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Participation of nuclear genes in chloroplast gene expression

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Abstract — The expression of the plastid genome is dependent on a large number of nucleus-encoded factors. Some of these factors have been identifie through biochemical assays, and many others by genetic screens in *Arabidopsis*, *Chlamydomonas* and maize. Nucleus-encoded factors function in each step in plastid gene expression, including transcription, RNA editing, RNA splicing, RNA processing, RNA degradation, and translation. Many of the factors discovered via biochemical approaches play general roles as components of the basic gene expression machinery, whereas the majority of those identifie by genetic approaches are specificall required for the expression of small subsets of chloroplast genes and are involved in post-transcriptional steps. Some of the nucleus-encoded factors may play regulatory roles and modulate chloroplast gene expression in response to developmental or environmental cues. They may also serve to couple chloroplast gene expression with the assembly of the protein products into the large complexes of the photosynthetic apparatus. The convergence of biochemical approaches with those of classical and reverse genetics, and the contributions from large scale genomic sequencing should result in rapid advances in our understanding of the regulatory interactions that govern plastid gene expression. © 2000 Société française de biochimie et biologie moléculaire / Éditions scientifique et médicales Elsevier SAS

chloroplast / plastid / RNA stability / translational control / group II introns

1. Introduction

The plastid genomes of green algae and vascular plants are circular molecules containing approximately 120 genes for plastid proteins, tRNAs and rRNAs. The vast majority of plastid gene products are components either of photosynthetic enzymes or of the plastid gene expression machinery. The thylakoid membrane complexes photosystem I (PSI), photosystem II (PSII), cytochrome b_6f, ATP synthase and NADH dehydrogenase, the soluble enzyme Rubisco, and plastid ribosomes each consist of both plastid and nucleus-encoded subunits. Thus, mechanisms must exist to ensure the stoichiometric accumulation of proteins derived from two physically separate genetic compartments. Furthermore, the rates of synthesis of plastid gene products change in response to developmental and environmental cues, and this regulation is mediated by nucleus-encoded factors. In this review, we will focus on genetic and biochemical studies that address the involvement of nucleus-encoded factors in regulating plastid gene expression.

2. Overview of chloroplast gene expression

Transcription of chloroplast genes is catalyzed by at least two distinct RNA polymerases. One resembles bacterial RNA polymerases and consists of a plastid-encoded core enzyme that assembles with nucleus-encoded accessory factors. The other is encoded entirely by nuclear genes and is related to certain bacteriophage polymerases (reviewed in [1]). Recent evidence suggests that both light and developmental signals influenc the relative activities of the different polymerases [1].

The products of transcription by both types of polymerase are typically polycistronic. Most primary transcripts then undergo extensive processing by splicing, endonucleolytic cleavage, 5’ and 3’-end maturation, and/or editing (reviewed in [2]). RNA editing, the post-transcriptional change of specifi cytidines encoded in the DNA to uridines, has been found in the plastids of vascular plants (reviewed in [3]). The fact that most edited codons specify amino acids that are more evolutionarily conserved than the corresponding unedited codon emphasizes the importance of this process, whose mechanism is not yet understood. The mature 3’ ends of chloroplast mRNAs are generated by processing rather than by transcription termination. There is evidence that correct 3’ processing can influenc translational efficiency [4], and is therefore important for optimal gene expression. Endonucleolytic processing between coding regions generates smaller, often monocistronic mature mRNAs. In land plants, the various processing intermediates accumulate to similar levels and are observed as complex transcript populations on RNA gel blots. In contrast, in the green

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alga *Chlamydomonas reinhardtii*, precursors and intermediates accumulate to low or undetectable levels, presumably as a consequence of more rapid processing and/or decreased precursor stability. The functional signif cance of a few such processing events has been explored. In some cases, cleavage may increase translational efficiency [5, 6]. In other cases, processing allows non-stoichiometric accumulation of mRNAs from cotranscribed genes [7], which may be important for synthesizing the gene products in optimal ratios.

The plastid genomes in land plants and algae contain numerous introns, which can be recognized as either group I or group II introns by conserved structural features. *C. reinhardtii* chloroplasts contain f ve group I introns (in the *psbA* and *rrnL* genes) and two group II introns (in the *psaA* gene) [8]. Land plant plastids have a very different intron distribution, with ≈ 17 group II introns (depending on the species) and only one group I intron. The chloroplasts of *Euglena gracilis* contain, in addition, a third type of intron (group III), which appear to be degenerate group II introns [9]. Evidence exists for the light regulation of group I intron splicing in *C. reinhardtii* chloroplasts [10] and for the developmental regulation of group II intron splicing in land plant chloroplasts [11]. Light and developmental signals also inf uence the stabilities of certain chloroplast RNAs (reviewed in [2, 12, 13]). Studies of decay mechanisms have revealed a role for 3' stem-loop structures in stabilizing mRNAs, and a polyadenylation-dependent decay pathway that resembles a pathway found in bacteria.

The chloroplast translation machinery resembles that of prokaryotes but also has distinct features (reviewed in [14, 15]). Chloroplast ribosomes are similar to bacterial ribosomes in size and antibiotic sensitivities, and the sequences of many ribosomal components resemble those of their bacterial ancestors. Despite these similarities, translation mechanisms in chloroplasts differ in several important ways from those in the model bacterium *E. coli* (reviewed in [16]). For example, Shine-Dalgarno sequences play a less prevalent role in chloroplast translation, and translation is commonly regulated by positively acting regulatory proteins. Translation rates in chloroplasts are inf uenced by a variety of factors, including light, redox poise, developmental status, and the assembly of enzymes containing plastid-encoded subunits (reviewed in [16, 17]).

This brief overview illustrates the complexity of chloroplast gene expression and the diversity of regulatory mechanisms. Examples exist for regulation at each step in plastid gene expression, but mechanisms of regulation are just starting to be explored. Given the simplicity of the plastid genome and the fact that plastid gene expression must be integrated with that in the nucleo-cytoplasmic compartment, it is anticipated that most factors that participate in chloroplast gene regulation are the products of nuclear genes.

3. Methods for identifying nuclear genes that function in plastid gene expression

Nuclear gene products have been implicated in chloroplast gene expression primarily through genetic studies or via biochemical assays for chloroplast proteins with relevantactivities (e.g., nucleic acid binding activity). However, with the recent burst of genome sequence data, it is increasingly common that a role in chloroplast gene expression is inferred solely from a protein's amino acid sequence: many proteins with predicted chloroplast targeting sequences harbor nucleic acid binding motifs or resemble bacterial proteins with well-def ned roles in gene expression. These proteins of unknown function will provide a rich source of material for future biochemical and genetic studies. Methodologies employed for the more classical biochemical and genetic approaches are summarized below.

