A Major Role for the Plastid-Encoded RNA Polymerase Complex in the Expression of Plastid Transfer RNAs

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Chloroplast transcription in land plants relies on collaboration between a plastid-encoded RNA polymerase (PEP) of cyanobacterial ancestry and a nucleus-encoded RNA polymerase of phage ancestry. PEP associates with additional proteins that are unrelated to bacterial transcription factors, many of which have been shown to be important for PEP activity in Arabidopsis (Arabidopsis thaliana). However, the biochemical roles of these PEP-associated proteins are not known. We describe phenotypes conditioned by transposon insertions in genes encoding the maize (Zea mays) orthologs of five such proteins: ZmPTAC2, ZmMurE, ZmPTAC10, ZmPTAC12, and ZmPRIN2. These mutants have similar ivory/virescent pigmentation and similar reductions in plastid ribosomes and photosynthetic complexes. RNA gel-blot and microarray hybridizations revealed numerous changes in plastid transcript populations, many of which resemble those reported for the orthologous mutants in Arabidopsis. However, unanticipated reductions in the abundance of numerous transfer RNAs (tRNAs) dominated the microarray data and were validated on RNA gel blots. The magnitude of the deficiencies for several tRNAs was similar to that of the most severely affected messenger RNAs, with the loss of tml-UAA being particularly severe. These findings suggest that PEP and its associated proteins are critical for the robust transcription of numerous plastid tRNAs and that this function is essential for the prodigious translation of plastid-encoded proteins that is required during the installation of the photosynthetic apparatus.

Transcription in land plant chloroplasts involves the interplay of two RNA polymerases with distinct evolutionary origins (for review, see Liere et al., 2011; Yagi and Shiina, 2012): a single-subunit, phage-like nucleus-encoded RNA polymerase (NEP) and a multisubunit plastid-encoded RNA polymerase (PEP) derived from the cyanobacterial enzyme. The core PEP subunits are encoded by the plastid rpoA, rpoB, rpoC1, and rpoC2 genes; this core polymerase is targeted to specific promoters by interaction with any of several nucleus-encoded sigma factors that are derived from bacterial sigma-70 (for review, see Lerbs-Mache, 2011). In addition, roughly 10 different nucleus-encoded proteins that are unrelated to bacterial transcription factors consistently copurify with PEP (Suzuki et al., 2004; Pfalz et al., 2006; Steiner et al., 2011). Genetic analyses in Arabidopsis (Arabidopsis thaliana) have shown that most of these PEP-associated proteins are important for the accumulation of transcripts derived from PEP promoters and for chloroplast biogenesis (for review, see Yagi and Shiina, 2012; Pfalz and Pfannschmidt, 2013). That the transcription of a small genome encoding fewer than 100 products requires such a diversity of novel components presents numerous evolutionary and mechanistic questions.

Pioneering studies that explored the contributions of PEP and NEP with a tobacco (Nicotiana tabacum) plastome mutant lacking RpoB concluded that plastid photosystem genes are transcribed primarily by PEP, whereas most other genes can be transcribed by either NEP or PEP (Allison et al., 1996; Hajdukiewicz et al., 1997). This question was subsequently addressed more comprehensively by analysis of the plastid transcriptome in a tobacco rpoA knockout (Legen et al., 2002), by comparison of transcription start sites in normal and ribosome-deficient barley (Hordeum vulgare) plastids (Zhelyazkova et al., 2012), and by profiling plastid transcriptomes in the presence of an inhibitor of PEP (Demarsy et al., 2006, 2012). These studies demonstrated that most plastid genes can be transcribed by either NEP or PEP. That being said, PEP and NEP preferentially influence mRNA levels from photosystem and genetic system genes, respectively, in a manner that resembles the original view (for review, see Yagi and Shiina, 2012).

