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Chloroplast gene expression: Recent advances and perspectives

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ABSTRACT

Chloroplasts evolved from an ancient cyanobacterial endosymbiont more than 1.5 billion years ago. During subsequent coevolution with the nuclear genome, the chloroplast genome has remained independent, albeit strongly reduced, with its own transcriptional machinery and distinct features, such as chloroplast-specific innovations in gene expression and complicated post-transcriptional processing. Light activates the expression of chloroplast genes via mechanisms that optimize photosynthesis, minimize photodamage, and prioritize energy investments. Over the past few years, studies have moved from describing phases of chloroplast gene expression to exploring the underlying mechanisms. In this review, we focus on recent advances and emerging principles that govern chloroplast gene expression in land plants. We discuss engineering of pentatricopeptide repeat proteins and its biotechnological effects on chloroplast RNA research; new techniques for characterizing the molecular mechanisms of chloroplast gene expression; and important aspects of chloroplast gene expression for improving crop yield and stress tolerance. We also discuss biological and mechanistic questions that remain to be answered in the future.

Key words: chloroplast, chloroplast gene expression, transcription, post-transcriptional processing, translation

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INTRODUCTION

Chloroplasts are a type of double membrane–bound cytoplasmic organelle found in plants, algae, and some protists. The ''energy factories'' of the cell, they are the site where photosynthesis takes place, converting light energy into chemical energy to sustain life on Earth. Chloroplasts are likely derived from an ancient photosynthetic cyanobacterium that was engulfed by a eukaryotic host cell, giving rise to an ancestral eukaryotic plant cell more than 1.5 billion years ago (Archibald, 2015). However, during subsequent endosymbiotic evolution, the chloroplast genome shrank markedly, as most genes of cyanobacterial origin were transferred into the nucleus or lost altogether (Martin et al., 2002; Daniell et al., 2016). In land plants, the chloroplast genome generally ranges in size from 120 to 160 kb and exhibits a highly conserved structure. It comprises a single circular DNA molecule with a quadripartite structure in which two copies of a 26-kb inverted repeat region separate it into one large and one small single-copy region (Daniell et al., 2016; de Vries and Archibald, 2018).

On average, the chloroplast genomes of land plants have retained about 120 genes with conserved content. Most of these genes are particularly important for plant viability because they encode core subunits of the photosynthetic apparatus and the gene expression system of chloroplasts (i.e., RNA polymerase subunits, ribosomal proteins, ribosomal RNAs [rRNAs], and transfer RNAs [tRNAs]). Substantial alteration of chloroplast gene expression can kill plants, underscoring the importance of these genes even in a reduced complement (Zhang et al., 2020).

Similar to those of bacteria, most chloroplast genes are organized as operons transcribed from single promoters and are separated by relatively small non-coding intergenic regions. However, coevolution of the chloroplast and nuclear genomes also allowed many chloroplast-specific innovations in the gene expression machinery to arise. Primary chloroplast transcripts must undergo complicated post-transcriptional processing steps to form mature mRNA, such as RNA editing, RNA splicing, 5′ and 3′ trimming, and intercistronic cleavage (Barkan, 2011). These post-transcriptional processing events ensure that transcripts in chloroplasts have proper stability and secondary structures more suitable for translation by chloroplast 70S ribosomes (Zoschke and Bock, 2018; Zhang et al., 2020). However, chloroplast genes are not constitutively expressed, and light is one of the most important

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environmental cues that switches on their expression through at least two layers of regulation, transcriptional and translational.

Numerous discoveries have substantially expanded our understanding of chloroplast gene expression over the last several years. However, systematic summary and discussion of chloroplast gene expression are generally lacking. Here, we highlight recent advances and emerging principles that govern chloroplast gene expression in land plants, especially at the levels of transcription, post-transcriptional processing, and translation. Because the activation of chloroplast gene expression is tightly controlled by light, we also discuss current proposed mechanisms for light-induced transcription and translation of these genes. Finally, we discuss a number of biological and mechanistic questions about chloroplast gene expression that remain to be answered.

TRANSCRIPTION

Chloroplast genes are transcribed by two distinct polymerases: nucleus-encoded polymerase (NEP) and plastid-encoded polymerase (PEP). NEP is a phage-type RNA polymerase composed of a single catalytic subunit, whereas PEP is a bacterial-type, multi-subunit RNA polymerase (Yagi and Shiina, 2014; Borner et al., 2015). Three typical NEPs have been identified in land plants, including plastid-targeted RpoTp, mitochondrion-targeted RpoTm, and dual-targeted RpoTmp that is localized to both chloroplasts and mitochondria (Borner et al., 2015). These NEPs share a strong evolutionary relationship with T3/T7 phage-type polymerase. Unlike chloroplasts that have both NEP and PEP, mitochondria contain only NEP for transcription, and the unique PEP of chloroplasts may be associated with their photosynthetic function.

The NEP promoter is characterized by a conserved YRTA motif upstream from the transcription start site. A subfamily of NEP promoters has an additional GAA box about 18- to 20-nt upstream of the YRTA motif (Weihe and Börner, 1999; Ortelt and Link, 2021). The PEP promoter is distinct from the NEP promoter and retains the –35 (TTGACA) and –10 (TATAAT) consensus sequence motifs, resembling bacterial σ^{70} promoters (Ortelt and Link, 2021). Except for the *rpoB* operon and the *accD* gene, which are exclusively transcribed by NEP, most genes in chloroplasts are transcribed by both NEP and PEP (Ortelt and Link, 2014). Currently, at least 215 transcription start sites of PEP and NEP have been determined in mature chloroplasts of Arabidopsis using Terminome-seq (Castandet et al., 2019).

The activities of both NEP and PEP increase considerably with chloroplast development. However, PEP activity undergoes a more pronounced increase than NEP activity. In mature chloroplasts, PEP serves as the major transcriptional machinery and produces more than 80% of all primary plastid transcripts (Cahoon et al., 2004; Zhelyazkova et al., 2012; Kindgren and Strand, 2015). The activities of NEP and PEP are much higher in young leaves than in old leaves, although old leaves still maintain substantial chloroplast transcription activity (Zoschke et al., 2007). Although NEP and PEP are still active in plastids of non-photosynthetic tissues, their activities are much lower compared with that in mature chloroplasts of photosynthetic tissues (Puthiyaveetil et al., 2021).

During evolution, chloroplasts of land plants inherited the PEP catalytic core from the ancestral cyanobacterium but lost all genes encoding transcription factors, several σ factors, and nucleoid-associated proteins (e.g., the HU protein that was first named the U factor in *Escherichia coli* strain U93 and later renamed HU because the U factor shared many characteristics with eukaryotic histones) (Drlica and Rouviere-Yaniv, 1987; Yagi and Shiina, 2014). With the acquisition of novel host cell–derived polymerase-associated proteins (PAPs), the PEP transcription apparatus, or PEP complex, of land plants became more complicated. In addition to transcriptional initiation, transcriptional pausing by the PEP complex has an essential role in transcriptional output. Transcription by the PEP complex is also highly induced by light, which helps chloroplasts synchronize their gene expression program to support the needs of photosynthesis. We focus below on our current understanding of the PEP complex, highlighting PAP functions, transcriptional pausing, and light-induced transcription.

The PEP complex is a hybrid system with a prokaryotic core and peripheral PAPs of eukaryotic origin

The PEP catalytic core is composed of α, β, β′, and β″ subunits that are encoded by the plastid genes *rpoA*, *rpoB*, *rpoC1,* and *rpoC2*, respectively. Similar to bacterial RNA polymerase, a σ factor is required for PEP to recognize its target promoters harboring the –35 and –10 motifs. Plant σ factors belong to the bacterial σ^{70} family and contain all four conserved σ domains also found in bacterial σ factors. In land plants, genes encoding σ factors were transferred to the nucleus and duplicated several times, yielding many nuclear copies (Fu et al., 2021). For example, there are as many as six σ factors in Arabidopsis (*Arabidopsis thaliana*), named SIG1 to SIG6 (Macadlo et al., 2020; Puthiyaveetil et al., 2021).

However, distinct from bacterial RNA polymerase, PEP has acquired many PAPs for executing RNA biosynthesis. Twelve PAPs have been identified in Arabidopsis chloroplasts, PAP1 to PAP12 (Steiner et al., 2011; Pfannschmidt et al., 2015). These PAPs bind specifically and tightly to the Rpo catalytic core, forming well-conserved protrusions as in other RNA polymerases, although the 3D map resolution of the PEP complex is currently insufficient to determine the relative positions of these PAPs within the PEP complex (Ruedas et al., 2022). All these PAPs are necessary for chloroplast transcription and show a low level of functional redundancy. Loss of function of each PAP results in similar phenotypes: a severe block of chloroplast transcription and an albino (cells devoid of chlorophyll) phenotype, resembling that seen in knockout mutants of chloroplast *rpo* genes (Pfalz et al., 2006; Garcia et al., 2008; Myouga et al., 2008; Arsova et al., 2010; Schroter et al., 2010; Steiner et al., 2011; Gao et al., 2011; Yagi et al., 2012; Chang et al., 2017).

