplast transcription occur in response to light, hormonal, and developmental signals (for review, see Liere and Börner, 2007). In barley (*Hordeum vulgare*), for example, chloroplast transcription peaks early during the differentiation of leaf cells (Baumgartner et al., 1989), and cytokinin and light act synergistically to stimulate the transcription of a subset of chloroplast genes in mature chloroplasts (Zubo et al., 2008). The *psbD* promoter is activated by blue light (Gamble and Mullet, 1989), and the relative rates of *psbA* and *psaAB* transcription change in response to different wavelengths of light, serving to optimize the ratio of PSII to PSI (Pfannschmidt et al., 1999).

Progress in elucidating the chloroplast transcription machinery has provided a basis for addressing the mechanisms underlying the control of chloroplast transcription (for review, see Liere and Börner, 2007). In addition to a bacterial-type, multisubunit plastid-encoded polymerase (PEP), chloroplasts in angiosperms and in the moss *Physcomitrella* harbor one or two nucleus-encoded RNA polymerases (NEPs). NEPs are single-subunit polymerases related to those in T7-type bacteriophage and in mitochondria. Initial data suggested a division of labor in which NEP transcribes "housekeeping" genes (e.g. genes for tRNAs, rRNAs, ribosomal proteins, PEP, ClpP, and AccD) and PEP transcribes genes involved in photosynthesis (Hajdukiewicz et al., 1997). The organization of chloroplast genes into two such regulons was proposed to comprise a developmental cascade, in which the activation of NEP early in chloroplast development supports the accumulation of chloroplast ribosomes and ultimately of PEP, which then transcribes photosynthetic genes at later stages (Mullet, 1993). This model in its simplest form has not stood the test of time. It is now clear that most chloroplast genes can be transcribed by either NEP or PEP, albeit from distinct promoters (for review, see Liere and Börner, 2007). Nonetheless, the absence of PEP does shift the balance of transcripts toward those involved in gene expression, and NEP-mediated transcription does predominate early in chloroplast development. Furthermore, neither NEP nor PEP is sufficient for the biogenesis of photosynthetically competent chloroplasts (Allison et al., 1996; Swiatecka-Hagenbruch et al., 2008), indicating that some chloroplast genes require one or the other polymerase for adequate expression.

PEP promoters are characterized by consensus sequences that resemble promoters recognized by σ-70 in *Escherichia coli*. Indeed, PEP promoters are recognized by nucleus-encoded proteins that are related to σ-70 (Lerbs-Mache, 2010; Schweer et al., 2010a). Chloroplast σ-factors are encoded by a small gene family in land plants. The presence of multiple σ-factors offers the potential to differentially regulate chloroplast gene subsets at the transcriptional level. In fact, there is evidence that different members of the σ-family are differentially regulated and target different chloroplast promoters. Nonetheless, the scope of this type of regulation seems to be limited, as reverse-genetic analyses in Arabidopsis demonstrate considerable functional redundancy among the different sigmas.

Evidence for nonredundant functions of each chloroplast σ-factor in Arabidopsis is summarized by Lerbs-Mache (2010). A compelling example involves SIG5. SIG5 is necessary for the use of the blue light-inducible *psbD* promoter, and SIG5 transcript levels are induced in response to blue light. Thus, the blue light regulation of SIG5 transcription may be sufficient to account for blue light induction of *psbD*. Another example involves the regulation of σ-factor activity by phosphorylation. SIG1 and SIG6 in Arabidopsis are substrates for phosphorylation in vivo (Shimizu et al., 2010) and in vitro (Schweer et al., 2010b), respectively. SIG1 phosphorylation changes in response to the oxidation state of the plastoquinone pool, and mutation of the phosphorylated amino acids alters the ratio of *psbA* to *psaAB* transcription (Shimizu et al., 2010). Several different protein kinases have been proposed to contribute to the regulation of σ factor activity (Ogrzewalla et al., 2002; Puthiyaveetil et al., 2008), but the signal transduction pathways that connect incident

light to changes in chloroplast transcription are largely uncharacterized.

Various other proteins have been detected in chloroplasts that bind DNA or that are associated with the chloroplast "transcriptionally active chromosome" (for review, see Liere and Börner, 2007; Lerbs-Mache, 2010). Mutations in several of the corresponding genes compromise chloroplast gene expression (Pfalz et al., 2006). However, more detailed analyses will be required to determine which of these proteins are true transcription factors and which function in other DNA-associated processes or in posttranscriptional processes that are coupled to transcription.

The termination of transcription in chloroplasts has received relatively little attention. Many 3′ RNA termini are not products of transcription termination but rather result from the processing of longer transcripts (for review, see Stern et al., 2010). However, PEP, NEP, and T7 RNA polymerases terminate in vitro at strong intrinsic bacterial terminators, which consist of GC-rich RNA hairpins followed by several contiguous uridines (Chen and Orozco, 1988; Jeng et al., 1990; Kühn et al., 2007). Thus, it seems likely that NEP and PEP respond to sequences with these features in vivo. As only a handful of 3′ regions have been assayed for transcription termination activity, more comprehensive assays may yet uncover efficient transcription terminators in chloroplasts.

