Nonessential Plastid-Encoded Ribosomal Proteins in Tobacco: A Developmental Role for Plastid Translation and Implications for Reductive Genome Evolution

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Plastid genomes of higher plants contain a conserved set of ribosomal protein genes. Although plastid translational activity is essential for cell survival in tobacco (*Nicotiana tabacum*), individual plastid ribosomal proteins can be nonessential. Candidates for nonessential plastid ribosomal proteins are ribosomal proteins identified as nonessential in bacteria and those whose genes were lost from the highly reduced plastid genomes of nonphotosynthetic plastid-bearing lineages (parasitic plants, apicomplexan protozoa). Here we report the reverse genetic analysis of seven plastid-encoded ribosomal proteins that meet these criteria. We have introduced knockout alleles for the corresponding genes into the tobacco plastid genome. Five of the targeted genes (*ribosomal protein of the large subunit2*, *rpl22*, *rpl32*, *ribosomal protein of the small subunit3*, *rps3*, and *rps16*) were shown to be essential even under heterotrophic conditions, despite their loss in at least some parasitic plastid-bearing lineages. This suggests that nonphotosynthetic plastids show elevated rates of gene transfer to the nuclear genome. Knockout of two ribosomal protein genes, *rps15* and *rpl36*, yielded homoplasmic transplastomic mutants, thus indicating nonessentiality. Whereas Δ*rps15* plants showed only a mild phenotype, Δ*rpl36* plants were severely impaired in photosynthesis and growth and, moreover, displayed greatly altered leaf morphology. This finding provides strong genetic evidence that chloroplast translational activity influences leaf development, presumably via a retrograde signaling pathway.

INTRODUCTION

Translation in plastids, such as chloroplasts, occurs on bacterial-type 70S ribosomes that are similar in structure and composition to bacterial ribosomes (Yamaguchi and Subramanian, 2000; Yamaguchi et al., 2000; Manuell et al., 2007). All four RNA components (*rRNAs*) of chloroplast ribosomes are encoded by the plastid genome (plastome): the 23S, SS, and 4.5S *rRNAs* of the large (50S) ribosomal subunit and the 16S *rRNA* of the small (30S) ribosomal subunit. By contrast, the protein components of the plastid ribosome, the ribosomal proteins, are partly encoded in the nuclear genome. In the model plant tobacco (*Nicotiana tabacum*), 12 out of the 21 proteins of the small ribosomal subunit that have homologs in *Escherichia coli* are encoded in the chloroplast genome, whereas the remaining 9 proteins are nuclear encoded. Similarly, 9 out of 31 proteins of the large ribosomal subunit are encoded by plastid genes, whereas the other 22 are encoded by nuclear genes. Plastid ribosomes also contain a small number of proteins that are not found in bacterial ribosomes, the plastid-specific ribosomal proteins. They are encoded by nuclear genes, and their functions in protein biosynthesis and/or ribosome assembly are still largely unknown (Yamaguchi and Subramanian, 2000; Yamaguchi et al., 2000; Manuell et al., 2007; Sharma et al., 2007).

The set of ribosomal protein genes retained in the plastid genome is highly conserved across seed plants. This suggests that the gene transfer of ribosomal protein genes to the nuclear genome was largely completed before the evolutionary diversification of seed plants and that a relatively stable situation has now been reached. Exceptions include *rpl22* (transferred to the nuclear genome in legumes) (Gantt et al., 1991), *rpl32* (transferred to the nuclear genome in poplar) (Ueda et al., 2007), and *rpl23* in spinach (*Spinacia oleracea*) (replaced by a eukaryotic L23 protein version encoded in the nucleus and imported into plastids) (Bubunenko et al., 1994).

The most notable exceptions to the otherwise high degree of conservation of the set of ribosomal protein genes are the highly reduced plastomes of parasitic plants, which usually lack several plastid ribosomal protein genes (Wolfe et al., 1992; Funk et al., 2007; Delannoy et al., 2011). Whether these missing genes are dispensable under nonphotosynthetic conditions or, alternatively, have been transferred to the nuclear genome is currently unknown. Although most plastid genome-encoded proteins in nonparasitic plants function in photosynthesis, plastid gene expression is also important for many other cellular functions. These include, for example, tetrapyrrole biosynthesis (which requires the chloroplast *tRNA*-Glu) (Schön et al., 1986), fatty acid biosynthesis (which requires the plastid genome-encoded D subunit of the essential enzyme acetyl-CoA carboxylase) (Kode et al., 2005; Kahla and Bock, 2008), and plastid protein homeostasis (which is critically dependent on the plastid-encoded ClpP1 protease subunit).
(Shikanai et al., 2001; Kuroda and Maliga, 2003). The involvement of plastid gene expression in these essential functions is probably why the loss of plastid translational activity is fatal in most plants (Ahlert et al., 2003; Rogalski et al., 2006; Rogalski et al., 2008a). Known exceptions are the grasses (Han et al., 1992; Hess et al., 1994a) and some Brassicaceae (Zubko and Day, 1998).