Limited functional information is available on each PAP based on experimentation. PAP5, also named PLASTID TRANSCRIPTIONALLY ACTIVE 12 (pTAC12) and HEMERA, can bind to single-stranded RNA and single-stranded DNA, suggesting that PAP5 may localize close to the active sites of the PEP complex and facilitate the positioning of tethered DNA and/or RNA (Pfalz et al., 2015). PAP5 and PAP3/pTAC10 are also required for formation and/or stability of the entire PEP complex (Pfalz et al., 2015; Chang et al., 2017). PAP8/pTAC6 contains an RNA-binding motif

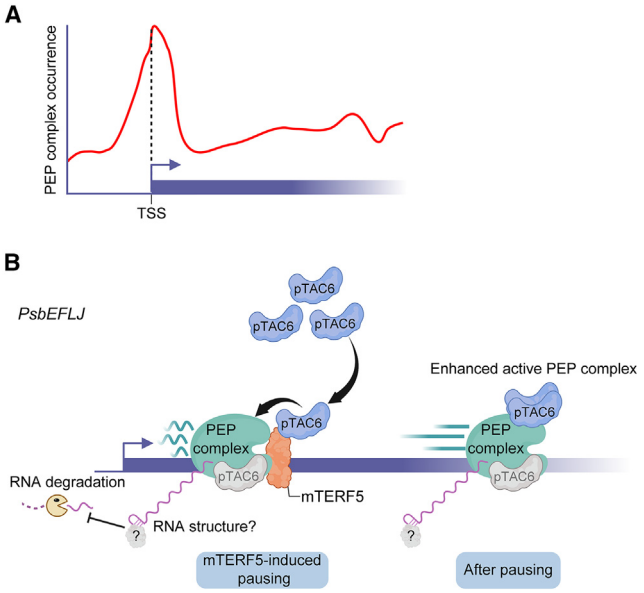

Figure 1. Proposed model for the function of mTERF5-induced pausing and preferential binding of the PEP complex to chloroplast promoters.

(A) PEP complex distribution as a function of position along chloroplast genes. The model for PEP occurrence was redrawn according to Palomar et al. (2022). TSS, transcription start site.

(B) A working model for mTERF5-induced pausing in regulating *psbEFLJ* transcription is proposed based on Ding et al. (2019) and Meteignier et al. (2020). The PEP complex represents the major transcriptional machinery in chloroplasts and is composed of the core enzyme and PAPs. PAP8/pTAC6 is one of 12 PAPs. mTERF5 serves as a transcriptional pausing factor and induces PEP pausing at the *psbEFLJ* polycistron by binding to the +30 to +51 region relative to the transcription start site of this gene. mTERF5 interacts with pTAC6 outside of the PEP complex and helps recruit additional pTAC6 proteins into the PEP complex to enhance its activity, thereby positively regulating *psbEFLJ* transcription. In addition, transcriptional pausing caused by mTERF5 may provide time and location for the action of unknown RNA-binding proteins or the folding of nascent RNA, thus improving RNA stability.

homologous to a rhinoviral RNA-dependent RNA polymerase and shows RNA-binding activity *in vitro*, suggesting a role in handling nascent RNA from the PEP catalytic core (Chambon et al., 2022). PAP10/THIOREDOXIN Z contains a thioredoxin domain; together with its interacting protein PAP6/FRUCTOKINASE-LIKE 1, PAP10 is thought to confer redox regulation of transcriptional activity (Arsova et al., 2010; Schroter et al., 2010; Diaz et al., 2018; Yang et al., 2021). Two other paralogous proteins with superoxide dismutase domains, PAP4/FE SUPEROXIDE DISMUTASE 3 and PAP9/FE SUPEROXIDE DISMUTASE 2, are implicated in protecting the PEP complex from reactive oxygen species produced by the photosynthetic apparatus (Myouga et al., 2008; Favier et al., 2021). Crystal structure analysis of recombinant PAP9 showed a fold similar to that of iron or manganese superoxide dismutases, although its catalytic center is coordinated with a zinc ion (Favier et al., 2021).

Transcriptional pausing exists in chloroplasts

In bacteria and metazoans, RNA synthesis by RNA polymerase is discontinuous. RNA polymerase tends to pause (from several seconds to minutes or even longer) at specific DNA regions, a phenomenon termed "transcriptional pausing" (Core and Adelman, 2019; Wissink et al., 2019). Transcriptional pausing is a major regulatory mechanism for transcription, as it allows time for paused RNA polymerases to make regulatory decisions at specific genomic locations and thereby modulate transcriptional output (Landick, 2021; Noe Gonzalez et al., 2021).

In metazoans, pausing of RNA polymerase II (Pol II) at promoter-proximal positions is a key rate-limiting step in transcriptional output and an essential point at which opposing or reinforcing signals are integrated (Core and Adelman, 2019). Promoter-proximal pausing by RNA Pol II also controls gene expression by opening chromatin architecture in the vicinity of promoters (Core and Adelman, 2019). Transcriptional pausing is also implicated in diverse gene regulatory mechanisms such as transcription–translation coupling in bacteria (Larson et al., 2014), splicing (Akhtar et al., 2019), RNA folding (Muniz et al., 2021), and genome stability (Saponaro et al., 2014; Lavigne et al., 2017). In Arabidopsis, RNA Pol II in the nucleus pauses at approximately 20%–40% of protein-coding genes, and pausing both over promoter-proximal regions and after the polyadenylation site influences transcriptional efficiency (Hetzel et al., 2016; Zhu et al., 2018; Kindgren et al., 2020).

Transcriptional pausing has also been examined in Arabidopsis chloroplasts. A global run-on analysis suggested that transcriptional pausing is a widespread phenomenon that occurs at about 30% of all chloroplast genes (Zhu et al., 2018; Ding et al., 2019). This conclusion was supported by a high-resolution map of PEP binding patterns along chloroplast genes, which showed that PEP is frequently non-uniformly distributed, with a tendency to accumulate in close proximity to and downstream of transcription start sites (Palomar et al., 2022). This result indicated that the early elongation of the PEP complex is more prone to pause over promoter-proximal regions than to move forward further downstream (Figure 1A). However, the mechanism that establishes this widespread promoter-proximal pausing is unclear. Moreover, decreasing transcriptional initiation of the PEP complex by knocking out *SIG2* or *SIG6* results in an overall reduction in PEP occurrence both over promoter-proximal regions and further downstream. This observation indicates that PEP pausing is tightly coupled with transcriptional elongation (Chi et al., 2015; Palomar et al., 2022) and suggests that it might generally regulate transcriptional output in chloroplasts.

Transcriptional pausing in chloroplasts can be regulated by proteinaceous *trans*-factors in a gene-specific manner. MITOCHONDRIAL TRANSCRIPTION TERMINATION FACTORs (mTERFs) were originally identified in mitochondria of metazoans. However, the mTERF family has expanded during the evolution of land plants, and its members are localized to mitochondria and/or chloroplasts (Kleine, 2012). mTERF5/MTERF DEFECTIVE IN ARABIDOPSIS1 is a member of the mTERF family that induces the PEP complex to pause at the *psbEFLJ* locus by binding to the +30 to +51 region relative to the transcription start site. mTERF5 also helps to recruit PAP8/pTAC6, an essential subunit of the PEP complex, into the paused PEP complex, yielding an enhanced PEP complex that may promote transcriptional elongation and thus positively regulate *psbEFLJ* transcription (Figure 1B) (Ding et al., 2019). In addition, stabilization of the 5′ ends of processed *psbE* RNA is clearly lower in *mterf5* mutants, suggesting that transcriptional pausing caused

by mTERF5 also regulates RNA stability (Meteignier et al., 2020). Considering that RNA stability is often maintained by RNA-binding proteins, mTERF5-induced pausing may facilitate the action of unknown RNA-binding proteins to improve the stability of *psbE* RNA. Overall, transcriptional pausing in chloroplasts represents an important point for the recruitment of regulatory proteins and for coupling transcription and post-transcriptional processes, such as RNA stability.

The PEP complex is activated by light-induced redox changes

Light is the major driver initiating transcription in chloroplasts of land plants. In the nucleus, light promotes the expression of genes encoding PAPs through histone H4 acetylation by the nucleosome acetyltransferase of the H4 complex at the early stage of light signaling (Barrero-Gil et al., 2022). In addition, light activates the formation of the PEP complex by eliminating PHYTOCHROME-INTERACTING TRANSCRIPTION FACTORs, which in turn synchronously generate a nucleus-to-plastid signal involved in σ factors (Yang et al., 2019; Yoo et al., 2019; Hwang et al., 2022). Thus, formation of the PEP complex is strongly controlled by light.