RNA SPLICING

A set of approximately 20 introns (one group I and approximately 19 group II introns) was acquired early during the evolution of land plants and is shared by most land plants today (Turmel et al., 2006). Algal chloroplast introns were acquired independently of those in land plants, so the chloroplast intron content in *Chlamydomonas* is entirely different. All chloroplast introns were derived from "self-splicing" group I or group II ribozymes. However, self-splicing has not been reported for any introns in land plant chloroplasts, and it is now abundantly clear that their splicing is protein dependent.

Group I and group II introns have distinct structures and splicing mechanisms. Both intron types are found in bacteria, albeit in small numbers, where they splice with the aid of conserved intron-encoded proteins called maturases (Pyle and Lambowitz, 2006). Chloroplast introns, however, are degenerate and require interactions with "host"-encoded proteins of diverse evolutionary origin. A parallel to the intron fragmentation that is believed to underlie the evolution of nuclear spliceosomal snRNAs can be seen in the transspliced group II introns in chloroplasts, two of which are found in *Chlamydomonas* and one in land plants.

Approximately 14 proteins in land plants and three in *Chlamydomonas* have been identified that are necessary to splice chloroplast group II introns and that associate with intron RNAs in vivo (de Longevialle et al., 2010; Stern et al., 2010). All but one of these proteins are encoded by the nuclear genome, the exception being the maturase encoded in the *trnK* intron in land plants. It is anticipated that many such proteins promote splicing by guiding intron folding into a catalytically active structure; biochemical data support this view for CRS1 (Ostersetzer et al., 2005), which is required for the splicing of the chloroplast *atpF* intron in angiosperms. Concepts to emerge from the characterization of chloroplast group II intron ribonucleoprotein particles (RNPs) are summarized below.

Chloroplast Group II Intron RNPs Are Complex

Studies of group I and group II intron splicing in bacteria and yeast revealed simple RNPs involving one or two proteins and an intron that is inherently self-splicing (Pyle and Lambowitz, 2006). By contrast, chloroplast group II intron RNPs are more protein than RNA. For example, six different proteins have been shown to associate with, and to be required for the splicing of, the maize *petD* intron (Fig. 2). This scenario is typical of the other group II introns in land plant chloroplasts.

There Is No Chloroplast "Spliceosome"

A spliceosome of uniform composition catalyzes the splicing of most nuclear pre-mRNA introns. By contrast, different chloroplast splicing factors act combinatorially to promote the splicing of different intron subsets. Furthermore, the proteins that participate in chloroplast splicing are unrelated to those that participate in nuclear splicing.

Invention of Novel RNA-Binding Domains, and Cooption of Preexisting RNA-Binding Domains for Plant Organellar Splicing

The CRM, PORR, and APO domains were initially recognized to be RNA-binding domains through the analysis of chloroplast RNA splicing, and all analyzed members of these protein families participate in intron splicing in organelles. Thus, the abundance of group II introns in plant organelles may have been the driving force for the evolution of these protein families. On the other hand, several chloroplast splicing factors maintain a strong resemblance to bacterial proteins. Examples include maize CRS2, which is related to bacterial peptidyl tRNA hydrolase, and *Chlamydomonas* Raa2, which is related to bacterial pseudouridine synthase. Minor evolutionary tinkering was sufficient to impart novel activities upon these ancient proteins.

Splicing as a Regulatory Step?

The regulation of splicing could, in principle, serve to adjust the balance of gene expression in chloroplasts. As for other steps in chloroplast gene expression, it is not known to what extent splicing efficiency