Targeted inactivation of essential components of the plastid translational apparatus typically results in heteroplasmic plants that, in the presence of selective pressure, maintain a mix of mutant and wild-type plastomes (Ahlert et al., 2003; Rogalski et al., 2006). This is explained by a balancing selection, in which copies of the mutant plastome are needed to provide the antibiotic resistance (conferred by the selectable marker gene used for selection of transplastomic plants) and copies of the wild-type plastome are needed to provide the essential gene function disrupted in the mutant plastome (Drescher et al., 2000). Although random sorting of plastid genomes can lead to the appearance of mutant cells that are homoplasmic, these cells are unable to survive and divide. This then results in the loss of entire cell lineages, which becomes phenotypically apparent as severe defects in organ development. Typically, leaves and flowers of these plants are missshapen, because of the random loss of tissue sectors (Ahlert et al., 2003; Rogalski et al., 2006; Rogalski et al., 2008a). Growth on Suc does not rescue this phenotype, demonstrating that plastid gene functions unrelated to photosynthesis are what make plastid translation essential.

For this reason and because many components of the translational machinery are conserved in the otherwise highly reduced plastomes of nonphotosynthetic plastid-bearing organisms, it is generally assumed that at least some translational activity is present in the plastids of pathogen protozoans (Apicomplexa) and holoparasitic plants. In view of the high synthesis rates of photosynthesis-related proteins in chloroplasts, it is conceivable that the lack of photosynthesis would result in a much lower demand for plastid translation. It, therefore, seems possible that the translational apparatus in plastids of nonphotosynthetic organisms is somewhat simpler and less efficient, because of the relaxed selection pressure for high translational capacity. Whether this makes some components of the translational machinery dispensable is currently not known. Alternatively, the relaxed pressure for high translational activity could make plastid ribosomal protein genes more prone to gene transfer into the nuclear genome, where at least initially their expression would be less efficient than in the plastid.

Here we have undertaken a systematic investigation of plastid ribosomal protein genes for their essentiality in the translation process. We focused on genes meeting at least one of two criteria that could indicate nonessentiality: (1) loss from at least one lineage of nonphotosynthetic plastid-bearing organisms, and/or (2) evidence of nonessentiality in bacteria.

RESULTS

Targeted Disruption of Seven Plastid Ribosomal Protein Genes

By applying the two criteria (nonessentiality in *E. coli* and gene loss from the plastomes of nonphotosynthetic plastid-bearing organisms, such as holoparasitic plants and apicoplast-containing protozoa) (Wilson, 2002; Barbrook et al., 2006; Krause, 2008), we identified seven plastid ribosomal proteins as candidates for being nonessential for translation (Table 1). As representatives of nonphotosynthetic plastid-containing species, we initially included the parasitic seed plants *Cuscuta reflexa* (Funk et al., 2007) and *Epipagus virginiana* (Wolfe et al., 1992), the colorless heterotrophic alga *Euglena longa* (Astasia longa) (Gockel et al., 1994), and the apicomplexan parasites *Eimeria tenella* (Cai et al., 2003), *Theileria parva* (Gardner et al., 2005), and *Toxoplasma gondii* (Wilson and Williamson, 1997; Wilson, 2002). Recently, the plastome of the parasitic orchid *Rhizanthella gardneri* was fully sequenced (Delannoy et al., 2011). It was found to be the most reduced plastid genome discovered to date in a seed plant, and its ribosomal protein gene content (kindly made available to us by the authors prior to publication) was also considered for the identification of potentially nonessential genes. Using the sequence information from these reduced plastomes and the information on essential and nonessential genes in the model bacterium *E. coli* (Baba et al., 2006), the following plastid ribosomal protein genes were identified as potentially nonessential: *rpl22*, *rpl23*, *rpl32*, *rps3*, *rps15*, and *rps16* (Table 1). For *rpl32*, *rps36*, and *rps15* (and the previously analyzed *rpl33*) (Rogalski et al., 2008b), evidence from mutant analyses in *E. coli* indicates that these could be nonessential ribosomal protein genes. *rpl22*, *rpl23*, *rpl32*, *rps3*, *rps15*, and *rps16* are missing from at least one plastome of the parasitic or pathogenic reference species (Table 1). (Another potential candidate gene could be *rpl14*, which is present as a putative pseudogene in the plastome of *E. virginiana*, but was not investigated here.)

