

The Translational Apparatus of Plastids and Its Role in Plant Development

Nadine Tiller and Ralph Bock¹

Max-Planck-Institut für Molekulare Pflanzenphysiologie, Am Mühlenberg 1, D-14476 Potsdam-Golm, Germany

ABSTRACT Chloroplasts (plastids) possess a genome and their own machinery to express it. Translation in plastids occurs on bacterial-type 70S ribosomes utilizing a set of tRNAs that is entirely encoded in the plastid genome. In recent years, the components of the chloroplast translational apparatus have been intensely studied by proteomic approaches and by reverse genetics in the model systems tobacco (plastid-encoded components) and *Arabidopsis* (nucleus-encoded components). This work has provided important new insights into the structure, function, and biogenesis of chloroplast ribosomes, and also has shed fresh light on the molecular mechanisms of the translation process in plastids. In addition, mutants affected in plastid translation have yielded strong genetic evidence for chloroplast genes and gene products influencing plant development at various levels, presumably via retrograde signaling pathway(s). In this review, we describe recent progress with the functional analysis of components of the chloroplast translational machinery and discuss the currently available evidence that supports a significant impact of plastid translational activity on plant anatomy and morphology.

Key words: plastid; translation; ribosome; ribosomal protein; evolution; plastid transformation; retrograde signaling; leaf development; palisade cell.

INTRODUCTION

Chloroplasts (plastids) are DNA-containing cell organelles that are bounded by a double membrane. They evolved from a photosynthetically active cyanobacterium by an endosymbiotic event that took place more than a billion years ago (Gray, 1993). During evolution, the vast majority of the genetic information of the initially genetically autonomous endosymbiont was relocated to the nucleus of the host cell (Timmis et al., 2004; Bock and Timmis, 2008). However, a specific set of genes was retained in the plastid compartment and is expressed by a dedicated gene expression machinery. In most photosynthetically active vascular plants, the circularly mapping plastid genome (plastome) encodes approximately 120 genes that are densely arranged on a single chromosome of 120–220 kb (reviewed in Wakasugi et al., 2001; Bock, 2007). The genes can be grouped into three major classes: genes encoding parts of the photosynthetic apparatus, genes encoding components of the genetic system, and genes encoding other functions (Shimada and Sugiura, 1991). Despite more than a billion years of separate evolution, the gene expression machinery of plastids still shares substantial similarities with that of its cyanobacterial ancestor, but also has acquired a number of novel organelle-specific features, components, and regulatory mechanisms. It seems reasonable to assume that many of these evolutionary novelties serve the purpose of optimizing the coordination of the expression

of the chloroplast genome with that of the two other genetic compartments in the cell: the nucleus and the mitochondrion. In addition, the evolution of multicellularity and, in particular, the evolution of complex body plans in vascular plants posed new regulatory challenges related to the tissue-specific and developmental stage-specific differentiation of plastids into specialized plastid types (e.g. proplastids of meristematic tissues, chromoplasts of fruits and flowers, amyloplasts of roots and tubers).

EXPRESSION OF THE PLASTID GENOME

Plastid-encoded genes are transcribed by two types of RNA polymerases, both of which are necessary for the biogenesis

¹ To whom correspondence should be addressed. E-mail rbock@mpimp-golm.mpg.de, fax +49 331 567 8701, tel. +49 331 567 8700

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of photosynthetically active chloroplasts (Allison et al., 1996; Swiatecka-Hagenbruch et al., 2008). The plastome itself encodes a bacterial-type multi-subunit RNA polymerase (plastid-encoded RNA polymerase, PEP) that requires nucleus-encoded sigma factors to facilitate promoter recognition. Additionally, a single subunit nucleus-encoded RNA polymerase (NEP) related to the RNA-synthesizing enzymes of T-type bacteriophages and mitochondria is present in plastids (Hedtkke et al., 2002). In plastids of vascular plants, two such NEP enzymes are present (dubbed RPOTp and RPOTmp; Liere and Börner, 2007).

Primary transcripts produced by both polymerases are usually polycistronic and undergo extensive posttranscriptional processing steps, including intron removal by splicing, processing of primary polycistronic RNA molecules into mature monocistronic or oligocistronic mRNAs, trimming of the 5' and 3' ends, and RNA editing—a process by which the identity of cytidine residues at highly specific sites is altered to uridine (reviewed, e.g., in Bock, 2000; Stern et al., 2010; Barkan, 2011; Stoppel and Meurer, 2012). Members of the RNA-binding pentatricopeptide repeat protein (PPR) family have been shown to play crucial roles in many of these posttranscriptional processes (Schmitz-Linneweber and Small, 2008; Barkan, 2011).

Although significant transcriptional regulation occurs in plastids (Mullet and Klein, 1987; Allison and Maliga, 1995; Liere and Börner, 2007), it is generally believed that, in the course of evolution, the regulation of plastid gene expression was shifted from a predominantly transcriptional to a predominantly posttranscriptional control level (Eberhard et al., 2002). Posttranscriptional control is exerted at the level of RNA stability and especially at the level of translation (Mullet and Klein, 1987; Staub and Maliga, 1993; Eberhard et al., 2002; Kahlau and Bock, 2008). Plastid mRNAs are stabilized by sequence elements located in their 5' and 3' untranslated regions as well as by RNA secondary structures and RNA-binding PPR proteins (Stern et al., 2010; Barkan, 2011). Degradation is mediated by a complex interplay of endo- and exoribonucleases with removal of protective terminal stem-loop-type RNA secondary structures and 3' polyadenylation being decisive initiating events (Stern et al., 2010; Stoppel and Meurer, 2012).

Translation of the genetic information in plastids is performed by ribosomes that are very similar to bacterial 70S ribosomes (Yamaguchi et al., 2000; Yamaguchi and Subramanian, 2000; Beligni et al., 2004b; Manuell et al., 2007). Almost all components of the plastid translational apparatus have close homologs in cyanobacteria, suggesting that the basic functions have been conserved throughout evolution. Translation initiation starts with the formation of a pre-initiation complex composed of the 30S ribosomal subunit and the initiator transfer RNA (tRNA) that selects the translation initiation site in the mRNA. AUG, and in rare cases the alternative triplets GUG or UUG, serve as initiation codons in plastid mRNAs (Sugiura et al., 1998). The precise

molecular mechanism by which the plastid pre-initiation complex recognizes the initiator codon is not fully understood. In bacteria, the most prominent mechanism of translation initiation is based on binding of the anti-Shine–Dalgarno sequence (residing at the 3' end of the 16S rRNA in the 30S ribosomal subunit) to a purine-rich sequence motif 7 ± 2 nucleotides upstream of the initiator codon in the mRNA. This motif has been dubbed the Shine–Dalgarno sequence (or ribosome-binding site, RBS). While some plastid genes clearly possess bacterial-type Shine–Dalgarno sequences and are likely to utilize them for translation initiation (Ruf and Kössel, 1988; Hirose et al., 1998; Drechsel and Bock, 2010), many genes lack properly positioned Shine–Dalgarno sequences. A recent study showed that, in both chloroplasts and bacteria, translation initiation regions lacking Shine–Dalgarno motifs are less structured than those harboring a canonical Shine–Dalgarno sequence (Scharff et al., 2011). Absence of secondary structure formation around the initiation codon was proposed to facilitate start site recognition and binding of the 30S ribosomal subunit.