3.1. Biochemical approaches

Because there are few open reading frames of unknown function in the chloroplast, it is likely that most biochemically-identif ed factors will be nucleus-encoded. Proteins have been purif ed from chloroplasts based on in vitro assays for a variety of activities, including nucleic acid binding, RNA maturation, transcription, and polyadenylation.

Both sequence-specif c and -nonspecif c RNA binding proteins have been purif ed by affinity chromatography methods. For example, general RNA binding proteins were purif ed from tobacco chloroplasts by single-stranded DNA affinity chromatography, leading to the identif cation of several abundant proteins with ribonucleoproteinconsensus motifs [18]. Antibodies against several of these proteins were used to immunoprecipitate material from stroma, revealing interactions with both mRNAs and unspliced tRNA precursors [19]. A related protein, 28RNP, had been purif ed previously from spinach chloroplasts based on an assay for mRNA 3' end processing [20]. Additional members of this protein family have since been discovered in a wide variety of organisms [21–25], but little functional information is available. These proteins may participate in a wide variety of processes and may play rather global roles, making their precise functions difficult to pin down.

Sequence-specif c RNA binding proteins have been identif ed by UV-cross-linking and gel mobility shift assays in the presence of competitors. A regime designed to identify proteins that interact specif cally with the 5' untranslated region (UTR) of the *C. reinhardtii psbA* mRNA led to the copurif cation of four proteins that have been proposed to form a complex that activates translation in a light-dependent fashion [26]. The genes encoding two of the proteins have been cloned. One encodes a protein called RB47, a poly(A)-binding protein that directly

interacts with RNA [27]; the other is a protein disulfide isomerase that has been proposed to influence the affinity of RB47 for its RNA target in a redox-dependent manner [28]. A protein that binds with specificity to the 5' UTR of the spinach *psbA* mRNA was purified with the goal of identifying factors that modulate *psbA* mRNA stability; this protein was found to be plastid ribosomal protein S1 [29]. S1 plays a central role in translation initiation in bacteria [30, 31], and may play a similar role in chloroplasts. It is striking that a search for proteins that bind to the 5' UTR of the *psbA* mRNA in spinach and *C. reinhardtii* led to such different outcomes and it will be interesting to see how this is resolved in the future.

Several proteins have been purified from chloroplasts based on assays for enzymatic activities involved in gene expression. For example, the spinach 28RNP cited above copurifies with a 3'-end processing activity [20], together with a complex of proteins that resembles the bacterial degradosome and that includes a homolog of the bacterial enzyme polynucleotide phosphorylase [12, 32]. It has been proposed that these proteins function as components of the general 3' end processing machinery. A spinach chloroplast protein called CSP41 binds specifically to the 3' terminal stem-loop of the chloroplast *petD* mRNA and also possesses an endonuclease activity [33], leading to the proposal that it functions more specifically in the processing or decay of *petD* mRNA. Similarly, a 54 kDa protein from mustard chloroplasts behaves as a sequence-specific RNA binding protein and is associated with an endonuclease activity that processes the 3' ends of several transcripts in vitro [34]. In the realm of chloroplast transcription, purification of plastid RNA polymerase activities from mustard chloroplasts led to the identification of two accessory proteins, one related to spinach CSP41 (implicated in RNA processing, as described above), and one resembling an Fe superoxide dismutase [35]. The fact that biochemical activities are associated with each of these proteins provides support for their proposed roles in a specific aspect of chloroplast gene expression. However, for none of these proteins have the in vivo roles been tested genetically.

Numerous other chloroplast proteins capable of sequence-specific interactions with RNA have been detected by UV-cross-linking and gel-mobility shift assays, but have not been purified sufficiently to obtain sequence information [34, 36–41]. However, in itself, the ability of a protein to bind a specific RNA sequence is not sufficient to conclude that it functions in any particular process. In this respect, correlation between the presence or absence of an RNA binding activity and the genotype of a nuclear locus implicated in chloroplast gene expression can provide further insight. For example, a polypeptide that can bind the *psbD* 5'UTR in *C. reinhardtii* is missing in extracts of the nuclear mutant *nac2-26*, in which *psbD* mRNA is unstable [42], suggesting that the polypeptide influences *psbD* RNA stability and that its activity is influenced by the *Nac2* gene. In another example, a protein that binds the *psbC* 5'UTR was detected in extracts of a *C. reinhardtii* mutant defective in *psbC* translation [37] but not in wild-type extracts, suggesting that the protein plays a negative role in *psbC* translation. Analogously, the abundance of RB47 in enriched fractions of *C. reinhardtii* cells was reduced in nuclear mutants defective in *psbA* translation [43], consistent with the hypothesis that the protein plays a positive role in translation.

3.2. Genetic approaches

3.2.1. Model organisms

Nuclear mutations that disrupt chloroplast gene expression define genes that participate, either directly or indirectly, in chloroplast gene expression. Most genetic studies addressing the role of nuclear genes in chloroplast gene expression have been carried out with either *Zea mays* (maize), *Arabidopsis thaliana* (Arabidopsis), or *C. reinhardtii*. *C. reinhardtii* is a unicellular green alga whose photosynthetic apparatus closely resembles that of land plants. Advantageous features of *C. reinhardtii* include the ease of obtaining and maintaining non-photosynthetic mutants, the fact that it is a microorganism with properties conducive to genetic analysis, and the well developed technologies for transforming both its chloroplast and nuclear genomes (reviewed in [44]). *C. reinhardtii* cells can grow in liquid culture or form colonies on agar plates and, when provided with a source of reduced carbon, non-photosynthetic mutants can be readily recovered and propagated [45]. Both haploid and diploid cells can be grown vegetatively, and the mating of haploid cells or the germination of zygotes can be induced at will by exposing cells to specific conditions. Mutations can be localized to either the nuclear, mitochondrial, or chloroplast genomes by the distinct transmission patterns characteristic of each genome. Transformation of the nuclear genome is rapid and efficient, allowing genes to be cloned by complementation of mutant alleles. Because foreign DNA is integrated at random sites, nuclear transformation has been useful for insertional mutagenesis [46]. Transformation of the chloroplast genome [47] is obtained using biolistic gene bombardment and proceeds by homologous recombination, resulting in precise gene targeting (reviewed in [48]).