To functionally analyze these seven plastome-encoded ribosomal proteins and to clarify the possible relationship between gene loss in nongreen lineages of plastid evolution and nonessentiality, we used reverse genetics in tobacco, which is both a higher plant species amenable to plastid transformation (Svab and Maliga, 1993; Maliga, 2004; Bock, 2007) and a model plant for chloroplast functional genomics whose complete chloroplast genome sequence is available (Shinozaki et al., 1986; Ruf et al., 1997; Hager et al., 1999; Hager et al., 2002). We constructed knockout alleles for all seven genes by either replacing or disrupting the reading frame of the ribosomal protein gene of interest with *aadA*, the standard selectable marker gene for chloroplast transformation, in a cloned plastid DNA fragment (see Methods for details). The *aadA* gene product (the enzyme aminoglycoside 3′-adenylyltransferase) confers resistance to the aminoglycoside antibiotics spectinomycin and streptomycin, which act as specific inhibitors of plastid translation. Knockout vectors for all genes of interest, which are part of operons, were produced by precisely replacing the coding region of the targeted ribosomal protein gene with the *aadA* coding region. This strategy ensures that selectable marker gene expression is driven by the endogenous expression signals of the ribosomal protein gene and thus avoids interference with the expression of neighboring genes in the operon.

All knockout alleles were then introduced into the tobacco plastid genome by particle gun-mediated (biolistic) transformation to replace the corresponding wild-type alleles by homologous recombination (Maliga, 2004; Maliga and Bock, 2011). For...
such transplastomic plants show characteristic deformations
of antibiotic selection were analyzed. All transplastomic plants
survived, the phenotypes of plants grown in soil in the absence
of the knockout allele and also investigated the phenotypes of
plant constructs upon growth in soil under standard green-
house conditions.

For each knockout construct, two to five independently gen-
erated transplastomic lines were selected for further analyses.
The lines will be subsequently referred to as Δrpl22, Δrpl23, Δrpl32, Δrps3, Δrps15, and Δrps16, respectively.

**rpl22, rpl23, rpl32, rps3, and rps16 Are Essential Plastid Genes**

Essentiality of plastid genes is typically revealed by two charac-
teristic features: (1) the wild-type plastid genomes cannot be fully
eliminated despite passage of the transplastomic lines through
several rounds of stringent antibiotic selection, and (2) leaves of
such transplastomic plants show characteristic deformations
causled by the lack of large sectors of the leaf blade (due to the
death of cell lines that segregated into homoplasmy) (Drescher
et al., 2000; Rogalski et al., 2006a; Rogalski et al., 2008a). To test
for essentiality of the targeted ribosomal protein genes, we
analyzed DNA samples from our transplastomic lines by restriction
fragment length polymorphism (RFLP) analyses for homoplasmy
of the knockout allele and also investigated the phenotypes of
transplastomic lines upon growth in soil under standard green-
house conditions.

When the Δrpl22, Δrpl23, Δrpl32, Δrps3, and Δrps16 transplas-
tomic lines were tested by RFLP analysis, all of them showed
hybridizing fragments that were larger than the hybridizing frag-
ments in the wild-type sample and corresponded in size to the
knockout allele carrying the aadA marker gene inserted into the
respective ribosomal protein gene locus (Figures 1 to 5). However,
all transplastomic lines had an additional hybridization signal for
the wild-type restriction fragment even after three to four rounds of
regeneration under spectinomycin selection (and supplementa-
tion with Suc), indicating that none of these five ribosomal protein
genes can be eliminated from all plastome copies. To provide
further evidence of these five genes being essential for cell
survival, the phenotypes of plants grown in soil in the absence
of antibiotic selection were analyzed. All transplastomic plants
showed characteristic defects in leaf development caused by
sectorial loss of leaf tissue (due to death of cell lines that
regenerated medium produced multiple independent antibiotic-
resistant lines. Successful transformation of the plastid genome
was preliminarily confirmed by double resistance tests on tissue
culture medium containing spectinomycin and streptomycin, a
standard assay suitable for eliminating spontaneous spectino-
mycin-resistant mutants (Svab and Maliga, 1993; Bock, 2001).
The primary transplastomic lines were subjected to two to four
additional rounds of regeneration and selection for spectinomyc-
in resistance to enrich the transgenic plastome and dilute out
the residual wild-type plastomes. Unless a gene on the wild-type
plastome is essential, this procedure typically results in homoplas-
omic transplastomic cell lines (i.e., lines that lack any residual
wild-type plastome copies) after two to three rounds of selection
and regeneration (Svab and Maliga, 1993; Bock, 2001; Maliga,
2004).