The recovery of PEP in a large complex whose mass is dominated by non-PEP proteins (Steiner et al., 2011) is intriguing, given that bacterial RNA polymerases form stable complexes with only a few proteins whose mass is negligible in comparison with the polymerase itself (for review, see Haugen et al., 2008). Furthermore, the PEP-associated proteins are not required for PEP-mediated transcription.
transcription in vitro (Hu and Bogorad, 1990), although their absence disrupts PEP-mediated transcription in vivo (Pfalz et al., 2006; Garcia et al., 2008; Myouga et al., 2008; Arsova et al., 2010; Gao et al., 2011, 2012; Steiner et al., 2011; Jeon et al., 2012; Yagi et al., 2012). A recent model posits that the PEP-associated proteins are required to establish a subdomain in the plastid nucleoid that is required for PEP-mediated transcription (Pfalz and Pfannschmidt, 2013).

In this report, we describe a set of maize (Zea mays) mutants lacking several PEP-associated proteins whose orthologs have been genetically characterized in Arabidopsis (PTAC2, PTAC12, MurE, and PRIN2; Pfalz et al., 2006; Garcia et al., 2008; Kindgren et al., 2012) or tobacco (PTAC10; Jeon et al., 2012). The phenotypes of these mutants in maize, both visible and molecular, are similar to one another and are consistent with those reported for the orthologous mutants. However, comprehensive analysis of the plastid transcriptomes in the maize mutants revealed defects that had not been reported previously. Most notably, we discovered a deficiency for numerous plastid tRNAs, several of which were as severely affected as the “classic” PEP-dependent mRNAs. Subsequent analysis of the orthologous Arabidopsis mutants revealed similar effects. These findings strongly suggest that PEP-associated proteins (and presumably PEP itself) play an important role in the transcription of many plastid tRNAs and that this activity is relevant to the reduction in plastid ribosomes and plastid-encoded proteins in mutants lacking PEP or its associated proteins. These results highlight the complex division of labor between NEP and PEP and have implications with regard to the regulatory cascade underlying the initiation of chloroplast development.

RESULTS

The mutants described here were recovered during our systematic effort to identify causal mutations in the Photosynthetic Mutant Library (PML), a large collection of Mutator (Mu) transposon-induced non-photosynthetic maize mutants (http://pml.uoregon.edu/photosyntheticml.html; Stern et al., 2004). Molecular phenotyping of mutants in this collection revealed several nonallelic mutants with similar defects in plastid transcript populations that were distinct from the pleiotropic effects commonly observed in nonphotosynthetic mutants (examples are shown below). The causal mutations were identified in a two-step process in which (1) cosegregating Mu insertions were identified by deep sequencing of Mu insertion sites (Williams-Carrier et al., 2010) and (2) independent alleles recovered in reverse genetic screens of the PML collection were used for complementation testing with the reference alleles. Four of the genes discovered in this manner proved to encode the maize orthologs of the PEP-associated proteins PTAC2, PTAC10, PTAC12, and MurE (Pfalz et al., 2006). The fifth gene encodes the maize ortholog of Arabidopsis PRIN2 (Kindgren et al., 2012), which has not been detected as a PEP-associated protein but which localizes to the plastid nucleoid and influences plastid transcript profiles in a manner that is similar to PEP-associated proteins (Kindgren et al., 2012). The maize PRIN2 ortholog, ZmPRIN2, likewise localizes to plastid nucleoids (Majeran et al., 2012), and we show here that the plastid transcriptome in Zmprin2 mutants is similar to that of mutants lacking PEP-associated proteins. For convenience, PRIN2 is referred to below as a PEP-associated protein, although the proteomics data suggest that it is not tightly associated with the PEP complex. The recovery of Zmptac12 mutants was reported previously (Williams-Carrier et al., 2010) but with little phenotypic data. To our knowledge, the other mutants are reported here for the first time. In each case, we recovered both strong (likely null) alleles harboring exon insertions and hypomorphic alleles with insertions in 5’ untranslated regions (Fig. 1); these condition ivory and yellow-green phenotypes, respectively (Supplemental Fig. S1). The heteroallelic progeny of allelism crosses exhibit intermediate phenotypes (ivory/yellow leaf blades with greening tips; Fig. 1A), demonstrating that these mutations fail to complement and confirming that the insertions in these genes underlie the chloroplast biogenesis defects.