Following selection of the start codon, recruitment of the 50S subunit converts the pre-initiation complex into an active initiation complex, which then can enter the elongation phase of protein synthesis. Similarly to bacteria, the process is likely to be assisted by translation initiation and elongation factors. Chloroplast homologs of bacterial initiation and elongation factors have been identified and, in part, characterized (Lin et al., 1996; Beligni et al., 2004a; Albrecht et al., 2006; Miura et al., 2007; Ruppel and Hangarter, 2007; Shen et al., 2013). During translation elongation, the ribosome slides along the coding sequence of the mRNA incorporating the amino acids corresponding to each codon into the growing polypeptide chain with the help of the translation elongation factors EF-Tu, EF-G, and EF-Ts. Plastids use the standard genetic code, but it must be borne in mind that single nucleotide deviations between DNA sequence and mRNA sequence can be caused by RNA editing (Bock, 2000). Translation is terminated when the ribosome reaches one of the three standard termination codons (UAA, UAG, or UGA). The termination process requires the assistance of ribosome release factors (Meurer et al., 2002; Motohashi et al., 2007). To prepare the ribosome for a new round of protein synthesis, the mRNA–ribosome complex is subsequently disassembled by the ribosome-recycling factor (RRF; Wang et al., 2010). RRF dissociates the 70S ribosome into its 30S and 50S subunits, thereby releasing the mRNA and making the free 30S subunit available for *de novo* translation initiation.

The translation rate of individual mRNAs is mainly regulated at the level of translation initiation. Suggested control mechanisms include redox regulation (that may couple translation to photosynthetic electron transfer) and the so-called control by epistasy of synthesis (CES), an autoregulation mechanism that couples translation to protein complex assembly (Choquet et al., 1998; Peled-Zehavi and Danon, 2007).

The extensive regulation of plastid gene expression at the translational level calls for experimental methods that are suitable to measure chloroplast translational activity. Pulse labeling of newly synthesized chloroplast proteins using radioactively labeled amino acids (^{35}S -labeled methionine and/or cysteine) represents one of the classical techniques that has been used in microorganisms and, to a much lesser extent, also in plants (e.g. Meurer et al., 1998b). However, with this method, only highly abundant proteins like Rubisco (RbcL), the large subunits of photosystem I (PsaA, PsaB), and photosystem II (D1, D2, CP43, CP47) can be readily detected. Also, in higher plants, it is difficult if not impossible to ensure the fast and homogeneous uptake of radiolabeled amino acids by multicellular tissues or even intact plants, making it hard to draw reliable quantitative conclusions from such experiments. Another frequently used technique to analyze plastid translational activity relies on the isolation of polysomes (Barkan, 1998). Polysomes are complexes of mRNAs and actively translating ribosomes. The number of ribosomes associated with a particular mRNA can serve as a proxy measure of the efficiency of its translation. Highly translated mRNAs are loaded with many ribosomes and can be separated from poorly translated and free mRNAs by analytical sucrose density centrifugation. The distribution of individual mRNAs across the gradient fractions is then assessed by Northern blot analysis (Barkan, 1988). By combining polysome isolation with microarray hybridization techniques, the method can be employed as a tool for the genome-wide analysis of translational regulation (also referred to as translomics; Kahlau and Bock, 2008). This analytical platform has been used in a number of studies to examine the translational regulation of plastid gene expression in response to developmental cues and genetic perturbations (Kahlau and Bock, 2008; Valkov et al., 2009; Walter et al., 2010). Recently, a novel technique combining ribosomal footprinting with an oligonucleotide tiling array of the plastid ORFeome has been developed and successfully applied to determine the abundance and translational status of all chloroplast mRNAs in translation mutants of maize (Zoschke et al., 2013). Future research on chloroplast translation should benefit greatly from combining ribosomal footprinting with next-generation sequencing techniques (RNAseq) for translomic analyses (Ingolia et al., 2009, 2012).

In view of the importance of translational regulation in the plastid (which can easily override even large changes in mRNA abundance; Eberhard et al., 2002), interfering with translation is the most appropriate reverse genetic method if down-regulation of a chloroplast gene or open reading frame is to be attempted. Knockdown of plastid genes by stable transformation of the plastid genome is a suitable strategy to study the function of essential genes whose knockout is lethal and therefore does not produce analyzable mutants (Drescher et al., 2000; Shikanai et al., 2001; Kode et al., 2005). It also provides a very valuable tool for the in-depth functional analysis of non-essential genes in that it can produce a spectrum of mutant phenotypes (Rott et al., 2011), which

often are more informative than a complete gene knockout. So far, two strategies have been proven to be suitable to down-regulate the efficiency of translation in plastids: (1) the change of the standard translation initiation codon AUG to less efficient start codons like AUU, GUG, or UUG (Majeran et al., 2000; Hirose and Sugiura, 2004; Rott et al., 2011), and (2) the introduction of point mutations into the Shine–Dalgarno sequence (Hirose and Sugiura, 2004). Unfortunately, the recognition efficiency of non-AUG start codons is sequence-context-dependent (Boeck and Kolakofsky, 1994) and the same is likely to hold true for the interaction between the Shine–Dalgarno sequence and the anti-Shine–Dalgarno sequence in the 16S rRNA. Therefore, it is currently not possible to predict the efficacy of point mutations that are introduced into the translational start codon or the Shine–Dalgarno sequence of a plastid gene of interest. Consequently, different mutations need to be tested and, in view of the laborious and time-consuming procedures involved in stable transformation of the plastid genome, this represents a serious limitation.

ESSENTIAL AND NON-ESSENTIAL COMPONENTS OF THE PLASTID TRANSLATION MACHINERY

Ribosomal RNAs

The 70S-type chloroplast ribosome (sometimes also referred to as the ‘chlororibosome’) consists of two multi-component subunits: the large (50S) and the small (30S) ribosomal subunit. Both subunits are ribonucleoprotein complexes comprising one or more ribosomal RNA species (rRNAs) and many proteins. All ribosomal RNAs fold into highly complex three-dimensional structures and each rRNA species is associated with a distinct set of ribosomal proteins. The RNA components of the plastid ribosome are strikingly similar to their bacterial counterparts—a finding that was one of the first compelling molecular evidences for the endosymbiotic origin of plastids (Schwarz and Kössel, 1979, 1980). The 30S particle harbors a single rRNA molecule, the 16S rRNA, whereas the 50S particle contains three rRNAs: the 23S, 5S, and 4.5S rRNAs. A 4.5S rRNA does not exist in bacteria, but the plastid 4.5S rRNA is homologous to the 3′-terminal part of the bacterial 23S rRNA (Edwards et al., 1981), suggesting that it evolved by fragmentation of the 23S rRNA. Whether or not this split has any functional significance is currently not known.

All plastid ribosomal RNAs are encoded in the chloroplast genome, transcribed from a single operon (*rrn* operon) as a long polycistronic RNA which then undergoes posttranscriptional processing to produce the mature rRNAs. This occurs by the action of a set of endoribonucleases and exoribonucleases, some of which have been identified and characterized (Bollenbach et al., 2005, 2007). In some other cases, it is not entirely clear whether the defects in plastid rRNA processing observed in loss-of-function mutants are a primary or a secondary consequence of the gene knockout (e.g. Bellaoui et al., 2003).