In land plants, most genetic studies of nuclear genes involved in plastid gene expression have involved either maize or Arabidopsis. Both organisms offer well-developed genetic tools and the ability to clone nuclear genes defined by mutations. In maize, gene cloning has been accomplished by transposon tagging, whereas in Arabidopsis, both map-based cloning and insertional mutagenesis methods have been employed. Although Arabidopsis offers the advantage of facile nuclear transformation, maize offers advantages for biochemical and developmental studies. The large maize seed supports the

growth of non-photosynthetic mutants to the point where they have developed approximately 0.5 gram of leaf tissue, providing quick and convenient access to ample material for biochemical analysis. In contrast, the growth of non-photosynthetic Arabidopsis mutants must be supported by sugars supplied in a growth medium. Given that exogenous sugars have profound effects on the expression of nuclear genes in plants [49], interpretation of mutant phenotypes must consider the potential pleiotropic effects caused by this artif cial growth regime. In maize, studies of the proplastid to chloroplast transition are facilitated by the fact that chloroplasts at different stages of development are physically separated along the length of the leaf, with proplastids at the leaf base and mature chloroplasts at the leaf tip [50]. In contrast, Arabidopsis leaves are a mosaic of cells and plastids at different stages of development that are not easily isolated from one another for analysis. The combined application of plastid gene transformation and nuclear genetics has been extremely fruitful in studies of *C. reinhardtii* chloroplast gene regulation (see below). Unfortunately, the analogous strategy is not yet possible in land plants. Although the plastid genome of Arabidopsis has been transformed, the transgenic plants have so far proven infertile and cannot be crossed into different nuclear backgrounds.

It has become clear that the distinct attributes of each of these three organisms engender different types of approaches that then lead to unique insights. Furthermore, because monocots, dicots and algae are widely divergent organisms, comparative studies will be important to determine to what extent mechanisms identif ed in one model organism are conserved between species.

3.2.2. Screening methodologies

Screens for nuclear mutations that disrupt chloroplast gene expression have generally relied on the fact that most chloroplast gene products are required for the formation of a functional photosynthetic apparatus. Consequently, lesions in chloroplast gene expression are typically ref ected by defects in photosynthesis and often by reduced chlorophyll accumulation. Defects in chlorophyll accumulation (e.g., pale green, yellow, or colorless/albino) are easily picked out by eye, and can result from defects in pigment synthesis, in the light harvesting antennae, or in the photosynthetic electron transport chain. Amongst this latter class of mutants, some have primary defects in chloroplast gene expression.

Another screening strategy is based on the f uorescent properties of chlorophyll [51]. Mutants impaired in photosynthetic electron transport show increased chlorophyll f uorescence because a high proportion of the energy that is captured by the antennae chlorophyll is not quenched by photochemistry, and is therefore re-emitted as f uorescence. Most high chlorophyll f uorescence (*hcf*) mutants have defects in the electron transfer chain in the thylakoid membrane. However, the loss of the thylakoid ATPase and the soluble enzyme Rubisco can also result in an *hcf* phenotype [51, 52]. In plants, the vast majority of mutations that cause the *hcf* phenotype also cause at least a slight reduction in chlorophyll content (reviewed in [53]). Consequently, the simple screen for pale green mutants can largely substitute for primary screens for the *hcf* phenotype in plants. Many interesting mutants with only a weak *hcf* phenotype have been picked up in this way.

Changes in f uorescence kinetics revealed by video imaging can be used to reveal photosynthetic defects and the site of the lesion [54, 55]. The f uorescence kinetics recorded after a shift from darkness to light allow a distinction between the wild type, mutants defective in PSII, and those defective in the cytochrome b_6f complex or PSI. Similarly, changes in luminescence following a transition from light to dark can reveal alterations in the *trans*-membrane potential of the thylakoids that result, for example, from mutations affecting the ATP synthase. Screening for mutants based on kinetic parameters such as these has been particularly fruitful with *C. reinhardtii* because large numbers of colonies can be rapidly screened on Petri plates.

Several screening strategies have been used specif cally with *C. reinhardtii*. One strategy takes advantage of the conditional nature of photosynthetic defects: non-photosynthetic mutants are viable on medium containing acetate, but fail to grow on minimal medium. Replica plating allows the identif cation of acetate-requiring mutants. Nuclear mutations in *C. reinhardtii* that suppress the phenotype of lesions in the chloroplast genome have also revealed loci involved in chloroplast gene expression. For example, strains with a *cis*-acting mutation that disrupts the translation of the chloroplast *psbC* mRNA were used to identify nuclear suppressors by selection for photosynthetic growth [16]. The analogous approach was used to identify a nuclear suppressor of mutations that alter the initiation codon of the chloroplast *petD* or *petA* mRNAs [57]. A nuclear gene involved in the 3' processing of several chloroplast mRNAs was identif ed by a nuclear mutation that suppresses the effects of a deletion of the 3'end of the chloroplast *atpB* gene [58]. In a related approach, nuclear suppressors of nuclear mutations have identif ed loci in *C. reinhardtii* that inf uence the accumulation of *rbcL* mRNA or the translation of *psbD* mRNA [59, 60]. Suppressor screens such as these are difficult in land plants because non-photosynthetic mutants do not produce gametes. Since mutations causing lesions in photosynthesis must be propagated by crossing heterozygous plants, one cannot simply mutagenize a population of homozygous mutants and then select progeny that can grow autotrophically. Alternative methods that take advantage of genetic markers linked to the parental mutation are possible in principle, but are problematic and have not been reported.

4. Nuclear loci involved in chloroplast gene expression

The genetic strategies summarized above have led to the identif cation of nuclear genes involved in many aspects of chloroplast gene expression in both land plants and *C. reinhardtii*. The insights gained from these studies are summarized below, and are placed in the context of a sampling of relevant biochemical data. More comprehensive summaries of the biochemical analysis of specif c aspects of chloroplast gene expression can be found in other reviews in this volume [1, 3, 16, 61].

4.1. Transcription

Genetic screens have revealed few if any nuclear genes involved in plastid transcription. Just one nuclear mutant, 76-5EN in *C. reinhardtii*, has been reported to have a defect in plastid transcription. This mutant fails to accumulate *rbcL* mRNA, and radiolabeled *rbcL* mRNA accumulated to reduced levels during in vivo pulse-labeling experiments [62]. However, the possibility remains that the *rbcL* RNA is synthesized at normal rates in the mutant, but is highly unstable. Despite the rarity of nuclear mutants with plastid transcription defects, many nuclear genes clearly play critical roles in chloroplast transcription. Nuclear genes contribute all of the subunits of the 'NEP' RNA polymerase and accessory subunits of the 'PEP' RNA polymerase (reviewed in [1]). A variety of nucleus-encoded sequence-specif c DNA binding proteins and sigma- like factors have been suggested to play a role in transcriptional regulation in the chloroplast (reviewed in [1]). The failure to f nd mutants with defects in genes encoding these proteins may be a consequence of screening strategies, which have focused on mutants lacking subsets of chloroplast proteins. It is possible that most nuclear genes that function in plastid transcription play more general roles.