Because albino phenotypes are associated with a suite of pleiotropic effects that can mask mutant-specific defects (Williams and Barkan, 2003), we used the hypomorphic progeny of complementation crosses (Fig. 1) for all molecular analyses described below. Heteroallelic mutants derived from strong and weak alleles of Zmwh1 (Prikryl et al., 2008) were analyzed in parallel for comparative purposes; ZmWHY1 is an abundant plastid nucleoid protein (Majeran et al., 2012) that is not tightly associated with PEP and that is required for the biogenesis of the plastid translation machinery (Prikryl et al., 2008; Maréchal et al., 2009; Melonek et al., 2010; Steiner et al., 2011). The severities of the chlorophyll, photosynthetic protein, and plastid ribosome deficiencies are similar in the Zmwh1, Zmptac2, Zmptac10, Zmptac12, ZmmurE, and Zmprin2 heteroallelic mutants used for the experiments described here (Fig. 1A; see below). Therefore, molecular defects observed in mutants lacking PEP-associated proteins but not in the Zmwh1 mutants are not simply secondary effects of defects in photosynthesis, chlorophyll deficiency, or plastid gene expression.

Zmptac2, Zmptac10, Zmptac12, ZmmurE, and Zmprin2 Mutants Have Reduced Levels of Plastid Ribosomes and Share Characteristic Defects in Plastid mRNA Metabolism

As an initial assessment of plastid gene expression in these mutants, the abundance of photosynthetic complexes that harbor plastid-encoded subunits was examined by immunoblot analysis of one core subunit of each complex (Fig. 2A). All of the mutants exhibit a more than 10-fold loss of each marker protein. This type of “global” protein deficiency is typical of mutants with defects in the biogenesis of the plastid translation machinery (Barkan, 1993). In fact, staining of gel-resolved...
total leaf RNA revealed a reduction in plastid ribosomal RNAs (rRNAs) in these mutants (Fig. 2B, bands marked 16S and 23S*), which implies a corresponding deficiency for plastid ribosomes. Despite this ribosome defect, the RpoB subunit of the PEP complex accumulates to increased levels in Zmptac2, Zmptac10, Zmptac12, ZmmurE, and Zmprin2 mutants (Fig. 2A), consistent with the increased levels of rpoB mRNA detected on microarrays (Supplemental Data Set S1). Two different antibodies were used to detect RpoB, and both indicate that RpoB accumulates to lower levels in Zmptac12 mutants than in the other mutants examined. This hints at a distinct function for PTAC12, but our use of hypomorphic rather than null alleles in this experiment precludes firm conclusions.

RNA gel-blot assays detected various plastid transcript defects that are shared by Zmptac2, Zmptac10, Zmptac12, ZmmurE, and Zmprin2 mutants (Fig. 3). For example, the rbcL, psaB, and psbA RNAs accumulate to reduced levels in these mutants but are less affected in the Zmwhy1 control, consistent with the known role of PEP in mediating rbcL, psaB, and psbA transcription in other species (for review, see Lerbs-Mache, 2011; Liere et al., 2011). However, whereas atpB RNA levels are not strongly reduced in mutants lacking PEP or its associated proteins in Arabidopsis and tobacco (Hajdukiewicz et al., 1997; Garcia et al., 2008) or in the Zmwhy1 mutant control (Fig. 3), atpB RNA is severely reduced in the maize mutants lacking PEP-associated proteins (Fig. 3). In addition, the blots revealed a loss of specific transcript isoforms from the polycistronic transcription units encoding psac, psaj, and petG. A similar effect at psaj was reported for an Arabidopsis sigma factor sig2 mutant (Nagashima et al., 2004) and was suggested to result from a SIG2-dependent promoter. However, the Zmwhy1 mutant exhibited a similar change in the psaj transcript pattern, suggesting that the loss of monocistronic psaj RNA in the maize mutants may be an indirect effect of compromised plastid biogenesis or translation. The isoform-specific defects for psac and petG are consistent with the discovery of operon-internal PEP promoters for these genes in barley (Zhelyazkova et al., 2012).