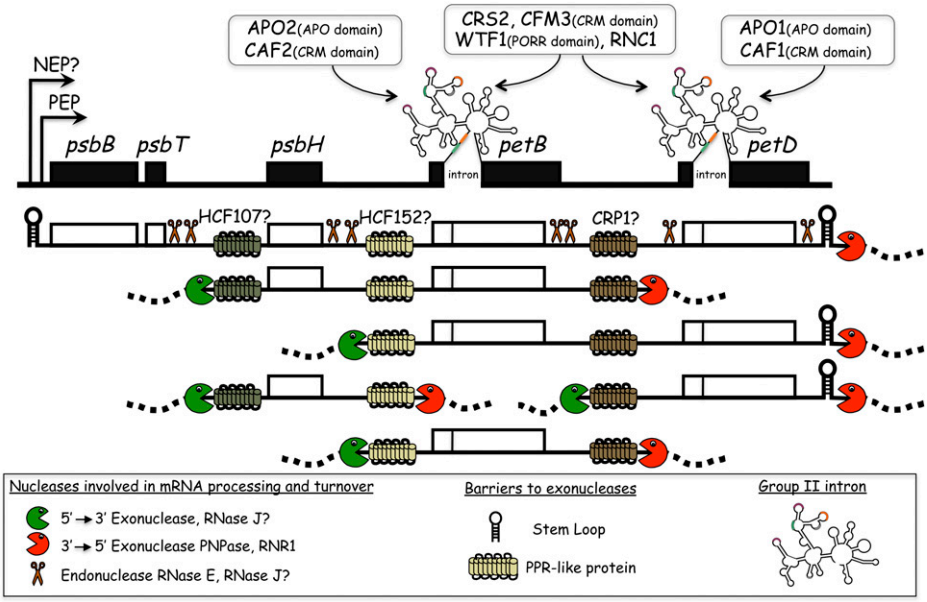

Figure 2. Model for expression of the chloroplast *psbB* gene cluster. The *psbB* gene cluster spans five genes and generates approximately 20 processed transcripts via intercistronic processing and the splicing of group II introns in the *petB* and *petD* genes (Barkan, 1988; Westhoff and Herrmann, 1988). The diagram shows a subset of the processed transcripts. Splicing factors for the *petB* and *petD* introns are indicated above, with their organelle-specific RNA-binding domains specified in parentheses (for review, see de Longevialle et al., 2010; Watkins et al., 2011). The position of a bound PPR-like protein defines the positions of processed RNA termini in each intercistronic region by blocking exoribonucleases; genetic data implicate HCF107, HCF152, and CRP1 in the indicated events (Barkan et al., 1994; Felder et al., 2001; Meierhoff et al., 2003), but these interactions have not been confirmed biochemically. The endonucleases RNAse E and RNAse J are posited to cleave rather nonspecifically at unstructured AU-rich regions to provide exonuclease access to internal RNA regions. A generic version of this model was presented previously, based on the effects of PPR10 on two other transcription units (Pfalz et al., 2009).

limits the final output of gene product. However, weak mutant alleles of several maize chloroplast splicing factors condition a distinct mutant phenotype (Watkins et al., 2007), indicating that these proteins and the splicing events they promote are limiting for gene expression. The degree to which these and other splicing factors perform a regulatory role remains to be determined.

RNA EDITING

Another distinguishing feature of gene expression in land plant chloroplasts is the posttranscriptional modification of mRNA sequences by RNA editing (for review, see Chateigner-Boutin and Small, 2010). mRNA editing has not been observed in bacteria or in algal organelles. However, this property is shared with plant mitochondria, and indeed, RNA editing in plant mitochondria and chloroplasts is believed to have a common evolutionary origin. In angiosperms, chloroplast mRNA editing is limited to the change of specific cytidine residues to uridine and occurs at approximately 40 positions. This is a rapidly evolving feature, as more than half of the edited sites differ between monocot and dicot plants. Chloroplast genomes that "lack" a particular edited site generally encode a uridine at the corresponding position. Most editing events in chloroplasts are important for gene function: some create start codons, and some modify the coding sequence such that a deleterious amino acid is changed to a conserved and functional one.

The chemistry of the editing reaction is believed to involve cytidine deamination (for review, see Chateigner-Boutin and Small, 2010). Thus, the editing machinery has been anticipated to consist of two types of component: (1) "specificity factors" that target a nucleotide for editing, and (2) a deaminase enzyme. Elucidation of the specificity factors began with the discovery that the PPR protein CRR4 is required to edit a nucleotide in the *ndhD* mRNA (Kotera et al., 2005). CRR4 binds with specificity to a short RNA harboring the edited site (Okuda et al., 2006). The position of the CRR4 binding site correlates well with in vivo and in vitro analyses of cis-elements required for editing: a region of approximately 30 nucleotides is sufficient to specify most edited sites, with the edited nucleotide found near the 3′ end of this region.

CRR4 belongs to a subfamily of PPR proteins that include appended motifs at their C terminus called E and DYW (Lurin et al., 2004). Following the discovery of CRR4, genetic analyses identified many more RNA-editing factors in chloroplasts and also in plant mitochondria. Almost all of these are either PPR-E or PPR-E-DYW proteins. These results strongly implicate both the E and DYW motifs in the editing process.

Mutagenesis experiments have confirmed the "specificity" function to reside in the PPR tract and have shown the E domain to be essential for editing (Okuda et al., 2007, 2009). The DYW motif, however, can be deleted from several editing factors without loss of activity (Okuda et al., 2009). It has been proposed that the E-DYW appendage recruits the editing enzyme and/or itself has enzymatic activity (Salone et al., 2007; Hammani et al., 2009; Okuda et al., 2009).

These results paint a picture in which the subset of PPR proteins harboring E or E-DYW extensions are the primary specificity factors for RNA editing in chloroplasts; the PPR tract binds approximately 20 nucleotides upstream of the targeted C residue, and the E-DYW motif promotes editing in an as yet undetermined manner. Some such proteins specify editing at a single site, but many target multiple sites and are proposed to recognize a degenerate sequence (Heller et al., 2008; Hammani et al., 2009). Thus, the approximately 40 PPR proteins of this type in land plant chloroplasts are more than sufficient to account for known editing events. Members of the abundant chloroplast ribonucleoprotein (cpRNP) family of RNA-binding proteins have also been shown to affect several editing sites in vitro (Hirose and Sugiura, 2001) and in vivo (Tillich et al., 2009). cpRNPs bind RNA rather nonspecifically, so the mechanism by which they influence RNA editing is likely to differ from that of PPR editing factors.