All four rRNA species represent essential components of the plastid translational apparatus (Scharff and Bock, 2014). A peculiarity of the plastid 23S rRNA is its fragmentation into three distinct pieces *in vivo*, due to the so-called 'hidden break' processing (Nishimura et al., 2010). The efficiency of 'hidden break' processing is known to be dependent on environmental factors and developmental cues (Rosner et al., 1974), but the possible functional relevance of this fragmentation is unclear.

As in bacteria, the assembly of ribosomes in plastids depends on auxiliary proteins that aid rRNA folding and association with ribosomal proteins. Although a number of such ribosome biogenesis factors have recently been identified (e.g. Bellaoui et al., 2003; Bang et al., 2012; Lee et al., 2013; Fristedt et al., 2014), the precise molecular mode of action of most of them currently remains unknown.

Ribosomal Proteins

Identification of the complete set of plastid ribosomal proteins revealed that the protein composition of the plastid ribosome is very similar to that of the *Escherichia coli* ribosome (Tables 1 and 2). However, five additional so-called plastid-specific ribosomal proteins (PSRPs) were identified (Yamaguchi and Subramanian, 2000; Yamaguchi et al., 2000) that lack homologs in *E. coli*. The chloroplast 30S ribosomal subunit comprises a total of 24 proteins (Table 2) of which 21 are orthologs of *E. coli* 30S ribosomal proteins (S1-S21) and three are specific to chloroplast ribosomes (PSRP2, PSRP3, PSRP4; Yamaguchi et al., 2000). In the 50S subunit, 31 out of 33 ribosomal proteins have orthologs in *E. coli* (L1-L6, L9-L24, L27-L29, and L31-L36; Table 1) and two proteins are specific to chloroplasts (PSRP5 and PSRP6; Yamaguchi and Subramanian, 2000).

Genome sequencing revealed that the genes encoding plastid ribosomal proteins are distributed between the plastid and the nuclear genomes. Twelve proteins of the small subunit are encoded in the nuclear genome, whereas 12 are encoded in the plastid genome (Yamaguchi et al., 2000). By contrast, the majority of the genes encoding ribosomal proteins of the large subunit (24 genes) were transferred to the nucleus and only nine ribosomal protein genes remain in the plastid genome (Yamaguchi and Subramanian, 2000).

Some chloroplast ribosomal proteins are larger than their bacterial counterparts due to the presence of N-terminal and/or C-terminal extensions, the functional significance of which is not clear (Yamaguchi and Subramanian, 2000; Yamaguchi et al., 2000). These size expansions and the presence of the PSRPs result in a significant alteration of the protein: RNA ratio of the ribosome, which is 2:3 in the chloroplast, but 1:3 in *E. coli*.

The rRNAs are only stable if integrated into ribosomal subunits. Therefore, rRNA accumulation can serve as a proxy for chloroplast ribosome content. In addition, determining the abundance of individual rRNA species provides information about ribosomal subunit accumulation (Walter et al.,

2010). For example, alterations in the ratio of 16S:23S rRNA are indicative of disturbances in ribosome assembly or stability that are specific to the 30S or 50S subunit. This indirect method of ribosome quantification has been employed in *Arabidopsis* and tobacco for the identification and characterization of mutants affected in chloroplast translation and ribosome biogenesis (Walter et al., 2010; Fleischmann et al., 2011; Tiller et al., 2012; Fristedt et al., 2014).

Comprehensive data concerning the essentiality of ribosomal proteins are available for *E. coli* (Dabbs, 1991; Baba et al., 2006; Shoji et al., 2011). Based on the strikingly similar overall composition of chloroplast and bacterial ribosomes, it seemed reasonable to assume that also the essentiality and non-essentiality of ribosomal proteins would be conserved between bacteria and plastids. However, recent studies indicate that this is not always the case (Tables 1 and 2).

The function of the nucleus-encoded plastid ribosomal proteins has been studied mainly in the model plant *Arabidopsis* by using publicly available T-DNA insertion lines (Pesaresi et al., 2001; Morita-Yamamuro et al., 2004; Bryant et al., 2011; Tiller et al., 2012; Romani et al., 2012), mutants generated by RNAi approaches (Tiller et al., 2012), or mutants identified in EMS mutagenesis screens (Yin et al., 2012). Due to the lack of a chloroplast transformation system for *Arabidopsis*, the essentiality of plastid-encoded ribosomal proteins has been exclusively investigated in tobacco (*Nicotiana tabacum*). These studies revealed that, similar to the results obtained in *E. coli*, the ribosomal protein genes *rpl20*, *rpl22*, *rpl23*, *rps2*, *rps3*, *rps4*, *rps14*, *rps16*, and *rps18* are essential genes in tobacco plastids (Tables 1 and 2). Targeted inactivation of these genes was attempted by insertional mutagenesis taking advantage of the active homologous recombination system present in chloroplasts. Disruption of an essential ribosomal protein gene with a selectable marker gene for chloroplast transformation that confers resistance to the aminoglycoside antibiotic spectinomycin produces plastid transformants (transplastomic lines) that remain heteroplasmic (i.e. maintain a mix of transformed and wild-type copies of the plastid DNA). This is indicative of a balancing selection in which the wild-type genome copies provide the essential ribosomal protein and the transformed genome copies provide the enzyme detoxifying the selecting antibiotic. When such heteroplasmic plants are grown in the absence of the antibiotic, they show a characteristic leaf-loss phenotype (Figure 1). Due to random genome segregation into homoplasmy, cell lineages that entirely lack the essential plastid gene die. This leads to misshapen leaves that can lack large sectors of their lamina (Figure 1; Ahlert et al., 2003; Rogalski et al., 2006, 2008b; Fleischmann et al., 2011).

Three plastid-encoded ribosomal protein genes turned out to be non-essential in reverse genetic studies: *rpl33*, *rpl36*, and *rps15* (Rogalski et al., 2008b; Fleischmann et al., 2011). The homologous genes were also shown to be dispensable in *E. coli* (Tables 1 and 2). While $\Delta rpl33$ and $\Delta rps15$ transplastomic knockout mutants show wild-type-like growth under

Table 1. Ribosomal Proteins of the Large (50S) Subunit of the Chloroplast Ribosome and Their Genes in Bacteria (*Escherichia coli*) and Seed Plants (<http://ppdb.tc.cornell.edu/dbsearch/subproteome.aspx>).