**Figure 1.** Mutants used in this study. A, Plants were grown for 7 d in soil. The mutants shown are the heteroallelic progeny of complementation crosses involving the two alleles diagrammed in B. The Zmwhy1 mutant is heteroallelic for an exon and a 5′ untranslated region insertion (Zmwhy1-1 and Zmwhy1-2) described previously (Prikryl et al., 2008). WT, Wild type. B, Positions of Mu insertions. Protein-coding regions are indicated by black rectangles, and transcribed but untranslated regions are indicated by white rectangles. The target site duplications flanking each Mu insertion are underlined.

**Genome-Wide Microarray Analysis Reveals a Major Role for PEP-Associated Proteins in the Expression of Plastid tRNAs**

To obtain a genome-wide perspective on the effects of PEP-associated proteins on the maize plastid transcriptome, total leaf RNA from the Zmptac2 and ZmmurE mutants was compared with that in their phenotypically normal siblings by hybridization to tiling microarrays that include strand-specific probes for every annotated maize chloroplast gene. Each protein-coding gene was represented across its length by overlapping 50-mers, each rRNA was represented by several 50-mers, and each tRNA was represented by a single 50-mer (Supplemental Data Set S1). The results are presented as the ratio of signal in the wild type versus the mutant (Fig. 4B) or as separate plots of the signal in the wild-type and mutant samples (Fig. 4C). The transcriptome profiles for the Zmptac2 and ZmmurE mutants were quite similar and included a set of dominating “peaks” representing severe deficiencies for a subset of RNAs. However, although
Pep is generally described as being particularly important for the transcription of genes encoding photosystem proteins (Hajdukiewicz et al., 1997; Pfalz et al., 2006; Gao et al., 2011; Kindgren et al., 2012; Yagi and Shiina, 2012), the psbA mRNA was the only such RNA among the dominating peaks. Other known Pep-dependent mRNAs (e.g. psaA/psaB and rbcL) were revealed in the microarray data, but the magnitude of the difference between wild-type and mutant samples appeared relatively small. It is important to note, however, that these plots underrepresent the magnitude of plastid mRNA deficiencies in the mutants due to the normalization method that was employed: values are represented as a fraction of total chloroplast RNA rather than total leaf RNA because the array contained only chloroplast probes, and the highly abundant plastid rRNAs and tRNAs are substantially reduced in the mutants. In any case, the major differences between the wild-type and mutant profiles were largely restricted to tRNAs and rRNAs. Pep is known to contribute to rRNA transcription (Sriraman et al., 1998; Suzuki et al., 2003) and to the transcription of several tRNAs (Kanamaru et al., 2001; Legen et al., 2002; Ishizaki et al., 2005). However, the most severe tRNA defects suggested by our microarray data (e.g. tmL-UAA, tmF-GAA, tmL-CAA, and tmL-UAG) as well as the widespread effects on tRNA abundance have not been highlighted previously. Microarray analysis of plastid transcriptomes in apical versus basal leaf tissue (representing mature and immature chloroplasts, respectively) yielded profiles that were very similar to those of the wild type versus Zmptac2 or Zmptac12 mutants (Supplemental Fig. S3). These results support the prevailing view that Nep-mediated transcription dominates in immature chloroplasts whereas Pep-mediated transcription dominates in mature chloroplasts and provide additional evidence that Pep stimulates the expression of many plastid tRNAs.

Quantification of abundant RNAs such as tRNAs and rRNAs by microarray hybridization can be problematic. Therefore, 12 plastid tRNAs were further assayed by RNA gel-blot hybridization (Fig. 5). The RNA gel-blot data showed that tmL-UAA is almost absent in the Zmptac12 and Zmpetr10 mutants and that it is severely reduced in Zmptac2 and Zmprin2 mutants. By contrast, tmL-UAA accumulates to much higher levels in the Zmptac12 mutant, indicating that its loss in mutants lacking Pep-associated proteins does not result solely from a deficiency for chlorophyll, plastid ribosomes, or photosynthesis. The tmL-UAA gene includes a group I intron; both the unspliced precursor and spliced product are reduced, as would be expected for a defect in transcription.