4.2. RNA editing

There is strong evidence that the editing of chloroplast RNAs involves factors encoded by nuclear genes. Analysis of plastid transformants has provided evidence for *trans*-acting factors that function in the editing of specif c sites (reviewed in [3]). Because plastid editing was detected in a barley mutant lacking plastid ribosomes and in the presence of inhibitors of chloroplast translation [63, 64], any protein components of the editing apparatus are likely to be nucleus-encoded. No factors involved in chloroplast RNA editing have been identif ed, but genetic screens should prove to be a powerful approach towards unraveling this mysterious process.

4.3. RNA splicing

Each of the introns in land plant and *C. reinhardtii* chloroplast genomes can be recognized as either a group I or group II intron by conserved structural features. Group I and group II introns are often referred to as 'self-splicing' introns because examples in each class have been shown to splice autocatalytically in vitro [65]. In fact, all f ve group I introns of the *C. reinhardtii* chloroplast are autocatalytic in vitro, but only under non-physiological conditions. Thus, like their counterparts in fungal mitochondria, they probably require protein factors for splicing in vivo (reviewed in [8]).

A nuclear mutant of *C. reinhardtii*, *ac20*, fails to splice the group I intron in the 23S rRNA. However, this is thought to be a secondary effect, with the primary defect being the failure to process an internal transcribed spacer (ITS-1) in the rRNA precursor [66]. No other nuclear mutations inf uencing group I intron splicing have been reported for either *C. reinhardtii* or land plants. Since the *C. reinhardtii psbA* gene contains group I introns and encodes an essential component of PSII, nuclear genes that function specif cally in *psbA* splicing should arise in screens for PSII mutants. It is interesting that among the large number of PSII mutants that have been reported in *C. reinhardtii*, none are defective in *psbA* splicing. This suggests that either nuclear gene products are not involved in its splicing or any nucleus-encoded proteins involved also participate in the splicing of the group I intron in the 23S rRNA or have other functions.

The genetic analysis of group II intron splicing in chloroplasts has been more fruitful. The *psaA* gene in the chloroplast of *C. reinhardtii* is composed of three exons that are scattered around the chloroplast genome. The mature *psaA* mRNA is assembled from three separate precursor RNAs by two steps of *trans*-splicing (reviewed in [8]). A small additional transcript, the product of the *tscA* gene, is required for the *trans*-splicing of intron 1, which is thus composed of at least three separate RNA components. The two split introns of *psaA* belong to group II, and share with other members of this class conserved sequence and structure elements. At least 14 nuclear genes required for *psaA trans*-splicing have been identif ed genetically [67]. Mutations at some of these loci prevent splicing of both introns (class B), but most loci function specif cally in the splicing of either intron 1 (class C) or intron 2 (class A). Two allelic mutations in class C prevent processing of *tscA* RNA from a polycistronic precursor and also block the splicing of intron 1. The effect on splicing in this case is probably indirect, as processing of *tscA* RNA may be required for its assembly with the other intron fragments during the splicing process [68, 69]. The class B mutant HN31 exhibits defects in the *trans*-splicing of both introns 1 and 2, and also in *tscA* processing; again, the *tscA* processing defect may be the cause of the block in intron 1 splicing [69].

Four of the nuclear genes required for *psaA trans*-splicing have been cloned: *Maa1*, *Maa3* and *Tr72* encode novel proteins (M. Goldschmidt-Clermont, C. Rivier and J.-D. Rochaix, unpublished; J. Nickelsen, personal com-

munication), while *Maa2* belongs to the family of pseudouridine synthases [70]. Site-directed mutagenesis has shown that the putative pseudouridine synthase enzymatic activity is not required for the splicing function of Maa2. Thus Maa2 may have been recruited during evolution to fulfill a new role as a splicing factor; it is not yet clear whether it has retained an activity as a pseudouridine synthase, and thereby has dual functions.

Genetic analysis in land plants has revealed two nuclear genes that are required for the splicing of chloroplast group II introns, the maize genes *crs1* and *crs2* (chloroplast RNA splicing) [71]. Mutations in *crs1* lead to a specific defect in the splicing of the *atpF* intron, whereas mutations in *crs2* disrupt the splicing of many group II introns [71, 72]. Interestingly, all of the introns that require *crs2* for efficient splicing fall into subgroup IIB, one of two group II intron subclasses predicted on the basis of differences in intron structure [73]. In contrast, nuclear mutants of barley and maize that lack plastid ribosomes fail to splice all of the chloroplast introns in subgroup IIA [71, 72, 74, 75]. Thus, a plastid gene product is required for group IIA splicing. It is likely that the critical plastid-encoded protein is the product of the chloroplast *matK* gene, given its similarity to maturases that facilitate group II intron splicing in fungal mitochondria (reviewed in [76]). However, a role for MatK in splicing has not yet been definitively demonstrated. It is noteworthy that the splicing of chloroplast group IIA and group IIB introns is facilitated by different factors and it will be interesting to decipher the structural basis for these distinct requirements. Furthermore, these findings demonstrate that most or all group II introns in chloroplasts do not self-splice in vivo: of the 17 group II introns in the maize chloroplast genome, only one is spliced in both *crs2* mutants and in mutants lacking plastid ribosomes [71, 72].

Both the *crs1* and *crs2* genes have been cloned by transposon tagging. The *crs1* gene gives rise to two different mRNAs by alternative splicing (B. Till et al., in preparation). The predicted gene products are highly basic, have predicted chloroplast transit peptides, and are related to a family of proteins of unknown function in Arabidopsis. Work with *crs2* has progressed somewhat further. CRS2 is a chloroplast-localized protein found primarily in the stromal compartment and is in a complex that includes an RNA component (B. Jenkins, A. Barkan, in preparation). The predicted amino acid sequence of CRS2 closely resembles a class of enzymes called peptidyl-tRNA hydrolases. Peptidyl-tRNA hydrolases (PTH) serve to recycle peptidyl-tRNAs released as abortive translation products [77]. It is not yet clear whether CRS2 maintains PTH activity. On the one hand, many of the amino acid residues that are critical for the activity of *E. coli* PTH are conserved in CRS2. On the other hand, one such residue is not conserved, and attempts to complement a temperature sensitive *pth* mutant of *E. coli* with *crs2* expression constructs have failed (B. Jenkins and A. Barkan, in preparation). This situation is reminiscent of that described above for the *C. reinhardtii* pseudouridine synthase-like protein involved in the *trans*-splicing of the *psaA* mRNA. In both cases, an enzyme that evolved to catalyze a biochemical reaction involving an RNA substrate was subsequently recruited to facilitate the splicing of group II introns.