In mature chloroplasts, light globally activates the association of the full PEP complex with promoters to start transcription (Finster et al., 2013). PLASTID REDOX INSENSITIVE2 (PRIN2) is a key component required for PEP complex activation in response to light. PRIN2 is a redox-regulated protein that localizes to chloroplasts (Kindgren et al., 2012) and forms a dimer via its Cys68–Cys68 disulfide bond in the dark. Exposure to light activates photosynthetic electron transport and triggers the reduction of thioredoxins within chloroplasts. Thioredoxins convert the PRIN2 dimer into the active PRIN2 monomeric form. PRIN2 monomers then activate the full activity of the PEP complex, potentially via their interaction with PAP10/THIOREDOXIN Z (Diaz et al., 2018). These findings highlight a signal transduction pathway that connects light-derived electron transport in the thylakoids to activation of the PEP complex. In addition, the activity of the PEP complex and of σ factors is tightly controlled by phosphorylation events that are mediated by redox-regulated plastid transcription kinases (Baginsky et al., 1999; Schweer et al., 2010; Türkeri et al., 2012; Ibrahim et al., 2020; Puthiyaveetil et al., 2021). The redox status of the plastoquinone pool affects the phosphorylation of SIG1, which in turn regulates transcription of the photosystem reaction center genes *psaA/B* and *psbA* (Shimizu et al., 2010). Hence, light-induced modification via phosphorylation of the PEP complex and σ factors may represent another mechanism by which transcription can be initiated in the light. Because mature chloroplasts have completed the biogenesis of the PEP complex, light-induced redox changes in chloroplasts would play a major role in starting or impeding transcription upon diurnal cycles, whereas light signaling in the nucleus is necessary for initiating chloroplast biogenesis and maintaining turnover of the PEP complex.

POST-TRANSCRIPTIONAL ALTERATIONS OF CHLOROPLAST RNAs

In cyanobacteria, gene expression is regulated mainly at the level of transcriptional initiation, whereas post-transcriptional control is more prominent in the chloroplasts of land plants. Primary chloroplast transcripts are produced from polycistronic operons and undergo extensive processing (e.g., 5′ and 3′ trimming, as well as intercistronic cleavage). In addition, over the course of chloroplast evolution, two eukaryotic features were recruited to chloroplasts: the acquisition of introns and the modification of specific nucleotides within RNA molecules, which is termed RNA editing (Germain et al., 2013). To accommodate these new features, numerous nucleus-encoded RNA-binding proteins were acquired in the context of nucleus–chloroplast coevolution, opening up new possibilities for more precise regulation of chloroplast genes and offering greater flexibility for chloroplast biogenesis and plant adaptation.

Intercistronic cleavage and terminal RNA processing

A particularly pronounced feature of chloroplast gene expression in land plants is the great complexity of mature RNA isoforms arising from polycistronic transcript processing by ribonucleases. The chloroplast contains two types of ribonucleases: exonucleases and endonucleases (Stoppel and Meurer, 2012). Similar to those of bacteria, exonucleases in chloroplasts remove successive nucleotides from the end of an RNA molecule in either the 5′→3′ or 3′→5′ direction, whereas endonucleases carry out intercistronic RNA cleavage, yielding discrete RNA fragments. To date, three chloroplast exonucleases have been identified in Arabidopsis: RNase J with 5′→3′ specificity and RNase R and polynucleotide phosphorylase with 3′→5′ specificity (Stoppel and Meurer, 2012; Manavski et al., 2018). Among the endonucleases, RNase E and RNase J are the most likely to execute internal RNA cleavage (Stern et al., 2010; Barkan, 2011). PROTEINACEOUS RNASE P and RNase Z (TRZ2) are required for maturation of tRNAs (Stoppel and Meurer, 2012). CHLOROPLAST STEM-LOOP BINDING PROTEIN OF 41 kDa a and b (CSP41a and CSP41b) are unique RNA-binding endoribonucleases in photosynthetic organisms. Arabidopsis CSP41 complexes are also thought to stabilize non-translated target mRNAs and precursor rRNAs in the absence of light (Qi et al., 2012; Leister, 2014). Two miniribonuclease III members (RNC3 and RNC4) cleave the 23S−4.5S rRNA precursor to produce the mature 5′ end of the 23S RNA and the 3′ end of the 4.5S RNA (Hotto et al., 2015). As with ribonucleases in other organisms, the substrate preferences of these chloroplast ribonucleases are determined largely by RNA structure and less by RNA sequence (Stern et al., 2010; Manavski et al., 2018).

Intercistronic processing of primary transcripts is initiated by chloroplast endonucleases (such as CSP41 and RNases E and J) cleaving the RNA in unprotected and unstructured regions. Next, the resulting 5′ and 3′ termini are trimmed by exonucleases. To form proper 5′ termini, the 5′ ends of chloroplast transcripts are predominantly protected by RNA protectors that serve as barriers against 5′→3′ exonucleases. By contrast, the 3′ ends of chloroplast transcripts are often protected by secondary structures in the 3′ untranslated region (UTR) of RNAs formed by short-inverted repeat sequences (Figure 2). In particular, most RNA protectors that act on chloroplast transcripts are pentatricopeptide repeat (PPR) proteins, which passively bind to terminal RNA regions to prevent their degradation by exonucleases. Typical PPR proteins harbor repeating 35-amino-acid motifs that present a long superhelical RNA-binding surface, whereby the exact sequence of each PPR repeat dictates the

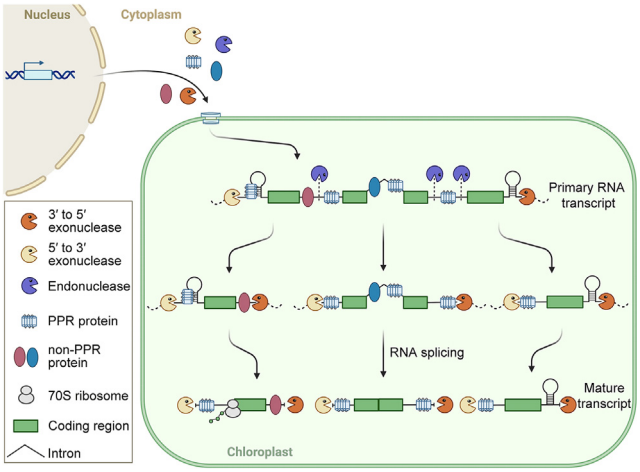

Figure 2. Model for intercistronic cleavage and termini processing of chloroplast RNAs.
In chloroplasts, primary RNAs are cleaved by endonucleases (such as RNases E and J, CSP41) often in intergenic regions, giving rise to new RNA precursors. The 5′ and 3′ ends of chloroplast RNAs are protected against degradation by exonucleases via the specific binding of RNA protectors, such as PPR proteins, and/or stem–loop RNA structures. Moreover, those factors bound to 5′ termini can also remodel RNA structures, which in turn stimulates translation initiation.

nucleotide to which it will bind (the one-repeat:one-nucleotide binding mode) (Barkan et al., 2012; Yin et al., 2013; Shen et al., 2016). PPR proteins bind to the 5′ ends of transcripts to prevent 5′→3′ exonuclease invasion and sometimes also compete with stem–loop structures and release ribosomal-binding sites (i.e., the Shine–Dalgarno [SD] sequence). For instance, SUP PRESSOR OF THYLAKOID FORMATION 1 in Arabidopsis and its ortholog PPR53 in maize (*Zea mays*) stabilize the 5′ ends of the 23S precursor RNA and *ndhA* (Wu et al., 2016; Zoschke et al., 2016; Zhou et al., 2017; Li et al., 2021). In addition, some PPR proteins also protect and define the 3′ ends of transcripts that lack short inverted repeat sequences (Figure 2). For example, PPR10 binds to the intergenic regions of *atpI–atpH* and *psaJ–rpl33* transcripts and serves as a barrier against RNA degradation from either the 5′ or 3′ direction (Pfalz et al., 2009). The PPR protein CHLOROPLAST RNA PROCESSING 1 cooperates with the S1-domain-containing RNA chaperone PETB/PETD STABILIZING FACTOR to bind to the intergenic regions of *petB–petD*, stabilizing the 3′ ends of *petB* transcripts and promoting *petD* translation (Jiang et al., 2019).

To date, numerous PPR and non-PPR protectors have been found in chloroplasts, most of them specific to land plants, reflecting tight nucleus–chloroplast coevolution since the initial endosymbiosis event. A summary of known chloroplast RNA protectors, including their preferential targets and classification of their domains, is given in Table 1.

RNA splicing

RNA splicing is an essential step for chloroplast gene expression in which removal of intronic sequences from precursor RNAs and connection of the remaining sequences produces mature RNAs and tRNAs with correct genetic information. The chloroplast genomes of land plants have 17–21 introns. Based on their conserved sequences and splicing mechanisms, most chloroplast introns can be classified as group II introns, with only one group I intron in *trnL* (de Longevialle et al., 2010; Germain et al., 2013).

All of these introns have lost the ability to self-splice owing to degeneration of RNA structures and loss of intron-encoded maturases over the course of evolution. Efficient splicing of these degenerated chloroplast introns must be stimulated by a set of additional proteins. Among chloroplast introns, only the *trnK* intron encodes the maturase MatK that is involved in splicing of several group IIA introns (Zoschke et al., 2010). Besides this intron-coded MatK, many nucleus-encoded splicing factors have been identified that localize to the chloroplast, such as the RNase III-domain protein RNC1 of maize (Watkins et al., 2007), the plant organelle RNA recognition protein what's this factor (WTF1), also from maize (Kroeger et al., 2009), and ACCUMULATION OF PHOTOSYSTEM ONE 1 from maize and Arabidopsis (Watkins et al., 2011). A summary of chloroplast splicing factors can be found in several detailed reviews (de Longevialle et al., 2010; Schmitz-Linneweber et al., 2015; Wang et al., 2022; Zeng et al., 2022).