Quantification of the RNA gel-blot data (Fig. 5B; Supplemental Table S1) revealed that tmK-UUU, tmQ-UUG, tmS-UCA, tmL-UAA, tmF-GAA, and tmL-UAG were decreased at least 2-fold more in all of the mutants lacking Pep-associated proteins than in the Zmptac12 mutant control. By contrast, the loss of tmS-GGA and tmL-CAA may be secondary effects, as these were reduced to a similar extent in Zmptac12 mutants as in mutants lacking Pep-associated proteins. Strong defects (in comparison with Zmptac1) were also observed for tmM, tmE-UUC, tmW-CCA, and tmA-UCC but only in a subset of the mutants lacking Pep-associated proteins. The differential effect of Pep-associated proteins on tmE

Figure 2. Chloroplast protein and ribosome deficiencies caused by mutations in maize genes encoding Pep-associated proteins. A, Immunoblots of total leaf extract (5 μg of protein and the indicated dilutions) were probed with antibodies against one core subunit of each photosynthetic enzyme complex: AtpB (ATP synthase), D1 (PSII), PsbD (PSI), and PetD (cytochrome b6). Duplicate blots were probed with antibodies to RpoB, a plastid-encoded subunit of Pep: the blot labeled RpoB1 was probed with an antibody raised to Arabidopsis RpoB, and the blot labeled RpoB2 was probed with an antibody raised to rice RpoB. The Porcneau S-stained blot at bottom served as a loading control and demonstrates the abundance of RbcL. B, Seedling leaf RNA (3 μg) was resolved on a denaturing agarose gel, transferred to a nylon membrane, and stained with methylene blue. 28S and 18S rRNAs are cytosolic rRNAs; 23S* is a fragment of 23S rRNA that is stained with methylene blue. 28S and 18S rRNAs are cytosolic rRNAs; 16S rRNAs are plastid rRNAs; 23S* is a fragment of 23S rRNA that is stained with methylene blue.
abundance is intriguing in light of the role for trnE in tetrapyrrole biosynthesis, which impacts retrograde signaling pathways that connect nuclear gene expression with chloroplast physiology (Woodson et al., 2012).

To determine whether these functions for PEP-associated proteins are conserved in Arabidopsis, several tRNAs were assayed in Arabidopsis mutants lacking PEP-associated proteins or the PEP sigma factor SIG6 (Fig. 5C; Supplemental Fig. S2B). As in maize, both spliced and unspliced trnL-UAA are severely reduced in the Arabidopsis ptac2, ptac10, and ptac12 mutants. Effects on the abundance of trnS-UGA, trnF-GAA, and trnE-UUC were also similar between the two species. SIG6 was shown previously to be important for transcription of the trnYED operon in Arabidopsis (Ishizaki et al., 2005). Our results confirm that observation and show further that SIG6 is important for the expression of trnF-GAA (Supplemental Fig. S2B).

DISCUSSION

Genetic analyses have shown that many of the proteins that copurify with PEP are important for chloroplast
development and for the accumulation of PEP-dependent mRNAs (Garcia et al., 2008; Myouga et al., 2008; Arsova et al., 2010; Gao et al., 2011, 2012; Steiner et al., 2011; Jeon et al., 2012; Kindgren et al., 2012; Yagi and Shiina, 2012; Pfalz and Pfannschmidt, 2013). It is intriguing that these proteins promote the activity of an enzyme that is closely related to its cyanobacterial ancestor, yet they are neither derived from bacterial transcription factors nor do they have apparent functional homologs in bacteria. Many fundamental questions about the PEP-associated proteins