4.4. RNA processing and stability

The maturation of the 3' ends of chloroplast mRNAs has been studied in spinach and *C. reinhardtii* chloroplast extracts, revealing a two-step mechanism involving endonucleolytic cleavage followed by exonucleolytic processing back to a hairpin loop (reviewed in [61]). A nuclear gene in *C. reinhardtii* likely to be involved in this process was identified in a screen for suppressors of a deletion of the *atpB* 3' hairpin loop [58]. Mutations in this gene, called *Crp3*, alter the 3' processing of several chloroplast transcripts [58, 78]. It was proposed that the *Crp3* gene product is a component of the general 3' end processing machinery [78].

Chloroplast mRNAs in land plants and in *C. reinhardtii* are typically processed at their 5' ends. For example, the *C. reinhardtii psbA* mRNA exists in two forms that differ at their 5'end, the shorter one being more prevalent and possibly resulting from the processing of the longer transcript. A correlation has been noted between the presence of the shorter transcript and the rate of synthesis of D1, the *psbA* gene product: the shorter form is missing in two nuclear mutants, *hf261* and *hf1085*, that exhibit reduced rates of D1 synthesis [79], and is also reduced in strains with *cis*-acting mutations in the *psbA* 5' UTR that block ribosome association. Therefore, translation initiation and formation of the shorter transcript are in some way intertwined. A similar phenomenon has been observed in two mutants in land plants. The maize *crp1* gene is required for cleaving *petD* coding sequences from upstream RNA in a polycistronic precursor. In *crp1* mutants, a defect in this processing event is accompanied by a decrease in the rate of synthesis of the *petD* gene product [5]. That the RNA processing defect may be the cause of the defect in *petD* translation is supported by secondary structure modeling, which suggests that the translation initiation region is masked in the unprocessed precursor but not in the final processed mRNA [5]. In a similar vein, mutations in the Arabidopsis nuclear gene *hcf107* cause a defect in the synthesis of the *psbH* gene product and a concomitant loss of those *psbH* transcripts with the fully processed 5' terminus (Felder, Meierhoff, Meurer, Driemell, Plücken, Klaff, Bechtold and Westhoff, personal communication). Again, RNA structure predictions suggest that the processing relieves an inhibitory RNA structure. These genetic data support the notion that 5' processing of chloroplast mRNAs serves, in general, to increase the translational efficiency of chloroplast mRNAs. This conclusion is further supported by a corre-

lation between 5' end processing and translational activity of two other chloroplast mRNAs: unprocessed *ndhD* mRNA is poorly translated in in vitro translation extracts from tobacco chloroplasts [6] and, in methyl-jasmonate treated barley seedlings, *rbcL* mRNA is aberrantly processed at its 5' end and is also inefficiently translated [80].

A variety of nuclear mutations have been described that alter the accumulation of chloroplast mRNAs due to post-transcriptional defects. In general, those in *C. reinhardtii* fail to accumulate a specif c chloroplast mRNA or several mRNAs derived from a single gene cluster, in contrast to those in land plants, which exhibit more global defects. In *C. reinhardtii*, the amount of *atpA* mRNA is decreased in *ncc1* mutants [81], *atpB* mRNA is absent in *thm24* mutants [81], *petA* mRNA is missing in the *MΦ11* mutants [82], *petB* RNA is missing in the *MΦ37* [82], *petD* RNA is missing in the mutant *mcd1* [83], *psbC* RNA is missing in the mutant 6.2Z5 [84], transcripts from the *psbB/T/H* cluster are missing in the mutants *mbb1*-222E and GE2.10 [84, 85], and *psbD* RNA is missing in the mutant *nac2-26* [86]. For each of these mutants, run-on transcription assays or in vivo pulse-labeling experiments have shown that the affected RNA is synthesized at normal rates, implying that its absence is due to its rapid degradation. In the mutant *ncc1*, the degradation of *atpA* RNA probably initiates in the 3' part of the transcript [81]. In contrast, the 5'UTR of the *atpB*, *petD*, *psbD* or *psbB* mRNA is sufficient to confer instability to reporter transcripts in the respective mutant backgrounds, implying that these 5' UTRs contain important determinants of RNA stability [42, 83, 87] (B. Rimbault, D. Drapier and F.A. Wollman, personal communication). The notion that these 5' UTRs contain the site of a rate-limiting step in mRNA decay is further supported by the f nding that a 5'→ 3' exonuclease is involved in the degradation of these RNAs [84, 87–89]. In the case of *psbB* and *psbD*, for which there are two alternative 5' ends, the short forms, but not the longer ones, are missing in the mutants. The inter-relationships among 5' processing, endonucleolytic cleavage, exonucleolytic degradation, and translation, and also the precise role of the nucleus-encoded factors in these processes, remain to be unraveled.

The *Nac2* and *Mbb1* genes, discussed above, have been cloned. In view of their similar roles, it is striking that both proteins contain tetratricopeptide repeats (TPR), a motif that mediates a wide variety of protein-protein interactions in various organisms and cellular compartments [90]. Nac2 and Mbb1 are found in large but distinct complexes in the chloroplast stroma (E. Boudreau, S. Lemaire, F. Vaistij, M. Goldschmidt-Clermont and J.-D. Rochaix, unpublished). It will be critical to identify the other components of these complexes to understand how these proteins inf uence the stabilities of specif c RNAs.

Results from the genetic analysis of chloroplast RNA stability in land plants are less extensive but do suggest that the regulatory circuits may be different from those in *C. reinhardtii*. All of the mutations thus far recovered affect the accumulation of transcripts from multiple transcription units. Arabidopsis *hcf109* mutants accumulate reduced levels of transcripts from four polycistronic transcription units (*psbB, psbD, ndhC, ndhH*) [91]. Run-on transcription analysis demonstrated that this is due to a defect in RNA stability rather than in transcription. Only a subset of the transcripts from each of these transcription units is affected in *hcf109* mutants, and the transcripts from several transcription units are unaffected. Therefore, the *hcf109* gene product plays a broad role in RNA metabolism but is not universally required. Analogous observations have been made for the Arabidopsis mutants *hcf5* [92] and *pac* [93], and for the maize mutant *hcf38* [94], although in these cases run-on transcription experiments have not been performed to test whether the defects are post-transcriptional. Maize *hcf38* mutants accumulate an aberrant form of the major *psbB* RNA and reduced amounts of *petA*, *psaA*, and *atpB* mRNAs [94]. Arabidopsis *hcf5* mutants accumulate reduced levels of *rbcL* and *psbH* transcripts [92]. Given the global loss of chloroplast-encoded proteins in *hcf5* mutants, it is very likely that other transcripts that were not examined are also affected. Arabidopsis *pac* mutants fail to accumulate normal levels of the chloroplast *psbA, psbB, psbC/D, rbcL, psbE/F, and psaA* RNAs, although other transcripts do accumulate normally [93]. The loss of the normal *rbcL*, *psbE/F* and *psaA* transcripts in *pac* mutants is accompanied by an increase in aberrant higher molecular mass forms. In the case of *rbcL*, the extension is at the 3' end, leading to the hypothesis that the *pac* gene product functions in the maturation of 3' termini. However, the possibility that the defect is in transcription termination has not been excluded.