Chloroplast RNA splicing and ribosome maturation (CRM) proteins are more general splicing factors and include CRS2-associated factor 1 (CAF1) and CAF2, chloroplast RNA splicing 1 (CRS1), and CRM family member 2 (CFM2) and CFM3 (de Longevialle et al., 2010). CAF1/2 bind to CRS2 to form complexes. CRS2–CAF1 complexes are necessary for the splicing of seven introns (*ycf3* intron 1, *trnG*, *rpl16*, *petD*, *rps16*, *clpP* intron 1, and *rpoC1*), and CRS2–CAF2 complexes are required for the splicing of five introns (*ycf3* intron 1, *ndhA*, *ndhB*, *petB*, and *rps12* intron 1) (Ostheimer et al., 2003; Asakura and Barkan, 2006). Because CRS2–CAF1/2 complexes are required for different sets of group IIB introns with only one overlapping intron substrate, *ycf3* intron 1, there must be some recognition mechanism by which CRS2–CAF1/2 complexes target distinct introns. The PPR protein PHOTOSYSTEM I BIOGENESIS FACTOR2 interacts with CAF1 and CAF2 and may contribute to the specificity of the CRS2–CAF1 and CRS2–CAF2 complexes toward *ycf3* intron 1 (Wang et al., 2020). A similar mode of action was also reported for the PPR protein EMBRYO DEFECTIVE 1270 (EMB1270), which appears to be required for the splicing of *clpP1* intron 2, *ycf3* intron 1, *ndhA*, and *ndhB* through its specific interaction with the splicing factor CFM2 (Zhang et al., 2021). Considering that PPR proteins bind to RNA in a sequence-specific manner, interactions between specific PPR proteins and general splicing factors such as CAF1/2 and CFM2 may help target their constituent complexes to the corresponding introns to form the full splicing complexes.

Distinct from other chloroplast introns, *rps12* intron 1 is the only intron that is *trans*-spliced in land plants, meaning that two partial *rps12* transcripts transcribed from different parts of the chloroplast genome are spliced together into one functional RNA. The *rps12* intron 1 is split into *rps12* introns 1a and 1b; *rps12* intron 1a lies between *rpl20* and *clpP1,* whereas *rps12* intron 1b is approximately 30 kb away and co-transcribed with *ndhB* and *rps7* (Glanz and Kück, 2009). Thus, the splicing of *rps12* intron 1 is more complex than that of other *cis*-splicing introns, requiring the *rps12* precursors to be cleaved and brought together prior to regular splicing. The PPR proteins

Name	Organism	Domain	Target(s)	Reference(s)
AtBFA2	*Arabidopsis thaliana*	PPR	*atpF–atpA* IGR	Zhang et al., 2019
AtCRP1; ZmCRP1	*A. thaliana*; *Zea mays*	PPR	*petB–petD* IGR; *psaC* 5′ UTR; *petA* 5′ UTR	Ferrari et al., 2017; Fisk et al., 1999; Schmitz-Linneweber et al., 2005
AtHCF152	*A. thaliana*	PPR	*psbH–petB* IGR; *petB* 5′ UTR; *psbH* 3′ UTR	Nakamura et al., 2003, 2004
AtPGR3; ZmPGR3	*A. thaliana*; *Z. mays*	PPR	*petL* 5′ UTR; *rpl14–rps8* IGR; *ndhA* 5′ UTR	Cai et al., 2011; Rojas et al., 2018; Yamazaki et al., 2004
AtPPR5; ZmPPR5	*A. thaliana*; *Z. mays*	PPR	*trnG* (UCC) intron	Beick et al., 2008
AtSVR7; ZmATP4	*A. thaliana*; *Z. mays*	PPR	*atpF–atpA* IGR; *psaJ–rpl33* IGR; *atpB/E* 5′ UTR	Zoschke et al., 2012, 2013b
ZmPPR10	*Z. mays*	PPR	*atpH* 5′ UTR; *psaJ* 3′ UTR	Pfalz et al., 2009; Prikryl et al., 2011
PpPPR_21	*Physcomitrium patens*	PPR	*psbI–ycf12* 5′ UTR	Ebihara et al., 2019
PpPPR_38	*P. patens*	PPR	*clpP–rps12* IGR	Hattori et al., 2007; Hattori and Sugita, 2009
AtCRR2	*A. thaliana*	PLS-class PPR-DYW	*rps7-ndhB* IGR	Hashimoto et al., 2003
AtEMB175; ZmPPR103	*A. thaliana*; *Z. mays*	PLS-class PPR-DYW	*rpl16* 5′ UTR	Hammani et al., 2016
AtSOT1; ZmPPR53	*A. thaliana*; *Z. mays*	PPR-SMR	*rrn23* 5′ end; *ndhA* 5′ UTR	Li et al., 2021; Wu et al., 2016; Zhou et al., 2017; Zoschke et al., 2016
AtBSF; ZmBSF	*A. thaliana*; *Z. mays*	S1 domain	*petB–petD* IGR; *petA* 5′ UTR	Jiang et al., 2019
28RNP	*Spinacea oleracea*	RRM motif	*psbA, rbcL, petD, rps14* 3′ UTRs	Schuster and Gruissem, 1991
AtCP31A	*A. thaliana*	RRM motif	3′ ends of *psbB*, *psbD*, *psaA/B*, *atpB*, *ndhB*, and *ndhF*	Kupsch et al., 2012; Tillich et al., 2009
AtCP29A	*A. thaliana*	RRM motif	3′ ends of *psbB*, *psbD*, *psaA/B*, *atpB*, and *ndhB*	Kupsch et al., 2012
AtCP33A	*A. thaliana*	RRM motif	multiple transcripts in chloroplasts	Teubner et al., 2017
AtHCF107; ZmHCF107	*A. thaliana*; *Z. mays*	HAT (half a TPR)	*psbH* 5′ UTR	Hammani et al., 2012; Sane et al., 2005
AtHCF145; PpHCF145	*A. thaliana*; *P. patens*	TMR domain; SRPBCC domain	*psaA* 5′ UTR	Lezhneva and Meurer, 2004; Manavski et al., 2015
AtPrfB1/AtHCF109	*A. thaliana*	ribosomal release factor; SPF and GGQ motifs	transcripts with UGA stop codons	Meurer et al., 2002
AtPrfB3	*A. thaliana*	homologous to PrfB1	*petB* 3′ UTR	Stoppel et al., 2011

Table 1. Summary of known chloroplast RNA protectors and their targets.
IGR, intergenic region

EMBRYO DEFECTIVE 2654 and PPR4 bind to the 3′ end of *rps12* intron 1a and the 5′ end of *rps12* intron 1b, respectively. Their binding was proposed to be an early event that is a prerequisite for the proper folding of domain 3 of *rps12* intron 1, which is ready for the formation of the splicing complex via general factors such as the CRS2–CAF2 complexes (Schmitz-Linneweber et al., 2006; Aryamanesh et al., 2017; Lee et al., 2019).

Chloroplasts have a rather small genome. However, numerous splicing factors are required for chloroplast gene expression. RNA splicing in plant chloroplasts appears to be particularly complex. Compared with those in their prokaryotic ancestors, introns in chloroplasts are newly invented and no longer have the ability to self-splice (Wang et al., 2022). In order to stimulate self-splicing of introns, numerous splicing factors may have arisen to compensate for the degenerated chloroplast introns in the

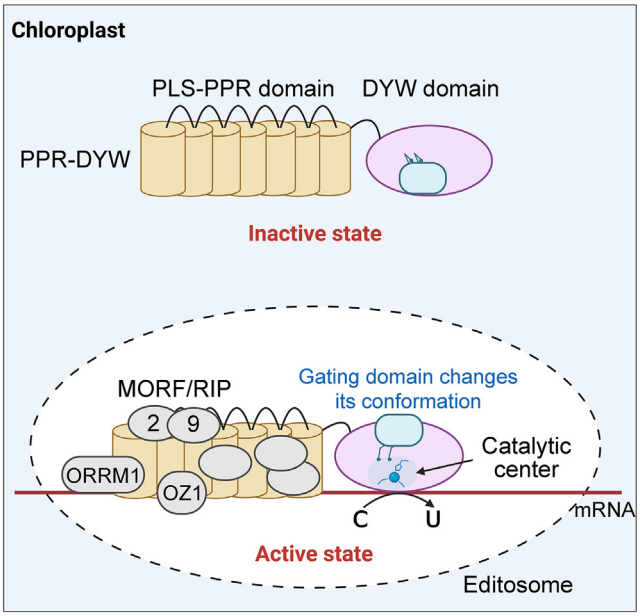

Figure 3. Proposed model for C-to-U RNA editing in the chloroplasts of flowering plants.