An open question concerns the role of RNA editing in chloroplast gene regulation. The results of a comprehensive analysis (Peeters and Hanson, 2002) suggested that the low editing efficiencies in nongreen maize plastids are unlikely to be a limiting factor in gene expression. Another question concerns the evolutionary forces that produced plant organellar RNA editing. An attractive model coined "genomic debugging" (Maier et al., 2008) posits that the ease of evolving new specificity factors for RNA editing (i.e. PPR-E class proteins) allowed the fixation of otherwise deleterious mutations in the chloroplast genome.

mRNA STABILIZATION AND DECAY

The stabilities of chloroplast mRNAs vary considerably and can change in response to light and during leaf development (Klaff and Gruissem, 1991; Baumgartner et al., 1993; Kim et al., 1993). The results summarized below support the view that the stabilizing influence of chloroplast RNA-binding proteins, especially PPR-like proteins, is layered upon an RNA turnover machinery borrowed from bacteria, to determine RNA half-life in chloroplasts.

Chloroplast mRNAs typically survive for many hours (Klaff and Gruissem, 1991; Kim et al., 1993) and are much more stable than are typical mRNAs in bacteria. Nonetheless, the chloroplast ribonucleases that are implicated most strongly in RNA decay are closely related to those in bacteria (Stern et al., 2010). The most thoroughly studied pathway for RNA decay in chloroplasts involves the 3′→5′ exonuclease polynucleotide phosphorylase (for review, see Schuster and Stern, 2009). As in bacteria, chloroplast polynucleotide phosphorylase activity is stimulated by 3′ polyadenylation of its RNA substrate, and it is blocked by stable 3′ RNA structures. Recent results show that chloroplasts also have a protein-based mechanism for stabilizing 3′ termini that has no apparent analog in bacteria: a bound PPR protein can block 3′ exonucleases in vivo and in vitro (Hattori and Sugita, 2009; Pfalz et al., 2009; Prikryl et al., 2011). There is strong genetic evidence for a 5′→3′ exonuclease activity in chloroplasts whose activity can likewise be blocked by a stable RNA structure or bound PPR-like protein (for review, see Stern et al., 2010). The 5′→3′ exonuclease activity has not been identified, but it is likely to reside in chloroplast RNAse J, whose ortholog in bacteria has both endonuclease and 5′→3′ exonuclease activity (Condon, 2007).

The rate-limiting step in RNA decay in chloroplasts, as in bacteria, is endonucleolytic cleavage, which generates products that are accessible to exonucleases by removing protective features at the RNA termini (for review, see Stern et al., 2010). However, the identities of the relevant endonucleases remain a mystery. A chloroplast protein called CSP41 exhibits endoribonuclease activity in vitro, but compelling evidence that CSP41 influences RNA decay in vivo has not emerged. RNAse E and RNAse J perform this function in bacteria, cleaving at unstructured AU-rich sequences (Condon, 2007). *E. coli* lacks RNAse J, whereas *Bacillus subtilis* lacks RNAse E, but cyanobacteria and higher plant chloroplasts harbor both enzymes. Arabidopsis mutants lacking chloroplast RNAse E do not exhibit a global increase in mRNA levels (Walter et al., 2010), indicating that RNAse E cannot be the sole activity that initiates chloroplast mRNA decay. Given the activities observed for chloroplast RNAse E in vitro (Schein et al., 2008) and the fact that bacterial RNAse E and RNAse J have similar endonuclease activities, it may be that RNAses E and J in chloroplasts act redundantly to initiate mRNA decay.

The parameters that determine the rate of the initiating endonucleolytic cleavages for chloroplast RNA decay are not known. These are likely to include the sequence and structure of the mRNA, its extent of ribosome association, and the presence of other proteins (particularly PPR-like proteins) that mask or expose potential RNase cleavage sites. In any case, it can be anticipated that regulation of the ribonucleases and stabilizing proteins underlies the regulation of chloroplast RNA stability, and that the details of these mechanisms will be forthcoming in the near future.

INTERCISTRONIC AND 5′ mRNA PROCESSING

A particularly striking feature of gene expression in land plant chloroplasts is the complexity of the RNA populations arising from most genes. This phenome-

non is exemplified by the *psbB* gene cluster (Barkan, 1988; Westhoff and Herrmann, 1988; Fig. 2). Multiple mRNA isoforms arise from the processing of polycistronic transcripts between coding regions ("intercistronic processing"), in the 5′ untranslated region (UTR; "5′ processing"), and by the removal of introns. The mechanisms and functional significance of these events in chloroplasts have been long-standing questions. Recent findings have begun to clarify these issues and have challenged models that have prevailed ever since these phenomena were recognized more than 20 years ago.

It had been widely assumed that intercistronic mRNA processing in chloroplasts results from site-specific endonucleolytic cleavages that simultaneously generate adjacent processed 5′ and 3′ termini. This hypothesis was based on low-resolution mapping data that placed processed 5′ and 3′ ends near one another in various intergenic regions. The first hint that this view may be incorrect came with the mapping of the processed RNA termini between the *petB* and *petD* open reading frames (ORFs) in maize (Barkan et al., 1994): the 5′ end of the processed RNA from the downstream ORF (*petD*) maps approximately 30 nucleotides upstream of the 3′ end of the processed RNA from the upstream ORF (*petB*), proving that this pair of processed termini do not result from a single endonucleolytic cleavage event (Fig. 2).