Figure 4. Analysis of plastid transcriptomes in Zmptac2 and ZmmurE mutants by hybridization to a high-density microarray. Total leaf RNA was fragmented, labeled with Cy5 (the wild type [WT]) or Cy3 (mutant), and competitively hybridized to a high-density, strand-specific synthetic oligonucleotide microarray covering all annotated plastid genes. For comparison, several RNA gel-blot hybridizations using these same RNA samples are shown in Supplemental Figure S2A. A, Map of the maize chloroplast genome created with OGDRAW (Lohse et al., 2013). Asterisks mark genes that correspond to peaks in the microarray data, which reflect RNAs that are more abundant in the wild type than in the mutant. B, Ratio of signal in the wild type relative to the mutant samples. The average median of ratios (F635:F532) in each analysis was normalized to 1, such that values reflect relative signal (wild type to mutant) for each array element as a fraction of the total chloroplast RNA in each sample. C, Normalized fluorescence intensities from each individual genotype (red, the wild type; green, mutant). The signal intensities for each channel were normalized to one another based on the average signal for both channels (F635 and F532) in the two assays. The underlying data are the same as those used in B.
remain, including the biochemical contributions of each protein to PEP-mediated transcription, whether they have additional functions beyond transcription, and how they contribute to the regulation of plastid gene expression by environmental, physiological, and developmental cues.

The results presented here highlight a function for PEP-associated proteins that has been hinted at in prior literature (see below) but that has not been fully appreciated: PEP-associated proteins are essential for the robust expression of numerous plastid tRNAs. The
expression of \textit{trnL-UAA} is particularly sensitive to the loss of PEP-associated proteins: very little expression remains even in the hypomorphic alleles analyzed here. \textit{trnL-UAA} and several other strongly affected tRNAs (e.g. \textit{trnF-GAA} and \textit{trnL-UAG}) are essential for plastid translation (Alkatib et al., 2012). These tRNA deficiencies, therefore, are expected to contribute to the reduction in plastid ribosomes in mutants lacking PEP-associated proteins: limiting tRNAs will reduce the synthesis of plastid-encoded ribosomal proteins, which, in turn, are needed to assemble a stable ribosomal structure. Thus, the reduced content of tRNA (and ribosomes) in mutants lacking PEP-associated proteins is likely due to a combination of reduced transcription from the PEP promoter upstream of the rRNA genes and increased rRNA degradation due to limiting ribosomal proteins. The relative contributions of these two effects cannot currently be assessed.

To our knowledge, prior studies of mutants lacking PEP-associated proteins have not assayed the expression of plastid tRNAs. “Genome-wide” array or quantitative reverse transcription-PCR assays were used in several cases (Nagashima et al., 2004; Kindgren et al., 2012; Yagi et al., 2012), but tRNAs were excluded. That being said, our results were foreshadowed by genetic analyses of PEP itself. A mutation in the Arabidopsis SIG2 gene (encoding a PEP sigma factor) was shown to reduce the expression of \textit{trnE-UUC}, \textit{trnD-GUC}, \textit{trnV-UAC}, and \textit{trnM-CAU} but to have no effect on \textit{trnG-GCC} or \textit{trnW-CCA} (Kanamaru et al., 2001). Mutation of the Arabidopsis SIG6 gene reduced the expression of \textit{trnQ-UUG} and the \textit{trnYED} transcription unit but did not affect \textit{trnV-UAC} (Ishizaki et al., 2005). In addition, the PEP-associated protein PTAC3 was shown to associate in vivo with the \textit{tmE/Y/D} promoter (Yagi et al., 2012). However, the tRNAs we found to be most severely affected in mutants deficient for PEP-associated proteins (\textit{trnL-UAA}, \textit{trnF-GAA}, \textit{trnL-CAA}, and \textit{trnL-UAG}) appear not to have been assayed in these studies. Importantly, a broad role for PEP in the expression of plastid tRNAs was detected in a thorough macroarray survey of plastid transcription and RNA levels in a tobacco plastome \textit{rpoA} knockout (Legen et al., 2002). Although reduced expression of many tRNAs was observed, the magnitude of these effects appeared small in comparison with the effects on PEP-dependent mRNAs. Perhaps it is for this reason that these widespread effects on tRNAs have not been incorporated into the generally accepted view of the repertoire of PEP functions.