The DYW domain alone tends to be in an inactive state, with a catalytic center closed by the gating domain. In editosomes, the deaminase activity of DYW is activated, which is accompanied by changes in the conformation of its gating domain and repositioning of the catalytic center architecture. In chloroplasts, the editosome is a large multi-protein complex that contains PLS-class PPR specificity factors and many non-PPR editing factors, such as MORF/RIP, ORRM1, and OZ1. Among these factors, MORF proteins can increase the RNA-binding activity of PLS-type PPRs, thus helping the editosome bind to the sequence upstream of its cognate target site.

context of nucleus–chloroplast coevolution. It remains unknown why so many splicing factors have arisen in plant chloroplasts. Presumably, these splicing factors may be required for plant growth and development and are regulated in response to environmental stresses.

The DYW domain is the catalytic domain for C-to-U editing in plants

In plants, RNA editing mainly involves deamination reactions that target specific cytidines (Cs) in organellar RNAs and convert them to uridines (Us). This phenomenon is nearly omnipresent in land plants but entirely absent in green algae (Takenaka et al., 2013; Yan et al., 2018; Small et al., 2020). Typical flowering plants edit 20–60 sites in their chloroplast transcripts, mostly located in protein-coding transcripts with only a few sites in tRNA, introns, non-coding regions, or UTRs (Ichinose and Sugita, 2016; Edera et al., 2018). These C-to-U editing events are necessary for the proper function of organellar proteins and optimal plant growth; they change the coding sequences of RNA, introducing translation start sites (AUG) and modulating secondary structures that influence the stability and/or splicing of RNAs (Shikanai, 2015; Small et al., 2020).

PLS-class PPR proteins are considered to be the core components for execution of RNA editing. They are distinguished from the typical PPR (P-class) proteins because they contain characteristic repeating triplets of P, L (long variants of P), and S (short variants of P) motifs (Lurin et al., 2004; Cheng et al., 2016). Approximately half of all PLS-class proteins harbor a C-terminal DYW domain, which was named after its conserved Asp-Tyr-Trp (D-Y-W) triplet residues (Lurin et al., 2004). Sequence alignments have demonstrated that the DYW domain exhibits some similarity to known cytidine deaminases from other kingdoms and contains a conserved $HxE(x)_nCxxX$ catalytic-site signature, suggesting that the DYW domain might be the deaminase catalytic entity of the organellar RNA editing complex (Salone et al., 2007; Schallenberg-Rüdinger et al., 2013). The PPR-DYW protein PPR56 from the moss *Physcomitrium patens* can execute effective C-to-U editing of its corresponding target *ccmFC* transcript on its own without additional cofactors (Oldenkott et al., 2019; Hayes and Santibanez, 2020). Considering the definitive function of PLS-class PPR domains in RNA substrate recognition, these findings show that the DYW domain serves as a cytidine deaminase during RNA editing.

Recently, the high-resolution structure of a DYW domain was resolved from the chloroplast editing factor ORGANELLE TRANSCRIPT PROCESSING 86 ($OTP86^{DYW}$) of Arabidopsis (Takenaka et al., 2021). Unlike deaminases in other organisms, the deaminase fold of $OTP86^{DYW}$ is interrupted by an inserted gating domain, which restrains entry of the substrate cytidine into the catalytic site. Upon activation, the $OTP86^{DYW}$ gating domain alters its conformation and triggers the repositioning of the active site architecture, which recruits a water molecule as a fourth ligand between a zinc ion and the catalytic residue E894 to attack the cytidine for deamination (Takenaka et al., 2021). In this way, the DYW domain alone tends to remain in an autoinhibited state, and its catalytic ability is only activated after transport into the organelle (Figure 3). This autoinhibition mechanism of DYW proteins would offer protection for RNAs in the cytosol from the damage of off-target editing events. In addition, the deaminase activity exerted by the DYW domain exhibits a different preference for the nucleotides close to the RNA editing sites, indicative of a complicated interplay between RNA substrate elements and the DYW domain during RNA editing (Maeda et al., 2022). In addition to canonical C-to-U editing, "reverse" editing (U-to-C) was also detected in the organelles of three land plant clades (hornworts, lycophytes, and ferns) (Small et al., 2020). This U-to-C editing is phylogenetically correlated with a subgroup of DYW domains, referred to as DYW:KP, which display a conserved Lys-Pro (KP) dipeptide at the start of the domain (Gerke et al., 2020; Gutmann et al., 2020). Designer DYW:KP proteins can exhibit U-to-C editing activity in bacteria and human cell cultures (Ichinose et al., 2022). This result raises the interesting question of how the relatively minor sequence differences between DYW:KP and the canonical DYW domain can lead to a complete reversal of a theoretically irreversible reaction. Based on the structures of $OTP86^{DYW}$, we hypothesize that minor differences in amino acid sequences between DYW:KP and canonical DYW domains affect the catalytic pocket to accept the presence of an amine acceptor or donor and execute the C-to-U deaminase or "reverse" reaction.

In addition to PPR-DYW proteins, many essential non-PPR editing factors have been acquired by flowering plants, such as RNA-EDITING FACTOR INTERACTING PROTEIN 1/MULTIPLE ORGANELLAR RNA EDITING FACTOR (MORF), ORGANELLE

RNA RECOGNITION MOTIF PROTEIN, the RNA helicase INCREASED SIZE EXCLUSION LIMIT 2, and ORGANELLE ZINC FINGER proteins (Takenaka et al., 2012; Sun et al., 2013, 2015; Bobik et al., 2017; Gipson et al., 2022). These PPR factors and non-PPR editing factors form diverse and large multi-protein complexes, also termed editosomes, that perform RNA editing (Sun et al., 2016; Sandoval et al., 2019). Among the non-PPR editing factors, MORF9 binds to the L motif close to the concave surface of the PLS triplet and therefore alters the angle between two helices of the L motif, ultimately leading to considerable compression of the PPR superhelix (Haag et al., 2017; Yan et al., 2017; Yang et al., 2018). This compressed conformational change by MORF9 markedly increases RNA-binding activity by PLS-type PPR proteins (Figure 3) (Yan et al., 2017; Royan et al., 2021). However, our understanding of the roles of other non-PPR components within editosomes remains rudimentary.

Plastid non-coding RNAs

Plastid non-coding RNAs (pncRNAs) range in length from 15 nt to >3 kilonucleotides (knt) and are abundant in chloroplasts (Hotto et al., 2011). The majority of pncRNAs derive from intergenic, intronic, and antisense regions and seem to be generated by readthrough from the adjacent genes followed by complicated RNA processing events. A small portion of pncRNAs are thought to be primary transcripts controlled by their own promoters (Zhelyazkova et al., 2012; Castandet et al., 2019; Anand and Pandi, 2021). Ribonucleases are involved in the biogenesis of pncRNAs. The chloroplast 3′→5′ exonuclease polynucleotide phosphorylase is required for the 3′ end maturation of many pncRNAs (Germain et al., 2011; Hotto et al., 2011). Loss of the 3′→5′ exonuclease RNase R results in almost complete deletion of both sense and antisense (as) *psbK* RNAs as well as a considerable decrease in AS5 asRNA. Virus-induced gene silencing of RNase J causes an accumulation of nc-*rbcL*-*accD*int precursor (a pncRNA corresponding to *rbcL* and *accD* intergenic regions) and minor alterations for other pncRNAs. RNase E stabilizes the accumulation of *aspsbK* and nc-*rbcL*-*accD*int RNAs (Hotto et al., 2011). Some PPR proteins, such as PPR10 and SUPPRESSOR OF THYLAKOID FORMATION 1/PPR53, are involved in generating chloroplast small pncRNAs (sRNAs), also termed "PPR footprints" (Pfalz et al., 2009; Wu et al., 2016; Zoschke et al., 2016).

pncRNAs play a role in regulating various aspects of chloroplast gene expression at the post-transcriptional level. The AS5 asRNA is antisense to the chloroplast *rrn5*–*trnR* region and may have a regulatory role in 5S rRNA maturation (Hotto et al., 2011; Sharwood et al., 2011). A long transcript antisense to *ndhB* is suggested to regulate *ndhB* RNA editing or stability (Georg et al., 2010). asRNAs for *psbT* may interact with *psbT* mRNA to prevent translation of *psbT* and protect *psbT* mRNA from nucleolytic degradation (Zghidi-Abouzid et al., 2011). In addition, a systematic analysis of chloroplast sRNAs indicates that chloroplast sRNAs might serve as competitors of mRNAs for PPR proteins to regulate RNA metabolism (Ruwe et al., 2016).

CHLOROPLAST TRANSLATION

In chloroplasts, translation is performed by a bacterial-type 70S ribosome that is composed of a 50S large subunit and a 30S small subunit. The chloroplast genome encodes a full set of tRNAs, all rRNAs, one quarter of all proteins comprising the 50S large subunit, and approximately half of all proteins forming the 30S small subunit. The remaining ribosomal subunits are encoded by the nuclear genome. Although the chloroplast and bacterial 70S ribosomes share a common ancestor, their structures and operational mechanisms have diverged during evolution. In addition, chloroplast translation is highly regulated by light and coordinated with the thylakoid membrane system to achieve optimum photosynthetic performance. Here, we discuss the variations in ribosomes from bacteria to chloroplasts, translation dynamics, and the pathways that activate translation in response to light.