Recent data show that this spatial relationship between processed 5′ and 3′ termini arising from the same intergenic region is common and provide strong evidence for a mechanism of intercistronic processing that does not involve site-specific endonucleolytic cleavage. During a study of the maize protein PPR10 (Pfalz et al., 2009), processed termini in the *atpI-atpH*, *psaJ-rpl33*, and *psbH-petB* intergenic regions were mapped precisely. In each case, the processed RNAs overlap by approximately 25 nucleotides, as had been shown previously for the *petB-petD* region (Figs. 1 and 2). By contrast, we are aware of only one instance in which processed 5′ and 3′ termini within an intergenic region have been shown unambiguously not to overlap in this manner, and even these termini appear to arise from independent processing events (Hashimoto et al., 2003).

There is strong evidence that the processed termini in the *atpI-atpH* and *psaJ-rpl33* intergenic regions arise in the following manner (Pfalz et al., 2009; Prikryl et al., 2011). PPR10 binds to these two intergenic RNAs at sites that have similar sequences, and blocks the progress of exoribonucleases approaching from either the 5′ or 3′ direction (Fig. 1). This results in the accumulation of processed RNAs whose 5′ or 3′ terminus is defined by the upstream or downstream edge, respectively, of bound PPR10. This model was validated by experiments showing that recombinant PPR10 is sufficient to block both 5′ and 3′ exoribonucleases in vitro (Prikryl et al., 2011). Furthermore, PPR10 in conjunction with a generic 5′ exonuclease is sufficient to generate a 5′ terminus that corresponds precisely to the PPR10-dependent processed end found in vivo, proving that no additional proteins are required. This mechanism is likely to be the rule rather than the exception, as genetic data link three other PPR proteins (CRP1, HCF152, and PPR38) to the accumulation of processed transcripts with 5′ or 3′ ends mapping in three other intergenic regions (Barkan et al., 1994; Meierhoff et al., 2003; Hattori and Sugita, 2009; Fig. 2). Thus, "processed" mRNAs might be more accurately described as metastable degradation intermediates resulting from a site-specific blockade to the exonucleases involved in bulk RNA decay. An alternative mechanism for intercistronic processing has also been proposed, involving a PPR protein harboring an appendage with RNA cleavage activity (Okuda et al., 2009). The site-specific barrier and site-specific cleavage mechanisms are not mutually exclusive, although current data suggest the barrier mechanism to be the predominant one.

This scenario for intercistronic RNA processing requires a means for exonucleases to bypass stabilizing elements at transcript termini. We proposed a model involving the same housekeeping endonucleases that trigger mRNA decay (Pfalz et al., 2009; Fig. 2). This model is supported by the phenotype of Arabidopsis mutants lacking chloroplast RNAse E, which have defects in the processing of several polycistronic transcripts (Walter et al., 2010). However, many processing events were not disrupted in these mutants, indicating that other endonucleases (perhaps RNAse J) contribute as well.

Genetic data implicate PPR-like proteins not only in intercistronic RNA processing but also in the stabilization of specific processed 5′ ends in the chloroplasts of land plants and *Chlamydomonas* (Stern et al., 2010). 5′ processing and intercistronic processing had appeared to be distinct processes, but it now appears that they involve the same basic mechanism: the stalling of exonucleases at specific sites by a bound PPR-like protein. 5′ processing results from site-specific protection from a 5′→3′ exonuclease (likely RNAse J) near the transcription start site, whereas intercistronic processing involves protection from exonucleases intruding from either direction. Taken together, the results to date suggest that the machineries for 5′ and intercistronic mRNA processing in chloroplasts arose by the superposition of newly evolved PPR-like proteins upon a mechanism for RNA turnover that was borrowed from bacteria.

Chloroplasts contain abundant cpRNP proteins that are related to nuclear hnRNPs and that have also been implicated in mRNA stabilization. cpRNPs can stabilize RNAs in chloroplast extracts (Schuster and Gruissem, 1991; Nakamura et al., 2001), and a role for a cpRNP in the stabilization of several chloroplast mRNAs was confirmed in an in vivo analysis (Tillich et al., 2009). That being said, genetic data point to PPR-like proteins as the primary protein class involved in chloroplast RNA stabilization (Stern et al., 2010), suggesting that the long RNA-protein interface pre-

sented by a long PPR tract (Prikryl et al., 2011) provides a particularly effective barrier to nucleases.

CHLOROPLAST TRANSLATION

Several observations have highlighted translation as an important control point in chloroplast gene expression (Peled-Zehavi and Danon, 2007). (1) The translation of some chloroplast mRNAs is rapidly induced by light. (2) Translation rate has been shown to be a rate-limiting step in the expression of many chloroplast genes in *Chlamydomonas* (Eberhard et al., 2002) and can be regulated by the assembly status of the multimeric complexes harboring plastid gene products (Choquet and Wollman, 2009). (3) Genetic screens have identified numerous nucleus-encoded proteins that are required for the translation of specific chloroplast RNAs, demonstrating a large investment of the host genome in promoting chloroplast gene expression at the translational level. Adding to the intrigue are hints that chloroplast translation may involve mechanisms that are distinct from those in bacteria. Shine-Dalgarno elements are not evident in many chloroplast mRNAs, leading to speculation about novel ribosome recruitment mechanisms. Furthermore, whereas translational modulators in chloroplasts are consistently activators of chloroplast translation, translational regulation in bacteria is generally mediated by negative regulators.