For each knockout construct, two to five independently gen-
erated transplastomic lines were selected for further analyses.
The lines will be subsequently referred to as Δrpl22, Δrpl23, Δrpl32, Δrps3, Δrps15, and Δrps16, respectively.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Essential in E. coli</th>
<th>Essential in Tobacco Plastids</th>
<th>Present in Plastomes of Nongreen Lineages</th>
</tr>
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<tbody>
<tr>
<td>rpl2</td>
<td>Yes</td>
<td>NA</td>
<td>Yes</td>
</tr>
<tr>
<td>rpl14</td>
<td>Yes</td>
<td>NA</td>
<td>Not in E. virginiana (Ψ)</td>
</tr>
<tr>
<td>rpl16</td>
<td>Yes</td>
<td>NA</td>
<td>Yes</td>
</tr>
<tr>
<td>rpl20</td>
<td>Yes</td>
<td>Yes b</td>
<td>Not in E. tenella, T. parva, and T. gondii</td>
</tr>
<tr>
<td>rpl22</td>
<td>Yes</td>
<td>Yes c</td>
<td>Not in E. virginiana, R. gardneri, and T. gondii</td>
</tr>
<tr>
<td>rpl23</td>
<td>Yes</td>
<td>Yes c</td>
<td>Not in C. reflexa</td>
</tr>
<tr>
<td>rpl32</td>
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<td>Yes c</td>
<td>Not in R. gardneri and C. reflexa (Ψ)</td>
</tr>
<tr>
<td>rpl33</td>
<td>No</td>
<td>No b</td>
<td>Not in T. gondii, E. longa, and R. gardneri (Ψ)</td>
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<tr>
<td>rpl36</td>
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</tr>
<tr>
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<td>Yes</td>
<td>Yes b</td>
<td>Not in T. gondii</td>
</tr>
<tr>
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<td>Yes</td>
<td>Yes c</td>
<td>Not in R. gardneri</td>
</tr>
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<td>rps4</td>
<td>Yes</td>
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</tr>
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<td>No</td>
<td>No c</td>
<td>Not in E. virginiana and R. gardneri</td>
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<td>rps16</td>
<td>Yes</td>
<td>Yes c</td>
<td>Not in C. reflexa and R. gardneri</td>
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<td>rps18</td>
<td>Yes</td>
<td>Yes b</td>
<td>Not in E. longa and T. gondii</td>
</tr>
<tr>
<td>rps19</td>
<td>Yes</td>
<td>NA</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Ψ, pseudogene; NA, not analyzed.

aData on E. coli ribosomal proteins are from Baba et al. (2006).
bData on plastid ribosomal proteins are from our previously published work (Ahlert et al., 2003; Rogalski et al., 2006; Rogalski et al., 2008b).
cData on plastid ribosomal proteins are from this study.
dCould not be confirmed in this study (see text for details).
In the absence of antibiotic selection, the knockout alleles in the transplastomic lines confer a selective disadvantage and, therefore, are usually rapidly lost due to random segregation of plastid genomes during cell division (Drescher et al., 2000). Consistent with this expectation, transmission of the transplastome into the next generation was very low when parent plants were grown without selection for spectinomycin resistance, as revealed by seed germination assays on spectinomycin-containing synthetic medium (Figures 1E, 2E, 3F, 4E, and 5E).

In contrast with the stable heteroplasmy observed in the \( \text{D} \text{rpl22}, \text{D} \text{rpl23}, \text{D} \text{rpl32}, \text{D} \text{rps3}, \text{and D} \text{rps16} \) transplastomic lines, no hybridization signal for the wild-type plastome was detectable in digested DNA samples from the \( \text{D} \text{rps15} \) plants after the lines had been grown without selection for spectinomycin resistance, as revealed by seed germination assays on spectinomycin-containing culture medium (Figures 1E, 2E, 3F, 4E, and 5E).