Two land plant mutants have the unique property of accumulating increased rather than decreased levels of specif c chloroplast mRNAs. The Arabidopsis *hcf2* mutant accumulates f ve-fold more *petA* mRNA than wild-type plants [95]; other transcripts examined accumulate normally. This is not a consequence of the loss of the cytochrome b_6f complex (to which the *petA* gene product belongs) because many plant mutants lacking this complex accumulate normal levels of *petA* mRNA [5, 61, 96]. Thus, the wild-type *hcf2* gene product may act to destabilize the *petA* RNA. In the maize mutant *bsd2-m1*, the chloroplast *rbcL* mRNA accumulates ectopically in mesophyll chloroplasts [97]. The *bsd2* gene has been cloned [98] and its gene product bears resemblance to DnaJ chaperones. It was proposed that BSD2 inf uences *rbcL* RNA stability indirectly by relieving the aggregation of nascent RbcL peptides emerging from the ribosome, which might otherwise stall ribosomes. According to this theory, it is the stalled ribosomes that stabilize the *rbcL* mRNA. A correlation between ribosome-association and stabilization of the maize *rbcL* mRNA had been observed previously [99], and is consistent with this hypothesis. The

ability of the chloroplast translation machinery to influence RNA metabolism is further illustrated by the fact that antibiotics that affect plastid ribosome behavior also influence RNA stability in spinach chloroplasts [100]. Dramatic and rather uniform defects in plastid transcript populations have been noted in a collection of albino maize mutants such as *iojap*, which lack plastid ribosomes [101, 102]. Some of these changes may be the consequence of transcription occurring solely via the nucleus-encoded RNA polymerase, but others may be the consequence of altered RNA stability or processing in the absence of plastid ribosomes.

4.5. Translation

Biochemical purification has been the primary means to characterize the plastid translation machinery, leading to the identification of nucleus-encoded plastid ribosomal proteins, initiation factors, elongation factors, and tRNA synthetases (reviewed in [15]). Although the majority of these proteins are related to their bacterial counterparts, several plastid-specific ribosomal proteins have been detected. The roles of these proteins in translation have not been established, but it is possible that they participate in the unique aspects of plastid translation. Several maize mutants exhibit a global loss of chloroplast-encoded proteins coupled with an overall reduction in the size of chloroplast polysomes [99], and several *C. reinhardtii* mutants have a reduced content of chloroplast ribosomes (reviewed in [15]). The genes defined by these mutations have not been cloned but may encode components of the plastid translation machinery or factors required for its assembly. Mutations in the *C. reinhardtii* nuclear gene *Sim30* can suppress the effects of initiation codon mutations in the chloroplast [103], suggesting that Sim30 participates in translation initiation. A genetic screen for genes involved in chilling resistance in Arabidopsis led to the identification of a nuclear gene, *DIM1*, that encodes a plastid rRNA methylase [104]. Interestingly, *DIM1* function is required for the assembly of functional ribosomes only when plants are grown at low temperatures.

The maize mutant *iojap* and the barley mutant *albostrians* have been widely studied because homozygous mutant plants lack plastid ribosomes in sectors or in their entirety [105, 106] and can be used to assess the contribution of plastid-encoded proteins to various processes. The basis for the ribosome loss in these mutants is not known. The *iojap* gene has been cloned [107] and encodes a protein that is found in association with 50S ribosomal subunits and also free in the stroma (C.-D. Han, R. Martienssen, personal communication) but the biochemical role of the protein has not been established.

Genetic approaches in *C. reinhardtii* have identified numerous nuclear loci required for the translation of specific chloroplast mRNAs. The *petA* mRNA is not translated in the mutant *tca1* [108], *psaB* mRNA is not translated in the mutant *tab1-F15* [109], *psbA* mRNA is not translated in the mutants *F35*, *hf149*, *hf233*, *hf261*, *hf859* and *hf1085* [43, 110], *psbC* mRNA is not translated in *tbc1-F34* and *tbc2-F64* [56], and *psbD* mRNA is not translated in the mutants *nac1-18* and *ac115* [111, 112]. Translation of chimeric reporter genes fused to the 5'UTRs of the *psaB*, *psbC* or *petA* mRNAs is also reduced in the respective nuclear mutant backgrounds, implying that these defects are at the level of translation initiation [37, 109] (K. Wostrikoff, J. Girard-Bascou, Y. Choquet and F.A. Wollman, personal communication). However, the mutations affecting *psbD* translation may act at a later stage, in elongation or stabilization of the nascent product [112]. A nuclear locus involved in *psbC* translation, *TBC3*, was identified by screening for suppressors of a *cis*-acting mutation in the 5'UTR of the *psbC* mRNA. Interestingly, the same mutation suppressed the effects of mutations in another nuclear locus involved in *psbC* translation, *TBC1* [113].

The *Ac115*, *Tbc2* and *Tab1* genes have been cloned by complementation rescue of the corresponding mutants. Ac115 and Tab1 are novel polypeptides with putative chloroplast transit sequences [112] (F. Laroche, J.D. Rochaix, personal communication). Tbc2 is a large chloroplast protein (1115 amino acids) with a region of approximately 400 residues that shares 37% similarity with CRP1, a maize chloroplast protein involved in translation and RNA processing (A. Auchincloss, W. Zerges and J.D. Rochaix, personal communication). The possible relationship between these two proteins is discussed below.

In land plants, only two nuclear genes have been found whose mutation disrupts the translation of subsets of chloroplast mRNAs, contrasting with the frequent recovery of this type of mutant in *C. reinhardtii*. The maize gene *atp1* is required specifically for the translation of the chloroplast *atpB/E* mRNA [114] and is the only nuclear gene in a land plant known to function in the translation of a single plastid mRNA. In *atp1* mutants, there is a reduction in the size of polysomes containing *atpB/E* mRNA, indicating that the defect occurs during translation initiation or early in elongation [114].

Mutations in the maize gene *crp1* also disrupt the translation of a subset of plastid mRNAs, but here the story is more complex. *crp1* mutants do not synthesize the chloroplast *petA* and *petD* gene products [5]. The defect in *petD* synthesis is accompanied by the failure to cleave a monocistronic *petD* mRNA from its polycistronic precursor; indeed, the RNA processing defect may be the cause of the reduced rate of *petD* translation. The defect in *petA* expression, however, is solely at the translational level as its mRNA is of normal size and abundance. Analysis of double mutants that lack both plastid ribosomes and *crp1* function provided evidence that the translation defects do not cause the *petD* RNA processing defect. These and other data led to the hypothesis that CRP1 acts independently in *petD* RNA processing and *petA* translation [115].