Chloroplast ribosomes are formed from rRNAs and proteins that retain a strong resemblance to those in bacteria, and they function in conjunction with bacterial-type initiation and elongation factors (Peled-Zehavi and Danon, 2007). With this as backdrop, the discussion below starts from the parsimonious viewpoint that translation and its regulation in chloroplasts and bacteria involve similar mechanisms. Features of chloroplast translation that seem at odds with this perspective are evaluated in this evolutionary context.

Plastid-Specific Ribosomal Proteins

Chloroplast ribosomes include several "plastid-specific ribosomal proteins" (PSRPs; Yamaguchi and Subramanian, 2003; Beligni et al., 2004), which have been invoked as candidates for mediators of light-regulated chloroplast translation. However, a cryo-electron microscopy study of the spinach (*Spinacia oleracea*) chloroplast ribosome suggested instead that several PSRPs play structural roles, compensating for the loss of specific rRNA elements (Sharma et al., 2007). That study and a related one in *Chlamydomonas* (Manuell et al., 2007) did highlight variants of conserved ribosomal proteins as candidates for participation in chloroplast-specific mechanisms. For example, chloroplast-specific extensions on ribosomal protein S21 in spinach (Sharma et al., 2007) and ribosomal protein S2 in *Chlamydomonas* (Manuell et al., 2007) are positioned to contact the mRNA 5′ UTR during translation initiation.

PSRP1 has proven to be neither a ribosomal protein nor plastid specific (Sharma et al., 2010). In fact, PSRP1 and its bacterial orthologs inhibit translation by blocking tRNA binding sites. Furthermore, the gene encoding the cyanobacterial ortholog is strongly repressed after illumination, providing a molecular link between incident light and cyanobacterial translation. This observation led the authors to propose the intriguing possibility that PSRP1 abundance or activity may likewise be repressed in the light and that this might underlie the global enhancement of plastid translation after a shift from dark to light.

Mechanism of Start Codon Recognition

The most familiar mode of ribosome recruitment in bacteria involves the Shine-Dalgarno interaction: the pairing of the 3′ end of the 16S rRNA with complementary sequences upstream of the start codon. The consensus bacterial Shine-Dalgarno element has the sequence GGAGG and is centered approximately 10 nucleotides upstream from the start codon. Approximately one-third of chloroplast genes in land plants are preceded by predicted Shine-Dalgarno elements at the consensus location, and several of these have been confirmed to enhance translation (Peled-Zehavi and Danon, 2007). However, the majority of chloroplast genes lack properly positioned Shine-Dalgarno elements, leading to speculation about alternative modes for start codon selection. Two hypotheses are often suggested: that mRNA-specific translational activators can substitute for Shine-Dalgarno elements, and that plastid-specific ribosomal proteins play a role in Shine-Dalgarno-independent translation.

It is useful to consider what is known about bacterial translation to assess whether unique mechanisms need to be invoked to explain observations in chloroplasts. One important point is that Shine-Dalgarno elements are far from universal in bacterial genes (Nakagawa et al., 2010): for example, only approximately 39% of genes in cyanobacteria have apparent Shine-Dalgarno elements. Another lesson from bacteria is that 30S ribosomal subunits bind nonspecifically to single-stranded RNA and that a structure-free region spanning approximately 60 nucleotides centered on the start codon is important for optimal translation (de Smit and van Duin, 2003; Kudla et al., 2009). Current data support the notion that translation initiation in bacteria involves an unstructured RNA landing pad from which the 30S subunit can slide bidirectionally to access the start codon (de Smit and van Duin, 2003).

Can fundamentally similar mechanisms account for observations in chloroplasts? Perhaps so. A recent study showed that in both chloroplasts and bacteria, translation initiation regions lacking a Shine-Dalgarno sequence are less structured than are those harboring a Shine-Dalgarno element, suggesting that start codon accessibility is particularly critical in the absence of a Shine-Dalgarno interaction (L. Scharff and R. Bock,

unpublished data). Furthermore, the chloroplast translational activators whose mechanisms are best understood seem to function by maintaining a structure-free zone for the ribosome (see below).

The possibility that 5′→3′ ribosome scanning contributes to start codon recognition in chloroplasts was raised in two studies, which reported preferential use of upstream start codons in reporter constructs (Hirose and Sugiura, 2004; Drechsel and Bock, 2010). It should be noted, however, that related observations have been made in *E. coli* (Adhin and van Duin, 1990), where ribosomes lack "specialized" structures and where polycistronic mRNAs are not generally processed prior to translation. Thus, these observations in chloroplasts may not reflect chloroplast-specific mechanisms but may instead be manifestations of differences in RNA structure resulting from differences in sequence, temperature, and intracellular milieu.