Differences between chloroplast and bacterial ribosomes

The structure of chloroplast ribosomes is generally similar to that of bacterial 70S ribosomes but has some distinct features (Zoschke and Bock, 2018). Chloroplast ribosomes contain a 4.5S rRNA in addition to a complete set of sequences homologous to bacterial rRNAs (23S, 16S, and 5S rRNA) with analogous functions. The 23S rRNA confers peptidyl transferase activity, and the 16S rRNA comprises the decoding center and acts as a scaffold during ribosome assembly (Shajani et al., 2011). An equivalent to chloroplast 4.5S rRNA does not exist in bacteria, but the chloroplast 4.5S rRNA is homologous to the 3′ terminus of the 23S rRNA, suggesting that the 4.5S rRNA may have arisen from fragmentation of the 23S rRNA during evolution (Edwards and Kössel, 1981; Maier et al., 2013). In addition, the chloroplast 23S rRNA is post-transcriptionally cleaved at two "hidden break" sites after assembly of the 50S subunit, generating three distinct fragments of ~0.5, 1.2, and 1.1 kb (from the 5′ to the 3′ end). These "hidden break" cleavages are required for chloroplast translation and are produced by specific enzymes such as RNA helicase RH39 (Nishimura et al., 2010; Bieri et al., 2017).

The protein composition of the chloroplast ribosome has also changed over the course of evolution since the initial endosymbiosis. The chloroplast ribosome lacks equivalents to the bacterial large ribosomal protein subunits Rpl25 and Rpl30. All orthologs of the *E. coli* ribosome are present in the chloroplast 70S ribosome but exhibit N- and C-terminal chloroplast-specific extensions compared with their bacterial counterparts (Yamaguchi and Subramanian, 2000; Ahmed et al., 2016). These extensions substantially reshape the mRNA entry and exit sites on the 30S small subunit as well as the polypeptide tunnel exit and the binding site of the signal recognition particle on the 50S small subunit (Bieri et al., 2017; Perez Boerema et al., 2018). These changes reflect specific chloroplast adaptations for translation initiation and protein targeting.

Chloroplasts also have several new components with no clear *E. coli* homologs, designated as plastid-specific ribosomal proteins (PSRPs) 1–6. PSRP1 was first identified as the ortholog of the bacterial cold-shock protein pY (Sharma et al., 2007, 2010), but a 2.9- to 3.1-Å resolution structure of the spinach (*Spinacia oleracea*) 70S ribosome obtained by cryoelectron microscopy revealed that PSRP1 binds to the mRNA channel of the small subunit and is highly similar to light-repressed transcript A of cyanobacteria from the long hibernation-promoting factor family (Perez Boerema et al.,

2018). PSRP2/RPS22, PSRP3/RPS23, and PSRP4/bTHXc are present in the small ribosome subunit, and PSRP5/RPL37 and PSRP6/RPL38 are present in the large ribosome subunit (Bieri et al., 2017; Perez Boerema et al., 2018). Based on the structure of the 70S ribosome, PSRP1 localizes to the mRNA channel and interacts with the 16S rRNA residues that form the decoding center, possibly preventing the binding of tRNAs, which suggests that it may have important roles in translation activity. Other PSRPs are genuine plastid-specific ribosomal proteins that structurally compensate for rRNA domains modified over evolution (Sharma et al., 2007; Bieri et al., 2017; Perez Boerema et al., 2018).

Translation mechanisms and ribosome pausing

In chloroplasts, translation is highly dynamic and entails four major steps: initiation, elongation, termination, and ribosome recycling. During initiation, the 30S small ribosome subunit is recruited to the ribosome-binding site with the help of the initiation factors IF1, IF2, and IF3 (Sijben-Müller et al., 1986; Campos et al., 2001; Miura et al., 2007; Zheng et al., 2016). About two thirds of chloroplast genes possess bacterial-type SD sequences and use them to initiate translation. The SD sequence complementarily interacts with the anti-SD sequence in the 16S rRNA to ensure proper positioning of the initiation complex (Bieri et al., 2017; Scharff et al., 2017). The remaining one third of chloroplast genes lack an SD sequence, suggestive of alternative mechanisms for chloroplast translation initiation. In these cases, a relative lack of RNA secondary structure at the 5′ UTR, which may be regulated by *trans*-acting factors, facilitates correct translation initiation (Scharff et al., 2011; Nakagawa et al., 2017). Binding of the 50S subunit converts the preinitiation complex into the complete 70S ribosome, which can then enter the elongation state of translation. Similar to that in bacteria, translation elongation in chloroplasts requires many prokaryotic elongation factors, such as EF-Tu, EF-G, and EF-Ts (Zoschke and Bock, 2018). During decoding of mRNA into an amino acid sequence, chloroplasts use the standard genetic code with a minimal set of tRNAs through mechanisms of wobble or superwobble base pairing (Alkatib et al., 2012). Translation termination occurs when the ribosome reaches a stop codon (UAA, UAG, or UGA) in the RNA and requires ribosome release factors RF1/PrfA (for UAA and UAG) and RF2/PrfB1 (for UAA and UGA) (Meurer et al., 2002; Motohashi et al., 2007). After termination, the chloroplast 70S ribosomes are subsequently disassembled by ribosome recycling factors to allow for their reuse in the next round of initiation (Tiller and Bock, 2014; Zoschke and Bock, 2018). However, our understanding of how these regulatory factors control translation in chloroplasts remains rudimentary.

The feedback regulation of translation represents an elegant means of robustly fine-tuning Rubisco levels. The translation of the plastid-encoded Rubisco large subunit RbcL is regulated by its nucleus-encoded partner, the Rubisco small subunit RbcS (Rodermel et al., 1988, 1996; Khrebtukova and Spreitzer, 1996). The downregulation of RbcS restrains the assembly of RbcL, and the resulting unassembled RbcL represses its own translation, possibly by direct RNA binding (Wostrikoff and Stern, 2007), thus ensuring the stoichiometry of Rubisco.

Chloroplast ribosomes are prone to pausing at conserved sites on RNAs in seed plants such as Arabidopsis and maize (Zoschke et al., 2013a; Gawronski et al., 2018). Such ribosome pausing is often induced by a combination of factors including RNA structure, internal SD sequences, and presence of positively charged amino acids. Moreover, ribosome pausing may help the insertion of transmembrane domains from photosynthetic apparatus components into the thylakoid membrane in the correct orientation, the integration of cofactors, and efficient protein folding, thereby ensuring correct assembly of photosynthetic complexes (Gawronski et al., 2018). The position features associated with ribosome pausing are conserved in photoautotrophic plants but less so in parasitic species with reduced photosynthetic capacity (Gawronski et al., 2018; Trosch et al., 2018), suggesting that ribosome pausing is a general mechanism that facilitates the biogenesis of photosynthetic complexes.

Chloroplast translation is triggered by light-induced pH changes and photosystem II (PSII) damage

In land plants, chloroplasts can dynamically change translation rates in response to environmental stimuli, especially light, throughout their whole life cycle. Light-induced regulation of translation couples the major energy-consuming step (protein synthesis) to the major energy-producing step (photosynthesis) in chloroplasts. In addition, as light causes constant damage to the reaction center protein D1 in PSII, light-induced translation ensures the coordination of D1 protein synthesis with the need to repair PSII.

Translation elongation in chloroplasts is activated rapidly after a shift from darkness to light (Chotewutmontri and Barkan, 2018). In lysed barley (*Hordeum vulgare*) chloroplasts, light-activated translation elongation is regulated by light-induced changes in lumen pH (Mühlbauer and Eichacker, 1998). The pH of the thylakoid lumen is affected by the cytochrome b_6f complex that pumps protons from the stroma. Light-stimulated translation elongation is considerably reduced in cytochrome b_{6f} mutants or upon treatment with pH uncoupler agents, but this plastome-wide translational response is not obviously affected by ATP -supply, reduced thioredoxin, or plastoquinone redox state (Chotewutmontri and Barkan, 2020). These findings demonstrate that light-induced pH changes globally activate chloroplast translation at the level of elongation, but how this pH change increases translation elongation is still unclear. A potential mechanism for general light-stimulated translation elongation in chloroplasts may rely on proteins that can orchestrate translation, such as the translation factor PSRP1 and/or EF-Tu (Perez Boerema et al., 2018; Zoschke and Bock, 2018).