The *petA* mRNA is associated with few ribosomes in *crp1* mutants, indicating a defect in translation initiation [5].

The *crp1* gene was cloned by transposon tagging. CRP1 is related to the fungal proteins Pet309p and Cya5p, which are required for the translation of the mitochondrial *cox1* mRNA [115], and to the *C. reinhardtii* protein Tbc2, involved in *psbC* translation (see above). The similarity in protein sequences and mutant phenotypes suggest an underlying mechanistic similarity between these proteins, but it is not known how any of them inf uence translation. Several predicted chloroplast-localized proteins of unknown function in maize and Arabidopsis also resemble CRP1. Given that Tbc2 in *C. reinhardtii* targets *psbC* translation and CRP1 in maize targets *petA* translation, it is tempting to speculate that within a species, different members of this protein family control the translation of different plastid mRNAs, possibly by interacting with different mRNA-specif c factors.

CRP1 is a stromal protein that is found in a stable complex of ≅ 300 kDa [115]. It contains a tandemly repeated motif that loosely resembles the TPR motif, which is found in proteins that inf uence the stabilities of chloroplast RNAs in *C. reinhardtii* (see above). These motifs in CRP1 may mediate the formation of the 300 kDa CRP1 complex and/or may mediate more transient interactions between CRP1, the target RNAs, and the translation/RNA processing machinery. *C. reinhardtii tca1* mutants, like *crp1* mutants, fail to translate the chloroplast *petA* mRNA. It will be interesting to learn whether Tca1 and CRP1 are related to one another.

The assembly status of multisubunit chloroplast enzymes may also inf uence the translation of specif c chloroplast mRNAs via undef ned feedback mechanisms. For example, deletion of the *C. reinhardtii petD* gene results in a defect in *petA* translation [116], and reduced synthesis of the small subunit of Rubisco in tobacco and *C. reinhardtii* causes reduced translation of the chloroplast mRNA encoding the large subunit [117, 118]. Therefore, loss of translation of a particular chloroplast mRNA might, in some cases, be a secondary effect of a primary lesion in enzyme assembly.

4.6. Interactions between RNA metabolism, translation and membrane assembly

The fact that transcription, transcript maturation, and translation occur in the same compartment in the chloroplast necessitates a consideration of how these processes might inf uence one another. Indeed, there is evidence that ribosome association can inf uence the stability of chloroplast RNAs (see above). Thus, the cause and effect relationship between defects in RNA processing, RNA splicing, RNA decay and translation can be difficult to decipher. For instance, two 5' ends have been mapped for mRNAs from the *C. reinhardtii psbA*, *psbB* and *psbD* genes, a minor long form and a much more abundant shorter form. Several different nuclear mutations, each of which act on just one of these genes, result in the absence of only the shorter form. In the case of *psbA*, the longer form accumulates to increased levels in the mutants so that RNA levels appear normal on Northern blots, but the D1 protein is not translated [43, 79]. However, for the mutants affecting *psbB* and *psbD*, the long forms are present in the same low amounts as in the wild-type, but no RNA is detected on Northern blots due to lack of the shorter forms [87, 89]. One interpretation of these f ndings is that the primary defect caused by these mutations is in translation for *psbA* and processing or decay for *psbB* and *psbD*. Alternatively, it is possible that the primary defect in all of these cases is in 5' processing, and that 5' processing is a prerequisite for stabilization of the mRNA or for its translation. These processes may also be coupled in some other way.

An intertwining of membrane targeting with chloroplast translation and RNA metabolism has been suggested by recent observations. Several chloroplast RNA-binding proteins and two factors involved in *psaA trans*-splicing (Maa1 and Maa2) are associated with a membrane fraction in *C. reinhardtii* [70, 119] (M. Goldschmidt-Clermont, unpublished results), suggesting that targeting of certain mRNAs to the membrane may be involved in their expression. Furthermore, mutations in maize that disrupt the nuclear gene encoding cpSecY, a component of the protein translocation machinery in the thylakoid membrane, lead to the loss of thylakoid membrane and to a defect in plastid translation [120]. The coordinate, membrane-localized synthesis of products of chloroplast genes such as *psaA* and *psaB,* which are chlorophyll-binding subunits of PSI, or *psbA*, *psbB*, *psbC*, and *psbD*, which are chlorophyll-binding subunits of PSII, may be necessary for their membrane insertion and/or assembly. It is not universally true, however, that proteins involved in chloroplast RNA metabolism and translation are membrane bound. Several RNA binding proteins in tobacco chloroplasts, the maize protein CRP1, and the *C. reinhardtii* factors Maa3, Mbb1 and Nac2 are not associated with membranes [19, 115] (E. Boudreau, S. Lemaire, J. Mickelsen, F. Vaistij, C. Rivier, M. Goldschmidt-Clermont and J.-D. Rochaix, unpublished results).

5. Perspectives

The nuclear mutations affecting chloroplast gene expression that have been recovered to date have two striking properties: many disrupt the expression of individual chloroplast genes, and most or all affect post-transcriptional steps of gene expression. It is not clear whether these properties ref ect the true nature of the interactions between the nucleus and the chloroplast, or whether the types of mutations recovered are biased by the screening strategies that have been employed. For example, in *C. reinhardtii*, a minimal level of chloroplast gene expression may be required for cell viability even

under growth conditions that are permissive for non-photosynthetic mutants. This is suggested by the fact that no mutants have been recovered that lack chloroplast gene expression entirely. Because numerous essential components of the chloroplast gene expression machinery (e.g., tRNAs, rRNAs, ribosomal proteins) are encoded by chloroplast genes, any nuclear mutation that disrupts the synthesis of any of these factors may not be recoverable. In contrast, the viability of albino *iojap* and *albostrians* seedlings illustrates that maize and barley seedlings germinate and grow normally in the complete absence of chloroplast translation, until such time that seed reserves are exhausted [105, 106]. Therefore, at least in the grasses, it should be possible to recover any mutant that fails to synthesize any or all chloroplast-encoded proteins. This may explain why the mutants described in land plants, in general, cause broader lesions than those recovered in *C. reinhardtii*.

The failure to recover mutants with defects in chloroplast transcription could well be due to biases in the genetic screens. The vast majority of mutants that have been analyzed in detail accumulate a signif cant amount of chlorophyll. In fact, the *hcf* phenotype requires the presence of chlorophyll, the source of the f uorescence. If most factors inf uencing plastid transcription affect large suites of chloroplast genes, then corresponding mutant phenotypes might be albino. However, few albino mutants have been analyzed in detail because their protein and RNA defects are so pleiotropic that it can be difficult to make meaningful hypotheses about the nature of the lesion. Now that chloroplast-localized sigma-like factors have been identif ed via biochemical and genomic approaches (reviewed in [1]), it will be interesting to use reverse genetics to discover their corresponding mutant phenotypes. These phenotypes may suggest approaches for identifying transcriptional defects via forward genetic screens.