Nucleus-Encoded Translational Activators

Analyses of nonphotosynthetic mutants in plants and *Chlamydomonas* have revealed numerous nucleus-encoded proteins that influence chloroplast translation (for review, see Peled-Zehavi and Danon, 2007). These proteins are invariably activators, and they act specifically on one or several chloroplast mRNAs. Many of them are PPR-like proteins and, where tested, they act via the 5′ UTR of the target mRNAs. Furthermore, many proteins that activate translation also stabilize the same mRNA.

Two well-characterized examples in *Chlamydomonas* act on the *petA* (Boulouis et al., 2011) and *psbD* (Schwarz et al., 2007) mRNAs. In both cases, genetic data provide evidence that a PPR-like protein binds the mRNA 5′ end and stabilizes the RNA downstream. These proteins each interact with a second protein that binds the adjacent RNA segment and enhances the translation of the downstream ORF. PPR-like proteins also activate the translation of specific mRNAs in the chloroplasts of land plants via interaction with specific 5′ UTRs (Barkan et al., 1994; Sane et al., 2005; Schmitz-Linneweber et al., 2005; Pfalz et al., 2009).

Two general mechanisms for translational activation can be envisioned: the recruitment of ribosomes or translation factors, or the maintenance of an RNA structure (or lack of structure) that is attractive to ribosomes. Phylogenetic arguments and the available mechanistic data for chloroplast translational activators support the latter view. In vitro assays revealed the likely mechanism by which PPR10 enhances translation of the *atpH* ORF (Prikryl et al., 2011): when PPR10 binds to the *atpH* 5′ UTR, it remodels the RNA such that the *atpH* ribosome-binding region is freed from a secondary structure (Fig. 1B). Genetic data support an analogous mechanism for two translational activators in *Chlamydomonas* chloroplasts (Stampacchia et al., 1997; Schwarz et al., 2007). Detailed study of additional examples will be necessary to determine whether other types of activation mechanism are also at play.

The light regulation of *psbA* translation has attracted particular attention. The *psbA* gene encodes the D1 reaction center protein of PSII, which is subject to light-induced damage that necessitates new D1 synthesis for PSII repair. Light activates the initiation of *psbA* translation via the *psbA* 5′ UTR (for review, see Peled-Zehavi and Danon, 2007). Genetic screens identified the Arabidopsis protein HCF173 (Schult et al., 2007) and the *Chlamydomonas* protein Tba1 (Somanchi et al., 2005) as being required specifically for *psbA* translation. HCF173 and Tba1 are unrelated, they are not PPR-like proteins, and their mechanisms of action are unknown. A biochemical approach in *Chlamydomonas* led to a model for the regulation of *psbA* translation via a set of RNA-binding proteins whose activity is modulated by redox poise (for review, see Peled-Zehavi and Danon, 2007). However, there is no evidence for a related system in land plants, and recent reports are at odds with several aspects of that model (for review, see Zerges and Hauser, 2009).

Relationship between mRNA Processing and Translational Efficiency

The enhancement of translational efficiency is often invoked as the raison d'être for the pervasive intercistronic mRNA processing in chloroplasts. This is an appealing possibility, but the body of evidence to date does not provide strong evidence in favor of this view. First, many chloroplast genes are represented solely by polycistronic mRNAs. Second, for several genes that are represented by processed monocistronic mRNAs, processing has been shown not to be necessary for translation. This was demonstrated to be the case in vivo for maize *petB* and *petD*: when antibodies to PetB and PetD were used to immunoselect polysomes engaged in PetB and PetD synthesis, all transcripts containing spliced *petB* or *petD* sequences were recovered regardless of the upstream or downstream sequences (Barkan, 1988). A similar conclusion was drawn for *atpH* based on results from a tobacco chloroplast in vitro translation system (Yukawa et al., 2007). Furthermore, downstream ORFs in engineered polycistronic transcription units in tobacco chloroplasts can be translated efficiently without processing (Staub and Maliga, 1995). There is also evidence, however, that some mRNA processing events do enhance translational efficiency. A compelling example comes from an analysis of the *psaC-ndhD* transcription unit (Hirose and Sugiura, 1997): in the tobacco chloroplast in vitro translation system, the *psaC* and *ndhD* ORFs were translated more efficiently as monocistronic than as dicistronic RNAs. This was shown to be due to an inhibitory interaction between a sequence in the *psaC* coding region and its complement in the *ndhD* 5′ UTR.