Gene	<i>E. coli</i>	Reference	A.t. gene	Seed plants	Reference
<i>rpl1</i>	Non-essential	Baba et al., 2006	At3g63490	Essential	Bryant et al., 2011; Romani et al., 2012
<i>rpl2</i>	Essential	Shoji et al., 2011	Atcg01310 Atcg00830	NA	
<i>rpl3</i>	Essential	Shoji et al., 2011	At2g43030	Essential (NC)	Unpublished
<i>rpl4</i>	Essential	Shoji et al., 2011	At1g07320	Essential	Romani et al., 2012
<i>rpl5</i>	Essential	Shoji et al., 2011	At4g01310	NA	
<i>rpl6</i>	Essential	Shoji et al., 2011	At1g05190	Essential	www.seedgenes.org
<i>rpl9</i>	Non-essential	Baba et al., 2006	At3g44890	NA	
<i>rpl10</i>	Essential	Shoji et al., 2011	At5g13510	Essential (NC)	Bryant et al., 2011
<i>rpl11</i>	Non-essential	Baba et al., 2006	At1g32990	Non-essential	Pesaresi et al., 2001
<i>rpl12</i>	Essential	Shoji et al., 2011	At3g27830 At3g27850	NA	
<i>rpl13</i>	Essential	Shoji et al., 2011	At1g78630	Essential (NC)	Bryant et al., 2011
<i>rpl14</i>	Essential	Shoji et al., 2011	Atcg00780	NA	
<i>rpl15</i>	Non-essential	Shoji et al., 2011*	At3g25920	NA	
<i>rpl16</i>	Essential	Shoji et al., 2011	Atcg00790	NA	
<i>rpl17</i>	Essential	Shoji et al., 2011	At3g54210	NA	
<i>rpl18</i>	Essential	Shoji et al., 2011	At1g48350	Essential (NC)	Bryant et al., 2011
<i>rpl19</i>	Essential	Shoji et al., 2011	At5g47190 At4g17560	NA	
<i>rpl20</i>	Essential	Shoji et al., 2011	Atcg00660	Essential	Rogalski et al., 2008b
<i>rpl21</i>	Non-essential	Shoji et al., 2011*	At1g35680	Essential	Yin et al., 2012
<i>rpl22</i>	Essential	Shoji et al., 2011	Atcg00810	Essential	Fleischmann et al., 2011
<i>rpl23</i>	Essential	Shoji et al., 2011	Atcg01300 Atcg00840	Essential	Fleischmann et al., 2011
<i>rpl24</i>	Non-essential	Shoji et al., 2011*	At5g54600	Non-essential	Tiller et al., 2012
<i>rpl25</i>	Non-essential	Baba et al., 2006	–	–	
<i>rpl27</i>	Non-essential	Shoji et al., 2011*	At5g40950	Essential	Romani et al., 2012
<i>rpl28</i>	Non-essential	Dabbs, 1991*	At2g33450	Essential	Romani et al., 2012
<i>rpl29</i>	Non-essential	Shoji et al., 2011*	At5g65220	NA	
<i>rpl30</i>	Non-essential	Shoji et al., 2011*	–	–	
<i>rpl31</i>	Non-essential	Baba et al., 2006	At1g75350	Essential (NC)	Bryant et al., 2011
<i>rpl32</i>	Non-essential	Baba et al., 2006	Atcg01020	Essential	Fleischmann et al., 2011
<i>rpl33</i>	Non-essential	Baba et al., 2006	Atcg00640	Non-essential	Rogalski et al., 2008b
<i>rpl34</i>	Non-essential	Shoji et al., 2011*	At1g29070	NA	
<i>rpl35</i>	Essential	Baba et al., 2006	At2g24090	Essential	Romani et al., 2012
<i>rpl36</i>	Non-essential	Baba et al., 2006	Atcg00760	Non-essential	Fleischmann et al., 2011
<i>psrp5</i>	–	–	At3g56910	Knockdown viable	Tiller et al., 2012
<i>psrp6</i>	–	–	At5g17870	Non-essential	Tiller et al., 2012

Reverse genetic data for plants are from *Arabidopsis* (nucleus-encoded genes) and tobacco (plastid-encoded genes). Plastid genes are indicated in bold. All gene identifier codes are from *Arabidopsis*. For genes in the tobacco plastid genome, see GenBank accession Z00044.2. A.t., *Arabidopsis thaliana*; NA, not analyzed; NC, not yet confirmed by independent alleles or genetic complementation; *, contradictory result published earlier; unpublished, unpublished data from the authors' laboratory; –, gene absent (from *E. coli* or plants).

Table 2. Ribosomal Proteins of the Small (30S) Subunit of the Chloroplast Ribosome and Their Genes in Bacteria (*Escherichia coli*) and Seed Plants (<http://ppdb.tc.cornell.edu/dbsearch/subproteome.aspx>).

Gene	<i>E. coli</i>	Reference	<i>A.t.</i> gene	Seed plants	Reference
<i>rps1</i>	Essential	Shoji et al., 2011	At5g30510	Knockdown viable	Romani et al., 2012
<i>rps2</i>	Essential	Shoji et al., 2011	Atcg00160	Essential	Rogalski et al., 2008b
<i>rps3</i>	Essential	Shoji et al., 2011	Atcg00800	Essential	Fleischmann et al., 2011
<i>rps4</i>	Essential	Shoji et al., 2011	Atcg00380	Essential	Rogalski et al., 2008b
<i>rps5</i>	Essential	Shoji et al., 2011	At2g33800	Essential	Bryant et al., 2011
<i>rps6</i>	Non-essential	Bubunencko et al., 2007	At1g64510	NA	
<i>rps7</i>	Essential	Shoji et al., 2011	Atcg00900 Atcg01240	NA	
<i>rps8</i>	Essential	Shoji et al., 2011	Atcg00770	NA	
<i>rps9</i>	Non-essential	Shoji et al., 2011*	At1g74970	Essential (NC)	Bryant et al., 2011 ; Ma and Dooner, 2004
<i>rps10</i>	Essential	Shoji et al., 2011	At3g13120	NA	
<i>rps11</i>	Essential	Shoji et al., 2011	Atcg00750	NA	
<i>rps12</i>	Essential	Shoji et al., 2011	Atcg00905 Atcg01230 Atcg00065	NA	
<i>rps13</i>	Non-essential	Bubunencko et al., 2007*	At5g14320	Essential	Bryant et al., 2011
<i>rps14</i>	Essential	Shoji et al., 2011	Atcg00330	Essential	Ahlert et al., 2003
<i>rps15</i>	Non-essential	Bubunencko et al., 2007	Atcg01120	Non-essential	Fleischmann et al., 2011
<i>rps16</i>	Essential	Shoji et al., 2011	Atcg00050	Essential	Fleischmann et al., 2011
<i>rps17</i>	Non-essential	Shoji et al., 2011*	At1g79850	Non-essential	Schultes et al., 2000 ; Romani et al., 2012
<i>rps18</i>	Essential	Shoji et al., 2011	Atcg00650	Essential	Rogalski et al., 2006
<i>rps19</i>	Essential	Shoji et al., 2011	Atcg00820	NA	
<i>rps20</i>	Non-essential	Bubunencko et al., 2007	At3g15190	Essential	Romani et al., 2012 ; Gong et al., 2013
<i>rps21</i>	Non-essential	Baba et al., 2006	At3g27160	Non-essential	Morita-Yamamuro et al., 2004
<i>psrp2</i>	–	–	At3g52150	Knockdown viable	Tiller et al., 2012
<i>psrp3</i>	–	–	At1g68590	Non-essential	Tiller et al., 2012
<i>psrp4</i>	–	–	At2g38140	Knockdown viable	Tiller et al., 2012

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standard growth conditions, they are more sensitive to chilling stress than the wild-type, suggesting that both proteins are required for maintaining sufficiently high chloroplast translational capacity in the cold ([Rogalski et al., 2008b](#); [Fleischmann et al., 2011](#)). By contrast, the loss of Rpl36 causes a severe mutant phenotype. Plants grow extremely slowly, display severe pigment deficiency, and show strongly altered leaf morphology ([Figure 2](#); [Fleischmann et al., 2011](#); see below).