It is not yet clear to what extent the networks linking the nucleus and chloroplast have been conserved between *C. reinhardtii* and land plants. These organisms diverged long ago, and this is ref ected by the entirely different organizations of their chloroplast genomes [121, 122]. Although many of the basic aspects of chloroplast gene expression are likely to have been conserved, there are hints emerging that some aspects have diverged. For example, *cis*-acting determinants of transcript stability in the 5' UTRs of two land plant chloroplast mRNAs did not stabilize mRNAs in transgenic *C. reinhardtii* chloroplasts [123]. The feedback mechanism in *C. reinhardtii* linking *petA* translation to the assembly of its gene product [116] differs in tobacco [61]. In addition, assays for proteins that bind to the 5' UTR of the *psbA* mRNA in spinach and *C. reinhardtii* revealed different proteins (see above). Finally, the results of the genetic screens suggest that nucleus-encoded factors in plants generally target multiple chloroplast genes, in contrast to the gene-specif city that is typical of *C. reinhardtii*. The low incidence of allelism to date indicates that many nuclear loci that inf uence chloroplast gene expression have yet to be identif ed in both land plants and *C. reinhardtii*. Only when genetic analyses are more saturating will it be possible to determine whether these early results ref ect biases in the genetic screens or are truly an indication that the regulatory circuits are less integrating in *C. reinhardtii* than in land plants.

5.1. Evolution of nucleus-encoded factors involved in chloroplast gene expression

Nucleus-encoded factors that inf uence chloroplast gene expression have several potential origins. They may have arisen from the genome of the organelle's ancestral endosymbiont or, alternatively, they may have been recruited from the ancestral host genome. In either case, their original role may or may not have been retained. Although only a handful of these genes have been cloned thus far, it is already clear that they have diverse evolutionary origins. Several genes are of bacterial origin, including maize *iojap* (R. Martienssen, personal communication) and maize *crs2*. Whether the original biochemical role of IOJAP has been retained cannot be evaluated, since the function of its bacterial homolog is unknown. However, the available data suggest that CRS2 may not have retained its ancestral function as a peptidytl tRNA hydrolase (B. Jenkins, A. Barkan, in preparation), and that it acquired a new function in facilitating group II intron splicing by a customizing of the protein. The analogous situation likely holds true for the *Maa2* gene of *C. reinhardtii*. Maa2 resembles pseudouridine synthases found in both prokaryotes and eukaryotes, yet this enzymatic activity is not required for its role in *trans*-splicing of *psaA* (see above). Thus both Maa2 and CRS2 may have been recruited during evolution to participate in mRNA splicing in the chloroplast, perhaps because their ability to interact with RNA enabled them to easily adopt a function as an RNA chaperone.

Other proteins involved in chloroplast gene expression appear to have been recruited from the host genome. Maize CRS1 and CRP1 do not have any close counterparts in bacteria, but related proteins in maize and Arabidopsis lacking predicted chloroplast targeting sequences have been identif ed (A.Barkan, unpublished results). In *C. reinhardtii*, the RB47 and RB60 proteins, purif ed following affinity chromatography to the 5'UTR of *psbA* mRNA, were derived from the ancestral host genome: RB47 belongs to the family of poly(A)-binding proteins (PABP) [27], and RB60 to the family of protein disulf de isomerases (PDI) [28]. Cytoplasmic PABPs participate in translation initiation by interacting with initiation factors that bind the 5' end of the mRNA. Thus, RB47 may have been recruited to play a similar role in chloroplast translation initiation, but by binding directly to the 5' UTR

[43]. RB60 has been proposed to modulate the RNA binding activity of RB47 in a redox-dependent fashion; this is consistent with the activities of PDIs, which catalyze the oxido-reduction of sulfhydryl groups [28]. These examples reveal that both endosymbiont and host proteins with other functions have been recruited and tailored to fulfill new roles as modulators of chloroplast gene expression.

5.2. Role of nucleus-encoded factors in chloroplast gene regulation

The genetic analyses summarized here have revealed factors that function in plastid gene expression, but little is known about whether they are required constitutively or play a truly regulatory role. In maize chloroplasts, the splicing of several group II introns is subject to developmental regulation [11]; it will be interesting to determine whether the products of the *crs1* and *crs2* genes, required for the splicing of these introns, are also responsible for this regulation. Analogously, the translation of the *psbA* and *psbD* mRNAs is enhanced in the light [14, 17, 124]. Among the genetically-identified nuclear genes required for *psbA* or *psbD* translation [43, 110–112], some may encode factors that mediate this light regulation. The biochemically-identified proteins RB47 and RB60 have been proposed to function in the light-regulated translation of the *psbA* mRNA in *C. reinhardtii* [26, 28, 43], but the relevance of these findings to the in vivo setting remains to be demonstrated. A joining of genetic and biochemical approaches, for example a demonstration that genetically-identified nuclear genes involved in *psbA* translation encode these proteins, will be important for testing this model.

5.3. Looking towards the future

The number of nuclear loci that participate in chloroplast gene expression is remarkably large. An extreme example is the *psaA* gene in *C. reinhardtii,* for which at least 14 nuclear genes are required simply for the *trans*-splicing of its mRNA. It is not unusual to find that several nuclear loci are involved in the expression of a single chloroplast gene in *C. reinhardtii*. Because many of these loci are represented by only one mutant allele, it is clear that many loci remain to be discovered. Assuming that an average of between two and five loci function specifically in the expression of 50 to 100 chloroplast genes, a total of several hundred nuclear genes may participate in the expression of subsets of chloroplast genes. Many additional loci encode the components of the gene expression machinery itself. A similar situation exists in land plants, where the low incidence of allelism indicates that the genes thus far identified represent only a small fraction of the total. A major challenge will be to identify all such genes, to understand the biochemical role of each protein, and to elucidate how their activities are intertwined in regulatory circuits. This will require the combined application of genetics and biochemistry, and will be facilitated by the development of genomic and proteomic tools in model organisms. Classical genetic studies can now be supplemented by approaches that take advantage of the massive genome sequence databases. For example, the complete sequence of the Arabidopsis genome, expected within a few years, can be used to rapidly identify genes encoding biochemically-identified proteins, as well as new genes whose sequence is suggestive of a role in chloroplast gene expression. Reverse genetic methodologies that can be used to assess the effects of eliminating such proteins in vivo are currently available in maize and Arabidopsis. It is anticipated that the use of these tools, in conjunction with transgenic chloroplast technologies and classical genetics, will lead to rapid advances in our understanding of the networks linking chloroplast gene expression with events in the host cell.

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