In chloroplasts, only *psbA* RNA experiences substantially increased ribosome occupancy in response to light–dark shifts and high light exposure (Chotewutmontri and Barkan, 2018; Schuster et al., 2020). In mature chloroplasts, this *psbA*-specific activation is triggered by signals from PSII repair and is distinct from the plastome-wide translation response triggered by light-induced pH changes (Chotewutmontri and Barkan, 2020; Chotewutmontri et al., 2020). Nascent D1 produced by *psbA* is transferred to the HIGH CHLOROPHYLL FLUORESCENCE 244 (HCF244)/ONE HELIX PROTEIN 1 (OHP1)/OHP2 complex with the help of HCF136. The presence of D1 in the HCF244 complex is thought to inhibit the ability of the HCF244 complex to activate the recruitment of ribosomes to *psbA*

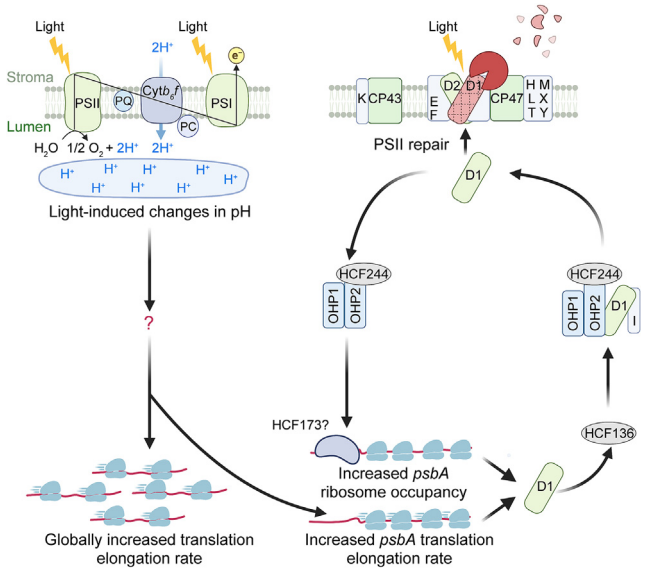

Figure 4. A schematic model showing chloroplast translation triggered by light-induced pH changes and PSII damage.
Chloroplast translation is rapidly activated after a shift from darkness to light. Upon illumination, the plastome-wide activation of translation elongation is triggered by light-induced changes in pH. Moreover, *psbA* translation is also activated by another light-induced regulatory pathway, the HCF244/OHP1/OHP2-centered autoregulatory circuit. Light causes constant damage to PSII, which requires replacement of damaged D1 by *de novo* synthesis of D1 proteins. HCF136 helps nascent D1 peptides insert themselves into the HCF244/OHP1/OHP2 complex, repressing its ability to activate ribosome recruitment to *psbA* RNA. The HCF244/OHP1/OHP2 complex delivers nascent D1 to photodamaged PSII, then releases the HCF244/OHP1/OHP2 complex to activate *psbA* translation. Given that HCF173 binds to the 5′ UTR of *psbA* RNA for translation initiation, a D1-less HCF244 complex is thought to communicate with HCF173 for the activation of *psbA* translation. The schematic model is drawn according to Chotewutmontri et al. (2020).

transcripts. It is unclear whether the interaction of the HCF244 complex with the nascent D1 occurs in the stroma or at the thylakoid membrane. Considering that OHP1 and OHP2 are tightly integrated at the thylakoid membrane (Li et al., 2019), it is most likely that the interaction of the HCF244 complex with the nascent D1 occurs at the thylakoid membrane. Light damages the PSII reaction center D1 protein, triggering delivery of nascent D1 by the HCF244/OHP1/OHP2 complex. A D1-less HCF244 complex recovers the ability to activate *psbA* translation (Figure 4). This autoregulatory mechanism contributes to explaining two long-standing questions in plant biology: (i) how chloroplasts perceive light to activate translation from *psbA* transcripts, and (ii) how the *psbA* translation rate is coupled to the need for nascent D1 during PSII repair.

POST-TRANSLATIONAL PROCESSING AND QUALITY CONTROL

Newly synthesized precursor proteins inside chloroplasts require N-formylated methionine excision, protein cleavage, and proper folding to generate fully functional products (van Wijk, 2015; Gao et al., 2023). The N-terminal methionine excision of precursor proteins in chloroplasts is initiated by deformylation, and methionine aminopeptidase then removes N-terminal methionine from precursor proteins (Meinnel and Giglione, 2022). In particular, the D1 precursor must undergo cleavage of the C-terminal tail by an endopeptidase named carboxyl terminal peptidase (Che et al., 2013).

Proteases are key components of the protein quality control systems in chloroplasts and play important roles in promoting correct protein folding and removing damaged proteins. Regulation of protein degradation in chloroplasts has recently been reviewed (Gao et al., 2023; Sun et al., 2023). The D1 protein of the PSII reaction center is encoded by the chloroplast *psbA* gene; it inserts into the PSII complex with its five transmembrane helix domains that are connected by lumenal and stromal loop domains (Inagaki, 2022). D1 is always photodamaged by light. Therefore, degradation of photodamaged D1 is essential for ensuring efficient D1 turnover and PSII repair. Here, we mainly discuss the role of proteases in degradation of photodamaged D1. A series of studies have demonstrated that Deg and FtsH proteases are involved in this degradation: Deg1, Deg5, and Deg8 cleave the lumenal-side loops of D1 (Kapri-Pardes et al., 2007; Sun et al., 2007), and Deg7 cleaves its stromal-side BC loop (Sun et al., 2010). Deg7 is also involved in degradation of the chloroplast-encoded PSII subunits D2, CP43, and CP47 (Sun et al., 2010). FtsH2 has been suggested to regulate D1 degradation at the CP43 formation step during PSII repair (Kato et al., 2009). However, the detailed processes and proteolytic sites of FtsH-mediated D1 degradation remain unclear and should be investigated further in the future (Kato et al., 2009; Chi et al., 2012; Kato and Sakamoto, 2018; Yi et al., 2022).

PPR PROTEINS COULD BE ENGINEERED AS TOOLS FOR MANIPULATING CHLOROPLAST GENE EXPRESSION

Characterization of the molecular mechanisms by which PPR proteins regulate chloroplast gene expression offers opportunities for designing PPR tools for RNA manipulation that could be used in chloroplast RNA research. Typical PPR proteins contain repeating 35-amino-acid motifs that stack into a long superhelical RNA-binding surface that interacts with RNA via an elegant one-repeat:one-nucleotide binding mode (Barkan et al., 2012; Yin et al., 2013; Shen et al., 2016). The combination of the 5th and 35th residues at each PPR motif is considered to be the "PPR code" that is responsible for specific RNA base recognition. PPR codes that are relatively specific for A, U, C, or G are fairly well understood, and numerous PPR codes have been deciphered (Barkan et al., 2012; Shen et al., 2016; Yan et al., 2019). Engineered PPR proteins with custom RNA-binding specificity have been created by either altering the PPR code of natural proteins (Zhou et al., 2017; Colas des Francs-Small et al., 2018) or completely redesigning the protein according to the consensus PPR motif (Coquille et al., 2014; Gully et al., 2015; Shen et al., 2015, 2016; Miranda et al., 2018; McDermott et al., 2019; Yan et al., 2019). Because natural PPRs often contain a C-terminal domain with specific functions, an engineered PPR can also be fused to a specific domain to fulfill a custom function. For example, a PPR domain with a C-terminal RNA endonuclease domain can act as an artificial sequence-specific RNA endonuclease that cleaves specific

RNA targets. Therefore, engineered PPRs might be used as tools to investigate the functions of chloroplast RNAs and thereby reveal molecular mechanisms of chloroplast gene expression.

Maize PPR10 binds upstream of the *atpH* ribosome-binding site and activates *atpH* expression by preventing 5′–3′ exonucleolytic degradation and improving its translational efficiency (Pfalz et al., 2009; Prikryl et al., 2011). On the basis of PPR10 characterization, an exciting on/off switch for plastid transgenes has been developed (Rojas et al., 2019). A PPR10 variant with new sequence specificity was designed, and its corresponding binding site was inserted upstream of a chloroplast transgene encoding a green fluorescent protein (GFP). Driving expression of this PPR10 variant by an ethanol-inducible promoter resulted in ~20-fold induction of the GFP reporter; GFP accumulated to ~15% of the soluble protein in leaf tissues. In the absence of ethanol, there was no detectable expression of the PPR10 variant, and protection of *GFP* mRNA within chloroplasts was abolished, leading to only trace expression of the GFP reporter (Rojas et al., 2019). A PPR10-based system was also applied to tissue-specific expression of a plastid-encoded GFP in potato tubers, and GFP accumulated to 1.3% of the total soluble protein in the tubers (Yu et al., 2019). Modified PPR proteins can thus be exploited as tools for controlling plastid gene expression in future research.

POTENTIAL ROLES OF NEW TECHNIQUES FOR ELUCIDATING MOLECULAR MECHANISMS OF CHLOROPLAST GENE EXPRESSION

The use of new techniques and approaches will be important for increasing our understanding of chloroplast gene expression. Cryoelectron microscopy can solve molecular structures with a resolution below 2 Å, and it is increasingly accessible and cost effective (Danev et al., 2019). Chloroplast gene expression machineries include PEP and NEP for transcription, "a possible editosome" for RNA editing, "a possible spliceosome" for RNA splicing, and the ribosome for translation. Elucidating the structures of these gene expression machineries by cryoelectron microscopy would reveal how they control chloroplast gene expression at the molecular level.