Contradictory results were obtained for *rpl32*. While Rpl32-deficient *E. coli* cells were reported to be viable ([Baba et al., 2006](#)), attempts to knockout the *rpl32* gene in tobacco strongly suggested its essentiality in plastids ([Fleischmann et al., 2011](#)). Re-analysis of the *E. coli* *rpl32* knockout strain from the Keio collection revealed that it contained an intact *rpl32* gene, raising the possibility that *rpl32* is also essential in *E. coli* ([Fleischmann et al., 2011](#)).



Figure 1. Typical Leaf Phenotypes of Transplastomic Plants Harboring a Knockout Allele for an Essential Component of the Translational Apparatus.

Leaves of transplastomic tobacco plants transformed with a knockout construct for the essential plastid gene *rps18* (encoding plastid ribosomal protein S18; Rogalski et al., 2006; Table 2) are shown. The plastid transformants are heteroplasmic and, in the absence of antibiotic selection, the plants randomly segregate into homoplasmies for the wild-type plastid genome or homoplasmies for the transplastome. Homoplasmies for the *rps18* knockout allele is lethal at the cellular level and results in loss of cell proliferation. Death of cell lineages during leaf development produces aberrantly shaped leaves that lack individual sectors or, in extreme cases, nearly the entire leaf blade (Ahler et al., 2003). Scale bar = 2 cm.

Much less is known about the essentiality of the nucleus-encoded subunits of the chloroplast ribosome. Results from the proteins studied to date by reverse genetic approaches do not suggest a strict conservation of essentiality and non-essentiality between *E. coli* and plastids (Romani et al., 2012). However, data for some *E. coli* proteins are controversial (Tables 1 and 2) and the *rpl32* case suggests that the results from high-throughput knockout projects may need to be treated with caution. *rpl11*, *rpl24*, *rps17*, and *rps21* null mutants are viable in *Arabidopsis*, which is in line with L11, L24, S17, and S21 being non-essential for the assembly of functional ribosomes in *E. coli* (Tables 1 and 2). Knockout plants for these ribosomal protein genes display retarded growth, reduced activity of plastid protein biosynthesis, and impaired photosynthesis (Pesaresi et al., 2001; Morita-Yamamuro et al., 2004; Romani et al., 2012; Tiller et al., 2012).

In agreement with data obtained for *E. coli*, the nucleus-encoded L4, L6, L35, and S5 proteins are essential in *Arabidopsis* (www.seedgenes.org; Tables 1 and 2). The genes for L3, L10, L13, and L18 are also likely to be essential in both *E. coli* and *Arabidopsis* (Table 1), but more rigorous experimental confirmation is still needed. A third category of nucleus-encoded plastid ribosomal proteins comprises L1 and S20, which were shown to be essential in *Arabidopsis* but are non-essential in *E. coli* (Tables 1 and 2). It is currently not clear whether L21, L27, L28, and S13 also fall in this

category, because conflicting data have been reported for *E. coli* (Tables 1 and 2).

The initially six plastid-specific ribosomal proteins (PSRP1-6) were suggested to play structural roles and regulate chloroplast translation in response to light (Yamaguchi and Subramanian, 2003; Manuell et al., 2007). The occurrence of PSRP2, PSRP3, PSRP4, PSRP5, and PSRP6 in stoichiometric amounts with the classical ribosomal proteins indicated that they could represent *bona fide* ribosomal proteins. Recently, PSRP1 has been proven to be neither a ribosomal protein nor plastid-specific. Instead, it appears to be a functional homolog of the *E. coli* cold-shock protein pY that acts as a ribosome-binding translation factor (Sharma et al., 2007, 2010). Cryo-electron microscopic studies localized PSRP2, PSRP3, PSRP4, and PSRP5 in the three-dimensional structure of the chloroplast ribosome (Manuell et al., 2007; Sharma et al., 2007) and suggest that these PSRPs might play structural roles, perhaps by compensating for the loss of specific rRNA sequences and/or secondary structural elements. PSRP6 might be only loosely associated with the plastid ribosome (Sharma et al., 2007).

In a recent study, a reverse genetic approach was taken in *Arabidopsis* to elucidate whether the remaining five PSRPs (PSRP2-6) are required for chloroplast translation or ribosome assembly (Tiller et al., 2012). Mutants were analyzed by describing their phenotypes, measuring their photosynthetic capacity, and analyzing their chloroplast translation efficiency. To determine the impact of reduced or abolished PSRP expression on ribosome assembly, ribosomal RNAs were precisely quantified. The data obtained suggest that PSRP3 and PSRP4 in the 30S subunit as well as PSRP5 in the 50S subunit play structural roles in the ribosome and thus qualify as genuine ribosomal proteins. Their down-regulation leads to decreased accumulation of the 30S or 50S subunits of the plastid ribosome. Although assigned as a protein of the small ribosomal subunit, PSRP3 also affects the accumulation of the 50S ribosomal subunit, possibly suggesting a dual role for PSRP3 in ribosome biogenesis and/or stability. By contrast, plants with reduced amounts of PSRP2 (in the 30S subunit) and plants without PSRP6 (in the 50S subunit) display no visible or measurable phenotypes, suggesting that they are non-essential proteins and dispensable for ribosome biogenesis and translation at least under standard conditions. PSRP2 contains two RNA-binding domains and recently was proposed to possess RNA chaperone activity and function as negative regulator in seed germination and abiotic stress responses (Yamaguchi and Subramanian, 2003; Xu et al., 2013).

The set of ribosomal protein genes that is retained in the chloroplast genome is highly conserved across seed plants. Known exceptions include *rpl32* which was transferred to the nuclear genome in poplar (Ueda et al., 2007), *rpl22* which was transferred to the nucleus in legumes (Gantt et al., 1991), and *rpl23* which is a pseudogene in the spinach plastid genome and whose gene product was replaced by a eukaryotic L23 protein that is encoded in the nucleus and imported into the chloroplast (Bubunencko et al., 1994).