A deep analysis of transcription start sites in barley plastids detected PEP but not NEP promoters for \textit{trnL-UAA}, \textit{trnS-UCA}, \textit{trnQ-UUG}, \textit{trnM-CAU}, \textit{trnN-GUU}, and \textit{trnT} (Zhelyazkova et al., 2012), but the assay employed did not address the possibility that these tRNAs can be transcribed from distal NEP promoters. However, a very recent study demonstrated a physical association between the RpoA subunit of PEP and numerous tRNA genes in tobacco chloroplasts (Finster et al., 2013). Those results in conjunction with our data and prior evidence that SIG2 and SIG6 enhance the expression of several plastid tRNAs (Kanamaru et al., 2001; Ishizaki et al., 2005) provide strong evidence that many plastid tRNAs are highly dependent on PEP and its associated proteins for their transcription. Notably, \textit{trnL-UAA} goes almost unexpressed in the absence of PEP-associated proteins. The PEP promoter inferred for \textit{trnL-UAA} in barley (Zhelyazkova et al., 2012) is conserved in dicots (Supplemental Fig. S4), in accord with our finding that \textit{trnL-UAA} expression is severely reduced in Arabidopsis mutants lacking PEP-associated proteins (Fig. 5C).

These results have implications for the regulatory cascade that activates the plastid gene expression machinery early in chloroplast development. The transcription of the \textit{rpoB}, \textit{rpoC1}, and \textit{rpoC2} genes, which encode PEP core subunits, is mediated primarily by NEP (Demarsy et al., 2012, and refs. therein). However, PEP synthesis also requires a functioning translation apparatus, including \textit{trnL-UAA} and the other tRNAs whose transcription relies primarily on PEP itself. This predicts that a basal level of PEP must be maintained in all cells that will give rise to photosynthetic tissues. Consistent with this view, it has been shown that PEP is present in the seeds of several dicot species (Demarsy et al., 2006, 2012). In addition, these findings predict that a small increase in PEP will initiate a positive feedback loop by increasing the concentration of limiting tRNAs and thereby accelerating the synthesis of PEP by plastid ribosomes. This may be of physiological importance, as the effect is predicted to be a responsive genetic switch to rapidly trigger chloroplast development upon a small burst in NEP-mediated transcription of the \textit{rpo} genes.

On a technical note, apparent levels of RNAs analyzed by both microarray and RNA gel-blot hybridization generally mirrored one another, but there were some major exceptions (Supplemental Table S1). For example, the microarray data suggested that \textit{tmA-UGC} and the \textit{psaB} mRNA accumulate to near-normal levels in the \textit{ZmmurE} and \textit{Zmptac2} mutants, but the RNA gel-blot data showed them to be strongly reduced. Differences of this nature could arise from artifacts due to signal saturation on the microarrays or to the fact that the microarray integrates transcripts of all sizes whereas the RNA gel blots do not. Although microarray data on their own should be interpreted with caution, it is striking that the microarray data for the wild-type samples span a much larger dynamic range than do the microarray data for the mutant samples (Fig. 4C). These results support the view that NEP promoters have rather uniform, low activity and that PEP activity at specific promoters is required to boost the transcription of a subset of genes to the exceptional levels required for the biogenesis and maintenance of the photosynthetic apparatus.

The direct biochemical functions of PEP-associated proteins remain unknown. Elucidation of their roles would be fostered by the ability to study biochemical defects in mutants that lack them. However, these mutants are nonphotosynthetic, and it is challenging to generate substantial quantities of nonphotosynthetic mutant leaf tissue in Arabidopsis. By contrast, growth of nonphotosynthetic mutants in large seeded plants...
Like maize is supported for several weeks by seed reserves; during this period, such mutants grow rapidly to substantial size (Fig. 1A), simplifying the recovery of nonphotosynthetic tissue for biochemical analysis. The gradient of chloroplast development along the length of the maize seedling leaf (Leech et al., 1973) is useful for developmental studies, and the fact that maize leaves develop in the absence of light can be used to discriminate the effects of light from the effects of developmental cues. Therefore, the functional analysis of PEP-associated proteins will be facilitated by their parallel study in maize and Arabidopsis, exploiting the advantages offered by each system.