Various high-throughput sequencing-based techniques have rapidly emerged, providing an opportunity for quantitative genome-wide assessments of chloroplast gene expression with unprecedented depth and resolution. Global run-on sequencing and native elongating transcript sequencing are powerful tools for characterizing nascent RNA to determine the positions, relative levels, and orientation of transcriptionally engaged RNA polymerases genome wide (Zhu et al., 2018). Transcriptional pausing in chloroplasts was found to be a regulatory point for gene expression (Ding et al., 2019). Thus, global run-on sequencing and native elongating transcript sequencing can be exploited to explore the functions of transcriptional pausing in chloroplasts by monitoring the dynamic responses of paused RNA polymerase to developmental and external stimuli. Structure sequencing is a quantitative and high-throughput method that offers genome-wide details on RNA structure at single-nucleotide resolution (Deng et al., 2018). Structure sequencing could be used to map the RNA structures of chloroplast transcripts and to investigate the roles of RNA structure in regulating RNA splicing, RNA stability, and translation within chloroplasts. In addition, genome-wide association studies are used to identify genetic variants that are statistically associated with a particular trait (Abdellaoui et al., 2023). Thus, changes in chloroplast gene expression, e.g., the abundance of *psbA* transcripts, could be used in genome-wide association studies to determine which genetic variants are involved in regulation of chloroplast gene expression.

Single-cell omics is revolutionizing our understanding from the tissue to the single-cell level, enabling us to access individual cell modalities, such as genomes, transcriptomics, and epigenomics (Wen et al., 2022). Leaves have a complex cellular architecture with heterogeneous cell types. For example, C4 photosynthesis is performed in mesophyll and bundle sheath cells of leaves, and single-cell sequencing could be used to characterize chloroplast gene expression in these distinct cell types. Single-cell sequencing also enables us to monitor chloroplast gene expression over the course of chloroplast biogenesis in specific cell types.

Chloroplast transformation is a powerful tool to investigate many aspects of chloroplast gene expression; it can identify the regulatory elements of chloroplast genes, reveal the mechanism and evolution of plant RNA editing, and characterize the functions of pncRNAs (Ruf and Bock, 2011; Sharwood et al., 2011). A new method for chloroplast transgene introduction was recently developed. Unlike classical chloroplast transformation, this method does not require foreign transgene insertion into the chloroplast genome; instead, the foreign transgene is amplified as an independent entity named the "minichromosome" (Jakubiec et al., 2021). This method could be used to reveal the functions of regulatory elements of chloroplast genes. In addition, two protein-only base editors have been developed that can induce targeted C-to-T and A-to-G base editing in chloroplast DNA (Kang et al., 2021; Mok et al., 2022). These base editors will enable us to explore regulatory elements of chloroplast genes *in situ* and provide an opportunity to optimize photosynthesis and improve crop yield by altering the coding sequences of chloroplast genes, e.g., modifying *RbcL* to enhance Rubisco activity and CO_2 assimilation.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Over the course of host–endosymbiont coevolution, the chloroplast has not only inherited some prokaryotic principles but also developed many new features of gene expression, such as highly complex RNA polymerases, as well as diverse RNA cleavage, splicing, and editing mechanisms. These combined factors ensure correct chloroplast gene expression. Over the past few years, extensive studies focused on chloroplast gene expression have advanced from describing phases to exploring their underlying gene expression mechanisms. Here, we discuss the next wave of questions that will improve our understanding in this field.

Chloroplast genes are mainly transcribed by the PEP complex that consists of bacterial-like core subunits and at least 12 additional components, the PAPs. All PAPs are required for the transcription

activity of the PEP complex. However, our understanding of PAPs remains rudimentary. Future studies should therefore address two major issues: where PAPs are located within the PEP complex, and how they coordinately control the transcriptional activity of the PEP complex. In addition, transcriptional pausing is widespread in chloroplasts and has an important role in regulating chloroplast transcription. As this mechanism is newly emerging, many gaps in our understanding remain. Most importantly, future research should aim to explore the causes and biological functions of transcriptional pausing by PEP.

The maturation of RNAs in chloroplasts requires a set of post-transcriptional processing events, such as intercistronic cleavage, RNA termini trimming, RNA splicing, and RNA editing (Zhang and Lu, 2019). Intercistronic cleavage and termini trimming of RNAs in chloroplasts are accomplished by ribonucleases and RNA-binding proteins, but how chloroplasts initiate the endonucleolytic cleavage and the interplay between ribonucleases and RNA-binding proteins remain largely unclear. In addition, the ion imbalance of K^+ and Na^+ significantly impairs chloroplast rRNA maturation by altering their secondary structure and reducing helper protein interactions with chloroplast rRNA transcripts (DeTar et al., 2021). Hence, how chloroplast ion homeostasis directly modulates RNA folding and RNA–protein interaction should be considered in the future.

To date, numerous splicing factors (e.g., PPR, CRM, plant organellar RNA recognition proteins) in chloroplasts have been identified that help remove introns from precursor RNAs (Wang et al., 2022). These splicing factors regulate the splicing of distinct but overlapping intron sets. Although chloroplast splicing events have been described over several decades, the specific mechanisms by which these splicing factors activate the self-splicing ability of introns have not yet been uncovered.

In flowering plants, C-to-U RNA editing is executed by multi-protein editosome complexes in which the PPR protein confers the RNA-binding specificity, MORF proteins regulate the binding activity of PPR domains, and the DYW domain catalyzes the deaminase activity (Yan et al., 2017; Small et al., 2020; Royan et al., 2021). However, the functions of other editosome components, such as the RNA helicase INCREASED SIZE EXCLUSION LIMIT 2 or ORGANELLE RNA RECOGNITION MOTIF PROTEIN and ORGANELLE ZINC FINGER proteins, remain largely unknown, and determination of their precise roles is an important future direction. In addition, a recent study revealed that there are complex interplays between RNA substrates and editosomes, whereby the DYW domains show distinct preferences for the neighboring nucleotides of RNA editing sites (Maeda et al., 2022). In light of these questions, the field anxiously awaits structural studies of RNA bound to a DYW domain and/or even the complete editosome.

Although chloroplast ribosomes show substantial deviations from the bacterial ribosome, a number of studies on their function and structure have demonstrated that the basic function and catalytic mechanism of the chloroplast translation apparatus are conserved. However, our understanding of the molecular mechanisms by which chloroplast translation is regulated remains rudimentary. In the future, we should focus on identifying novel RNA *cis*-elements and proteinaceous *trans*-factors and exploring their roles in regulating translation in chloroplasts.

In contrast to that of bacteria, the coupling of translation and transcription in the chloroplasts of land plants is believed to be relaxed, perhaps because chloroplast RNAs are stable when not covered by ribosomes and exhibit a longer half-time than bacterial RNAs, and translation initiation of numerous mRNAs is activated by post-transcriptional processing. However, there is also evidence that transcription–translation coupling has not been fully lost in chloroplasts. Electron micrographs from 1982 showed ribosomes associated with nascent transcripts (Rose and Lindbeck, 1982). In addition, several ribosomal proteins co-purify with the PEP complex (Pfalz et al., 2006), and many translation-related factors are enriched in plastid nucleoids in a ribonuclease-sensitive manner, which is a hallmark for proteins tethered to nascent transcripts (Majeran et al., 2011). Moreover, pTAC13/NusG associates with the PEP complex via PAP9 as well as two 30S ribosome subunits, RPS5 and RPS10. Hence, pTAC13/NusG might link the translational apparatus to the main chloroplast RNA polymerase, PEP (Xiong et al., 2022). Taken together, these observations suggest that further analysis of the relationships between the transcriptional and translational apparatus will be necessary to improve our understanding of chloroplast gene expression.

Light is one of the most important environmental cues required to start transcription. Despite several fresh insights into the signaling steps of the activation of transcription and translation by light, our knowledge of how light switches on gene expression within chloroplasts is still limited. Therefore, identifying new signaling components that link light and chloroplast gene expression would be a very important step forward. Another complementary approach could be to explore the influence on chloroplast gene expression mediated by other internal and external triggers, such as temperature, ion balance, osmotic status, phytohormones, and circadian rhythms.

Recent studies suggest that manipulation of RbcL and D1 with enhanced abundance can improve crop yield and stress tolerance. Supplementing RbcL of nuclear origin together with the RbcS and Rubisco assembly chaperone RAF1 results in a >30% increase in Rubisco content, a 15% increase in CO_2 assimilation, and a 27% increase in fresh weight in maize (Salesse-Smith et al., 2018). The artificial nuclear D1 imported into the chloroplast through the chimeric RbcS signal peptide leads to enhanced thermotolerance and a considerable increase in biomass accumulation in Arabidopsis, tobacco, tomato, and rice (Chen et al., 2020). Presumably, this artificial nuclear D1 synthesis complements nascent D1, which helps the rapid repair of damaged PSII under conditions of heat and high-light stress. In addition, as discussed above, modified PPR proteins might be used as a tool to improve crop yield and environmental stress resistance by regulating the expression of chloroplast genes. Clearly, it will be a challenge to elucidate the role of chloroplast gene expression in improving crop yield and stress, and these topics should be investigated extensively in future work.

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