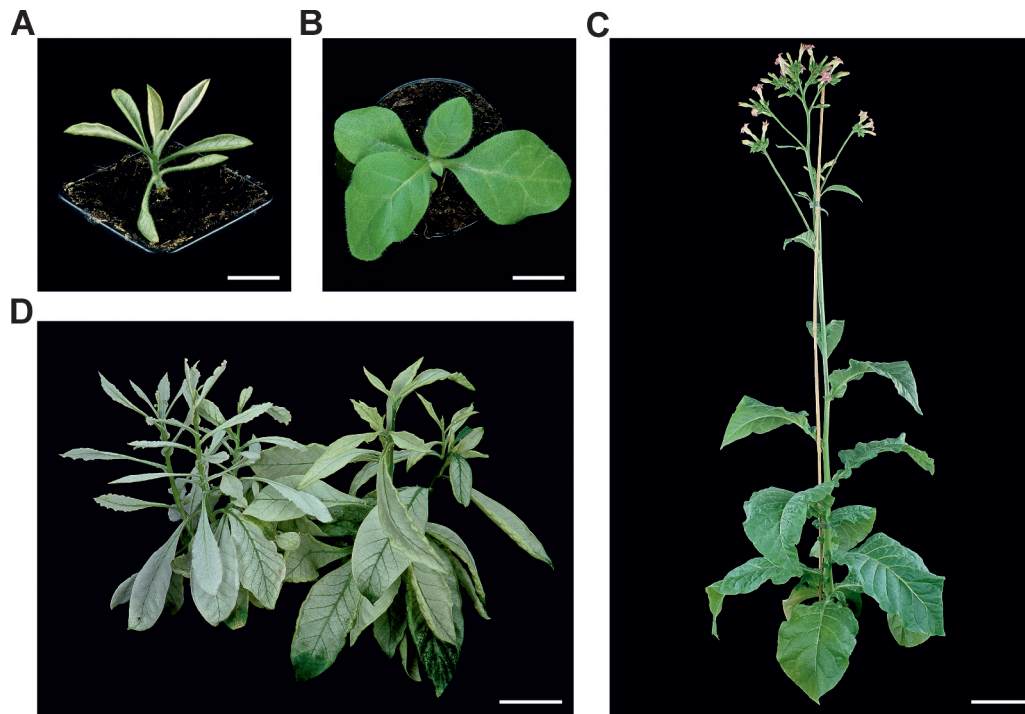


Figure 2. Knockout of a Non-Essential Plastid Gene Involved in Translation.

The large ribosomal subunit protein L36 is not essential for plastid translation and, therefore, homoplasmic knockout mutants can be obtained (Fleischmann et al., 2011). Tobacco $\Delta rpl36$ plants grow autotrophically in soil, but suffer from severe photo-oxidative damage due to low levels of plastid protein biosynthesis which results in low photosynthetic activity. Moreover, the mutants display striking alterations in leaf morphology and plant architecture (Fleischmann et al., 2011).

(A) A young $\Delta rpl36$ plant two month after transfer from *in vitro* culture to the greenhouse. Note the much narrower leaf blade compared to the wild-type shown in (B). Scale bar = 2 cm.

(B) Leaf shape of a young wild-type plant at approximately the same developmental stage as the $\Delta rpl36$ plant shown in (A). Scale bar = 2 cm.

(C) A flowering wild-type plant (after 9 weeks of growth in the greenhouse). Scale bar = 10 cm.

(D) A $\Delta rpl36$ plant after 1.3 years of growth in the greenhouse. Note the narrow leaves, the extensive atypical shoot branching (indicative of reduced apical dominance), and the lack of floral induction. Scale bar = 10 cm.

Transfer RNAs

The genetic code comprises 64 triplets, 61 of which specify the 20 different proteinogenic amino acids. Due to the relaxed base pairing between the third codon position in the mRNA and the first position of the anticodon in the tRNA, fewer than 61 tRNA species are needed to read all 61 triplets. This was early recognized by Francis Crick and has become known as the wobble hypothesis (Crick, 1966). According to Crick's wobble rules, 32 tRNA species should constitute the minimum set required for translation. The plastid genome of most seed plants encodes only 30 tRNA genes (Sugiura and Wakasugi, 1989; Alkatib et al., 2012b). In the absence of experimental evidence for the import of nucleus-encoded tRNAs into plastids, this raised the question how chloroplasts synthesize their proteins with fewer than 32 tRNAs.

A number of recent studies addressed this issue by employing reverse genetics in the model system tobacco. Systematic knockout analysis of individual tRNA genes revealed that a process called superwobbling facilitates translation with reduced tRNA sets (Rogalski et al., 2008a; Alkatib et al., 2012b).

Superwobbling refers to the capability of a single tRNA species to read an entire codon box. (A codon box is defined as set of four codons sharing the first two nucleotides.) According to the classical wobble rules, at least two tRNA isoacceptors are needed to read all four triplets of a codon box. For example, the four glycine codons GGC, GGU, GGA, and GGG are decoded by two tRNAs: tRNA-Gly(GCC) and tRNA-Gly(UCC). While tRNA-Gly(GCC) reads GGC (by Watson–Crick base pairing) and GGU triplets (by wobbling), tRNA-Gly(UCC) recognizes GGA (by Watson–Crick base pairing) and GGG codons (by wobbling). However, knockout of the *trnG-GCC* gene in the tobacco plastid genome demonstrated that translation is sustained in the absence of tRNA-Gly(GCC). This suggests that a single glycine tRNA, tRNA-Gly(UCC), is sufficient to read all four glycine triplets. Apparently, the (unmodified) uridine in the first position of the anticodon of tRNA-Gly(UCC) can superwobble by engaging in base-pairing interactions with all four nucleotides (Rogalski et al., 2008a). Interestingly, the *trnG-UCC* gene encoding tRNA-Gly(UCC) is essential and tRNA-Gly(GCC) cannot read all four glycine codons. This is in

agreement with theoretical considerations (Crick, 1966) that, for steric reasons, the small pyrimidine base U can base pair weakly with U and C, but the bulky purine base G cannot base pair with A and G. Superwobbling provides a straightforward explanation for the ‘missing tRNAs’ in chloroplast genomes (Alkatib et al., 2012b). It, however, cannot explain all cases of missing tRNA genes in the highly reduced plastid genomes of non-photosynthetic holoparasitic plants (Morden et al., 1991; Delannoy et al., 2011; Krause, 2011) and how these parasites perform protein biosynthesis in their plastid compartment currently remains a mystery.

Systematic reverse genetic analysis of tRNA genes in the tobacco plastome demonstrated that superwobbling occurs in all codon boxes where it is theoretically possible (Rogalski et al., 2008a; Alkatib et al., 2012a,b). These studies also defined the minimum set of tRNA species that is required to sustain protein biosynthesis. Upon maximum use of wobbling and superwobbling, the minimum tRNA set comprises 25 tRNA species (Alkatib et al., 2012b). However, this minimum set is only rarely found in nature because, in most cases, superwobbling appears to be considerably less efficient than conventional base pairing and wobbling. Consequently, most knockouts of non-essential plastid tRNA genes displayed mutant phenotypes characterized by reduced photosynthetic activity and retarded growth, as caused by their lowered levels of plastid translation (Rogalski et al., 2008a; Alkatib et al., 2012b). The phenotypes of mutants for essential plastid tRNA genes are very similar to those described above for essential plastid genes encoding ribosomal proteins (Alkatib et al., 2012a,b).

As in all other biological systems, plastid tRNAs are transcribed as precursors, undergo posttranscriptional processing (Vogel et al., 1997; Gutmann et al., 2012), and are subject to extensive nucleotide modifications, which are thought to improve decoding accuracy or influence tRNA folding and/or stability (Delannoy et al., 2009; Karcher and Bock, 2009).

PLASTID TRANSLATION AND PLANT ANATOMY

Characterizations of plants with defects in chloroplast gene expression revealed the importance of plastids for leaf architecture (Pyke et al., 2000; Wang et al., 2000; Wycliffe et al., 2005). The leaf architecture is defined by mesophyll and bundle sheath cells enclosed by a layer of epidermal cells on both sides of the leaf (i.e. the adaxial side and the abaxial side). The mesophyll is composed of palisade cells and more loosely packed spongy mesophyll cells. The cylindrically shaped palisade cells reside below the adaxial epidermis and are major contributors to the plant’s photosynthetic performance. The bundle sheath cells are embedded in the mesophyll and transport water, minerals, sugars, and other organic compounds. Analysis of mutants in plastid gene expression, including translation mutants, suggested a tight connection between chloroplast function and palisade cell development. Many mutants in plastid gene expression show not only defects in

chloroplast biogenesis, but also disturbed palisade parenchyma development (Chatterjee et al., 1996; Wang et al., 2000; Wycliffe et al., 2005; Tiller et al., 2012).