**MATERIALS AND METHODS**

**Plant Material**

The recovery of maize (Zea mays) Zmsthy1 and Zmptac12 mutants was described previously (Prikryl et al., 2008; Williams-Carrier et al., 2010). The other mutants employed here were initially recognized as recessive mutations that condition chlorotic, seedling-lethal phenotypes and distinctive plastid RNA profiles during the systematic phenotyping of mutants in the PML mutant collection (http://pml.uoregon.edu/photosyntheticml.html). Mut insertions that cosegregate with these phenotypes were identified with an Illumina-based method (Williams-Carrier et al., 2010). Candidate genes were then validated by the recovery of second alleles in reverse-genetic screens of the PML collection, followed by complementation tests involving crosses among heterozygous plants harboring each allele. The progeny of the allelic crosses segregated in mutant progeny (of intermediate phenotype when the parental alleles differed in strength), demonstrating a lack of complementation and validating the gene identifications. The maize genes were named according to nomenclature established for their Arabidopsis (Arabidopsis thaliana) orthologs (evidence for orthology is summarized at the POGs2 Database; http://cas-pogs.uoregon.edu/; Zmutor1, maize locus GRMZM2G122116, orthologous to At1g74850; Zmptac12, maize locus GRMZM2G091419, orthologous to At1g48580; Zmptac12, maize locus GRMZM5G897926, orthologous to At2g34640; and Zmprim2, maize locus GRMZM2G119906, orthologous to At1g10522).

**RNA and Protein Analyses**

RNA was extracted from the second leaf of 1-week-old seedlings. For the developmental analysis in Supplemental Figure S3, RNA was extracted from the base (1.5–3.5 cm above the basal node) referred to as “young” or the tip (apical 3 cm, referred to as “mature”) of the second seedling leaf. RNA gel-blot hybridizations were performed as described previously (Barkan, 1998) using the methods described previously (Zoschke et al., 2015). Each microarray data set came from one competitive hybridization (i.e., one mutant and one wild-type sample combined to probe the same array), but all conclusions are based on results that were validated by RNA gel-blot hybridization. The elements on the array and a summary of the data are provided in Supplemental Data Set S1.

**Corrections to tRNA Annotations**

During the course of this work, we discovered errors and ambiguities in the annotations of several tRNAs in the reference plastid genome for maize (Maier et al., 1995). Therefore, the identity of each tRNA was checked with tRNAscan (http://lowelab.ucsc.edu/tRNAscan-SE/). For clarity, the residue number of the first nucleotide of each tRNA in the reference maize plastid genome (Maier et al., 1995) is included in figures and tables, where relevant. The corrections to annotations are indicated in the footnotes to Supplemental Table S1.

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Figure S1.** Phenotypes conditioned by the individual mutant alleles used to generate the heteroallelic plants shown in Figure 1.
- **Supplemental Figure S2.** Additional RNA gel-blot analyses of plastid RNAs in maize and Arabidopsis mutants lacking PEP-associated proteins.
- **Supplemental Figure S3.** Comparative analysis of the plastid transcriptome in apical (mature) versus basal (immature) maize leaf tissue.
- **Supplemental Figure S4.** Multiple sequence alignment showing the similarity between the barley PEP promoter for trnl-UAA (Zhelezaykova et al., 2012) and orthologous sequences in other species.
- **Supplemental Table S1.** Quantification of northern and microarray data for those RNAs assayed by both methods.
- **Supplemental Table S2.** Probes used for RNA gel-blot hybridization.
- **Supplemental Data Set S1.** Plastid tiling microarray design and transcriptome data.

**ACKNOWLEDGMENTS**

Antibody to rice RpoB was generously provided by Congming Lu (Chinese Academy of Sciences). We are grateful to Bobby Coalter and Dylan Udy (formerly of the University of Oregon; currently at the University of California [Davis and San Francisco, respectively] for contributing to surveys of RNA defects in PTAC mutants by RNA gel-blot hybridization, to Tiffany Kroeger (University of Oregon) and Nick Stiffler (University of Oregon) for expert technical assistance, and to David Stern and his former laboratory members (Boyce Thompson Institute) for the survey RNA gel blots that originally brought to our attention several of the mutants described here.

Received September 16, 2013; accepted November 16, 2013; published November 18, 2013.

**LITERATURE CITED**


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