A key argument that has been used to support the idea that intercistronic processing enhances transla-

tion derives from the genetic analysis of the PPR-like proteins CRP1, HCF107, and CRR2: in *crp1*, *hcf107*, and *crr2* mutants, the loss of specific processed mRNAs correlates with reduced translational efficiencies (Barkan et al., 1994; Felder et al., 2001; Hashimoto et al., 2003). However, recent results with PPR10 warrant consideration of an alternative explanation for those correlations. The *ppr10* mutant phenotype parallels those of *crp1*, *hcf107*, and *crr2* in that specific processed *atpH* transcripts are absent and the *atpH* translation rate is also reduced. Both of those effects result from the stable association of PPR10 with the *atpH* 5′ UTR: bound PPR10 simultaneously blocks 5′→3′ RNA degradation and remodels the adjacent translation initiation region to expose the ribosome-binding site (Pfalz et al., 2009; Prikryl et al., 2011; Fig. 1). These findings undermine the notion that the loss of processed RNAs in *crp1*, *hcf107*, and *crr2* mutants is the sole cause of reduced translational efficiency. Instead, it may be that it is the presence of CRP1, HCF107, and CRR2 proteins on their target 5′ UTRs that enhances translation, and that the reduced translation and loss of processed mRNAs in the mutants are independent effects of the absence of these proteins.

On balance, the current evidence argues against translational enhancement as a significant driving force for the evolution of chloroplast mRNA processing, although processing likely enhances translational efficiency in some cases. In fact, it can be anticipated that a newly acquired mRNA processing event will, over evolutionary time, become increasingly important for optimal translation due to relaxed constraints on flanking RNA sequences: If an ORF can be separated from cotranscribed sequences by processing, then it will be released from prior evolutionary constraints that would have limited inhibitory interactions with sequences found elsewhere on the RNA precursor. Although it is possible that chloroplast-specific mechanisms are at play during translation initiation and translational activation, perhaps it is too soon to let go of the conservative view that differences between chloroplasts and bacterial translation lie in the types of RNA-binding proteins that are available to modulate RNA structure and processing (e.g. PPR-like proteins) rather than in the translation process itself.

COUPLING OF CHLOROPLAST TRANSCRIPTION WITH DOWNSTREAM EVENTS

Bacterial transcription and translation are said to be "coupled" in that ribosomes initiate translation soon after the start codon exits RNA polymerase. The long lifetime of chloroplast mRNAs and the fact that they can be translated after separation from downstream RNA sequences imply that translation is not obligatorily coupled to transcription in chloroplasts. Nonetheless, translation in chloroplasts may generally initiate on nascent transcripts during the process of transcription. Chloroplast DNA is in contact with the stroma, which is the location of many RNA-binding proteins and ribosomes. Therefore, it can be expected that RNA-binding proteins and ribosomes begin to associate with nascent transcripts in a cotranscriptional manner. This view is supported by the fact that the chloroplast splicing factor APO1 (Watkins et al., 2011) colocalizes with the nucleoid (Amann et al., 2004). The degree to which RNA processing and translation are "cotranscriptional" versus "posttranscriptional" may simply reflect the underlying kinetics: events involving RNA-protein interactions that form more slowly will generally be posttranscriptional, whereas those involving interactions that form rapidly will generally be cotranscriptional.

There is evidence for the cotranslational insertion of some chloroplast-encoded proteins into the thylakoid membrane via a conserved "Sec" machinery (Zhang et al., 2001). In addition, translational pausing has been shown to accompany the integration of D1 into the membrane (Kim et al., 1991; Zhang et al., 2000). Whether this pausing is a consequence of the engagement of the nascent peptide with the membrane integration machinery or serves as a means to facilitate membrane integration remains to be resolved.

PERSPECTIVE

It is gratifying to look back upon the past decade and to recognize the remarkable progress that has been made in understanding the mechanisms of chloroplast gene expression. Some processes that had appeared to be complex now appear to have rather simple underlying mechanisms. For example, the complexity of intercistronic RNA processing now appears to distill down to a set of PPR-like proteins that bind specific RNA sites and block housekeeping exonucleases. Likewise, the process of RNA editing appears to involve a set of PPR proteins that target a multitude of sites but that recruit a shared enzymatic machinery. Other aspects of chloroplast gene expression that had been anticipated to involve simple mechanisms have turned out to be surprisingly complex. A prime example is chloroplast RNA splicing, which involves "self-splicing" RNAs whose splicing requires a multitude of different proteins. This complexity can also be seen in the interplay between NEP, PEP, and the various PEP-associated σ-factors that promote chloroplast transcription.

The evolution of chloroplast gene expression systems continues to present a set of intriguing puzzles. Proteins of bacterial ancestry serve in core gene expression processes (transcription, translation, and RNA turnover). Acquired aspects of chloroplast gene expression (RNA editing, protein-facilitated group II intron splicing, and intercistronic RNA processing) were imparted by the superposition of newly evolved proteins upon these ancient core machineries. The environment of plant organelles seems to have been particularly permissive for the evolution of novel

RNA-binding motifs, including the multitude of diversified PPR proteins. In fact, it seems likely that the complex RNA metabolism and complex RNA-binding protein repertoires in plant organelles arose through an as yet mysterious coevolutionary process.

Another set of unanswered questions concern the regulation of chloroplast gene expression. There is no single step in gene expression that is the primary regulated step; rather, each step can contribute to different patterns of chloroplast gene expression under different conditions. Now that the nuts and bolts of chloroplast gene expression are understood in considerable detail, the field is poised to understand how chloroplast gene expression responds to light quality and quantity, stress, and developmental cues. A current challenge is to identify the subset of nucleus-encoded factors whose activity is limiting for gene expression and the signal transduction pathways that link these regulators to external signals.

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