Almost all levels of plastid gene expression have been implicated in the differentiation of palisade cells, including transcription (Hricová et al., 2006), RNA maturation (Reiter et al., 1994; Meurer et al., 1998a), and translation (Tiller et al., 2012). For example, defective palisade cell development has been reported in a wide spectrum of translation mutants, including mutants affected in components of the ribosome (Tiller et al., 2012), factors involved in ribosome biogenesis (Bang et al., 2012), and translation factors (e.g. elongation factor G and RRF; Ruppel and Hangarter, 2007; Wang et al., 2010). This suggests that sufficient translational capacity in the plastid (rather than specific components of the translational apparatus) is required for proper formation of the palisade parenchyma. This interpretation has gained further support from studies using specific inhibitors of chloroplast protein biosynthesis (Pyke et al., 2000).

It was suggested that the developmental and/or metabolic status of the plastids regulates cell differentiation and leaf morphogenesis by plastid-to-nucleus communication (Streatfield et al., 1999; Rodermel, 2001; Naested et al., 2004; Tan et al., 2008). Plastid retrograde signaling enables the chloroplast to communicate its functional and metabolic status to the host cell and to adjust nuclear gene expression according to the needs of the organelles (for recent reviews, see, e.g. Lepistö et al., 2012; Barajas-López et al., 2013; Estavillo et al., 2013; Jarvis and López-Juéz, 2013). With the currently available data, it is difficult to judge whether or not the defects in palisade cell development caused by impaired plastid translation represent a secondary consequence of disturbed chloroplast biogenesis. Preliminary evidence for the possibility to uncouple defects in plastid translation from impaired chloroplast biogenesis has recently come from the analysis of a set of plastid ribosomal protein mutants. When grown under comparable conditions, different mutants showed varying defects in palisade cell differentiation which, however, did not correlate well with the severity of the mutant phenotype (Tiller et al., 2012).

How does the protein biosynthesis capacity of the plastid affect mesophyll development? It seems conceivable that the deficiency in a specific plastid genome-encoded protein is responsible for the effect on palisade parenchyma development upon impaired plastid translation. Alternatively, the reduced expression capacity of the chloroplast could be sensed and transmitted by retrograde signaling to the nucleus, where it alters the gene expression program required for palisade cell differentiation. Whether the proposed plastid retrograde signal controlling mesophyll development is distinct from the classical (GUN-type) retrograde signaling remains to be clarified. Expression of *LHCB*, a favored marker gene of retrograde signaling (Susek et al., 1993; Sullivan and Gray, 1999), was investigated in some of the plastid gene expression mutants showing altered mesophyll organization.

While *LHCB* expression appears to be unaffected in some mutants (e.g. in the *pac1* mutant which is defective in plastid mRNA maturation; Reiter et al., 1994; Meurer et al., 1998a), it was found to be reduced in others (Hricová et al., 2006; reviewed, e.g., in Rodermel, 2001). This may provide preliminary evidence for the existence of a GUN-independent communication pathway influencing leaf anatomy, which vice versa is also supported by deregulated *LHCB* expression in the absence of disturbed palisade parenchyma development in retrograde signaling mutants, such as *gun1* (Rodermel, 2001).

PLASTID TRANSLATION AND PLANT MORPHOLOGY

Plastid Influence on Leaf Development in Evening Primroses

Early genetic studies in evening primroses (genus *Oenothera*; Onagraceae) provided evidence for the genotype of the plastid controlling leaf shape (Schwemmle, 1938, 1941, 1943). This discovery was facilitated by three salient features of the evening primrose system: (1) biparental chloroplast inheritance, (2) crossability among species and formation of fertile interspecific hybrids, and (3) a genetic phenomenon referred to as permanent translocation heterozygosity (Rauwolf et al., 2008; Greiner and Bock, 2013). These features make it relatively straightforward to produce interspecific hybrids with identical nuclear genomes but different plastid genomes. Performing interspecific crosses between *Oenothera odorata* and *Oenothera berteriana*, German botanist Julius Schwemmle was the first to realize that the leaf shape in evening primroses co-segregates with the genotype of the plastid. Leaves of *Oe. berteriana* are wider and more serrated than *Oe. odorata* leaves. Hybrids of *Oe. odorata* and *Oe. berteriana* with *berteriana* plastids have *berteriana*-like (wider and more strongly toothed) leaves, hybrid plants with *odorata* plastids have *odorata*-like (narrower and less toothed) leaves. This work clearly established that the genotype of the plastid determines the shape of evening primrose leaves (Schwemmle, 1938, 1941, 1943; Hagemann, 1964), which at that time was referred to as an 'extraplasmidic trait', because it was unrelated to leaf pigmentation and photosynthesis.

Plastid Translation and Leaf Development in Tobacco

Investigation of chloroplast gene expression by transplasmic approaches in tobacco revealed that plastid protein synthesis is absolutely required for plant development (Ahlert et al., 2003; Figure 2). Leaf and flower development are dependent on the expression of plastid genes in that loss of translation results in arrested cell division. This leads to a characteristic phenotype in which the death of entire cell lineages causes severely misshapen organs (see above; Figure 1). This phenotype was observed not only with knockout alleles for essential components of the plastid translational apparatus (Tables 1 and 2; Rogalski et al., 2008a; Alkatib et al., 2012b), but was also seen in heteroplasmic knockout mutants

for other essential chloroplast genes, such as *accD* (encoding the D subunit of acetyl-CoA carboxylase, an enzyme required for fatty acid biosynthesis; Kode et al., 2005), *clpP* (encoding an essential subunit of the Clp protease; Shikanai et al., 2001), and the two large open reading frames *ycf1* and *ycf2* (Drescher et al., 2000). This raises the possibility that plastid translation is only essential because it is required to express the few essential plastid genes that function outside of plastid gene expression.

In addition to the essentiality of plastid translation for leaf formation, the translational activity in plastids also affects leaf shape. For example, loss of the (non-essential) gene for tRNA-Gly(GCC) strongly reduces plastid translational activity and, interestingly, also causes an altered shape of the cotyledons (which are slender in the mutant compared to round-shaped cotyledons in the wild-type; Rogalski et al., 2008a). A similar narrow leaf phenotype was observed in true leaves of tobacco plants carrying a knockout allele for the (non-essential) plastid ribosomal protein gene *rpl36* (Fleischmann et al., 2011; Figure 2). Although both the *trnG*(GCC) and the *rpl36* mutant also have a photosynthetic phenotype, reduced photosynthetic activity cannot cause the alterations in leaf shape, because none of the many photosynthesis mutants (including many mutants that entirely lack photosynthesis) has been reported to display leaf shape phenotypes (Hager et al., 1999, 2002; Krech et al., 2012).

The altered leaf shape seen in tobacco mutants with reduced plastid translational activity is reminiscent of the control of leaf shape by the plastid genotype in evening primroses (Schwemmle, 1938, 1941, 1943). However, whether or not there is a direct mechanistic link between these two phenomena (in that altered translation of a plastid gene causes the phenotypic difference between *Oe. odorata* and *Oe. berteriana* leaves; see above) remains to be established.

The size and shape of leaves are pivotal factors determining plant performance and photosynthetic activity under ever-changing environmental conditions. Leaf morphology influences key photosynthetic parameters, such as the efficiency of light absorption and gas exchange. Some aspects of leaf morphology (e.g. leaf area, leaf thickness, and leaf index, i.e. the ratio of leaf length to leaf width) respond to environmental cues, including light intensity, growth temperature, and water availability. It seems tempting to speculate that the altered leaf morphology upon impaired plastid translation is part of such an adaption mechanism. In this scenario, changes in the environmental conditions induce changes in the translational activity of the chloroplast which in turn generate a signal to modify leaf morphology. It is well established that the translational apparatus of plastids responds sensitively to abiotic stress, especially temperature stress (Hanson and Bogorad, 1978; Büniger and Feierabend, 1980; Hopkins and Elfman, 1984; Xu et al., 2013). Since reduced chloroplast translation has a direct impact on photosynthetic activity (Fleischmann et al., 2011; Tiller et al., 2012), it seems conceivable that low chloroplast translational activity generates a

plastid signal that causes the development of more narrow leaves that absorb less light energy, thereby avoiding photo-oxidative damage.

Altered Leaf Development in *Arabidopsis* Mutants with Defects in Plastid Translation

The impact of plastid protein biosynthesis on leaf development was also confirmed by the identification and characterization of plastid gene expression mutants in the model plant *Arabidopsis thaliana*. As with the effects of plastid gene expression on leaf anatomy, mutations in any gene expression processes upstream of plastid translation are likely to also affect translation. For example, general defects in transcription or RNA processing are likely to have an impact on rRNAs, tRNAs, and/or mRNAs for ribosomal proteins and, therefore, unavoidably will result in lower levels of plastid translation. Consequently, mutants in plastid RNA metabolism and plastid translation mutants can have very similar phenotypes. For example, the *Arabidopsis* mutants *scabra3* (defective in the gene for the nuclear-encoded plastid RNA polymerase RpoTp; Hricová et al., 2006) and *hfp108-1* (defective in the chloroplast RRF; Wang et al., 2010) both have aberrantly shaped, strongly serrated leaves. A similar phenotype was also observed in a mutant defective in chloroplast ribosome biogenesis and, interestingly, correlated with reduced amounts of the phytohormone abscisic acid (Lee et al., 2013). In view of the crucial role of abscisic acid in leaf development in other systems (Chen et al., 2011; Wanke, 2011), this observation certainly warrants further investigation.

Since the functions of nearly all plastid genome-encoded gene products have been elucidated (Scharff and Bock, 2014) and none of them appears to be directly involved in leaf development, it seems reasonable to assume that the effect of plastid translation on leaf development is the result of retrograde signaling. This, however, remains to be firmly established. Also, the signaling molecule(s) and nuclear target genes of such a retrograde communication pathway will need to be identified. The development of the leaf blade is dependent on polar cell proliferation and polar cell expansion (reviewed, e.g., in Tsukaya, 1995; Sylvester et al., 1996; Byrne, 2012; Gonzalez et al., 2012). Leaf growth by cell proliferation is influenced by a number of genes in *Arabidopsis*, including the *ROTUNDIFOLIA* and *ANGUSTIFOLIA* genes, among others. While *ANGUSTIFOLIA* gene products are required for leaf expansion in the lateral direction, *ROTUNDIFOLIA* proteins mediate expansion in the longitudinal direction (Kim and Cho, 2006; Kawade et al., 2010). Whether or not these two groups of genes represent targets of signaling from plastids with impaired translational activity is currently not known. A recent study has provided evidence for plastid retrograde signaling influencing the spatial expression patterns of key genes involved early in expansion of the leaf lamina (Tameshige et al., 2013). In leaf primordia, the expression of the gene *FILAMENTOUS FLOWER* (*FIL*) and the activity of a microRNA (miR165/166) are specific to the abaxial side. Upon

inhibition of plastid translation, the spatial restriction of *FIL* expression and miR165/166 activity is retarded and the leaf lamina becomes narrow (Tameshige et al., 2013).

Interestingly, genetic screens for mutants with alterations in leaf shape have revealed not only genes involved in plastid translation (Moschopoulos et al., 2012), but also several genes for ribosomal proteins of the cytosolic (80S) ribosome (Pinon et al., 2008; Yao et al., 2008). Why mutations in some cytosolic ribosomal protein genes cause leaf phenotypes while mutations in many others do not is currently unknown. Ribosome heterogeneity in the cytosol or formation of aberrant ribosomes has been proposed as a possible explanation, but more work is needed to resolve this conundrum (Byrne, 2012; Horiguchi et al., 2012). Nonetheless, the striking mutant phenotypes caused by defects in both the cytosolic and the plastid protein biosynthesis machineries suggest an intimate relationship between translation in the cytosol and translation in the plastid compartment in controlling leaf shape. To elucidate the underlying molecular mechanisms and to determine the physiological significance of this regulation (and, in particular, its possible role in optimizing photosynthesis at the level of organ size and organ shape) represent major challenges for the future.

The Impact of Plastid Translation on Flower Development and Other Morphological Traits

Transplastomic experiments have established that plastid protein biosynthesis is also required for flower development (Ahler et al., 2003). Consistently with this finding and similarly to the observed effects of the plastid genotype on leaf shape, early genetic evidence from interspecific crosses in evening primroses also suggested an influence of the plastid genome on flower morphology. Reciprocal crosses between *Oe. odorata* and *Oe. berteriana* revealed that the length of the hypanthium is under genetic control of the plastid (Schwemmler, 1938, 1941, 1943). Unfortunately, this potentially very interesting finding was never followed up on and its interpretation remained controversial (Rhoades, 1955; Hagemann, 1964). The same holds true for the reported quantitative effect of the plastid genotype on stem growth (Hagemann, 1964). Interestingly, a potentially related observation was made in transplastomic knockout mutants of the gene for ribosomal protein L36 (Fleischmann et al., 2011). These mutants display strongly reduced apical dominance leading to excessive stem branching, thus giving the plants a bushy appearance (Figure 2C and 2D). As apical dominance is mainly determined by concentration gradients of the phytohormone auxin, this finding could suggest that impaired plastid translation influences auxin biosynthesis and/or transport. This could occur either by a plastid genome-encoded gene product influencing the activity or turnover of one of the (nucleus-encoded) enzymes of auxin biosynthesis or, alternatively, via a retrograde signaling pathway controlling the expression level of nuclear genes involved in auxin synthesis, transport, or degradation.

In summary, although it has long been known that the fidelity of plastid gene expression influences gene expression in the nucleus (Börner et al., 1986; Hess et al., 1994) and much has been learnt about the underlying signaling pathways (Susek et al., 1993; Koussevitzky et al., 2007), only recently phenotypic traits have been firmly associated with the fidelity of plastid gene expression. It appears likely that the translational activity of the plastid can generate a retrograde signal that influences specific aspects of plant anatomy and morphology, but how this signal fits into the increasingly complex landscape of plastid-to-nucleus communication (Leister, 2012; Barajas-López et al., 2013) remains to be elucidated. Future work should be directed towards (1) the discovery of components of the signaling pathway that act downstream of plastid translation and (2) the identification of nuclear target genes that produce the ultimate morphological output of this pathway.

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