**Rps15 is a Nonessential Plastid Ribosomal Protein**

In contrast with the stable heteroplasmy observed in the \( \text{D} \text{rpl22}, \text{D} \text{rpl23}, \text{D} \text{rpl32}, \text{D} \text{rps3}, \text{and D} \text{rps16} \) transplastomic lines, no hybridization signal for the wild-type plastome was detectable in digested DNA samples from the \( \text{D} \text{rps15} \) plants after the lines had been grown without selection for spectinomycin resistance, as revealed by seed germination assays on spectinomycin-containing culture medium (Figures 1E, 2E, 3F, 4E, and 5E).

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Essentiality of \( \text{rpl32} \) in plastids was somewhat surprising, because the gene has been identified as nonessential in \( \text{E. coli} \) (Baba et al., 2006). This prompted us to order the \( \text{E. coli} \) knockout strain from the Keio collection (Baba et al., 2006) and reinvestigate it. Sequencing of the \( \text{rpl32} \) locus in the mutant strain that we received revealed that the gene was undisrupted. Whether this means that the \( \text{E. coli} \) data on \( \text{rpl32} \) are incorrect or, alternatively, a mix-up in strains has occurred upon storage or shipping remains to be clarified by the curators of the Keio collection.
passed the third regeneration round (Figures 6A to 6C). This tentatively suggested that the lines are homoplasmic and that the \(S15\) protein may not be essential for maintenance of plastid translation. Homoplasmy of the \(D_{rps15}\) knockout lines was ultimately confirmed by inheritance assays. Germination of seeds from \(D_{rps15}\) plants on spectinomycin-containing medium yielded a uniform population of homogeneously green seedlings (Figure 6D). Lack of segregation in the T1 generation provides strong genetic proof of homoplasmy of the transplastomic lines and thus of the nonessentiality of the plastid \(rps15\) gene.

When \(D_{rps15}\) knockout plants were raised under a variety of different light intensities (ranging from 100 to 1000 \(\mu \text{E m}^{-2} \text{s}^{-1}\)), they displayed no obvious mutant phenotype (Figures 6E and 6F). In fact, their growth rates were almost identical to the wild-type plants, although young plants grew slightly more slowly (Figure 6E), and flowering was a bit delayed (Figure 6F).

To confirm that chloroplast ribosomes in the \(D_{rps15}\) mutants indeed function without an S15 subunit, we purified plastid ribosomes and subjected them to mass spectrometric protein identification (Rogalski et al., 2008b). Although S15 was readily detectable in the wild-type ribosomes, no S15 protein was found in ribosomes from \(D_{rps15}\) knockout plants (see Supplemental Table 1 online). These data confirm that the knockout leads to a complete loss of Rps15 and, moreover, demonstrate that the loss of the plastid-encoded S15 is not compensated by import of a nuclear-encoded S15 protein. This is consistent with our failure to identify putative nuclear genes for a chloroplast S15 protein when we searched the available genome and EST databases for tobacco and other Solanaceous plants.

**Photosynthesis in \(D_{rps15}\) Plants**

The efficiency of photosynthetic electron transport and the accumulation levels of the protein complexes in the thylakoid membrane are highly sensitive indicators of plastid translational capacity (Rogalski et al., 2008b). Because of the very mild growth

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**Figure 2.** Targeted Inactivation of the \(rpl23\) Gene Encoding Plastid Ribosomal Protein L23.

(A) Physical map of the region in the tobacco plastid genome containing the \(rpl23\) gene. Genes above the line are transcribed from the left to the right, genes below the line are transcribed in the opposite direction.

(B) Map of the transformed plastid genome produced with plastid transformation vector \(p_{Δrpl23}\). The selectable marker gene \(aadA\) replaces the \(rpl23\) gene in the operon of ribosomal protein genes. Restriction sites used for cloning, RFLP analysis, and/or generation of hybridization probes are indicated. Expected sizes of restriction fragments detected by hybridization are indicated by black bars below the maps.

(C) RFLP analysis of five plastid transformants. All lines are heteroplasmic and show the 2.3-kb wild type–specific hybridization band in addition to the 2.7-kb band diagnostic of the transplastome. WT, wild type.

(D) Phenotype of a typical (heteroplasmic) \(Δrpl23\) transplastomic plant. Arrows point to examples of misshapen leaves that lack part of the leaf blade.

(E) Segregation of the \(rpl23\) knockout allele in the progeny as determined by seed germination assays on synthetic medium containing spectinomycin. Two examples of green antibiotic-resistant seedlings (that still contain the transplastome) are denoted by arrows.

Bar in (D) = 13 cm.
Figure 3. Targeted Inactivation of the rpl32 Gene Encoding Plastid Ribosomal Protein L32.

(A) Physical map of the region in the tobacco plastid genome containing the rpl32 gene. Transcriptional orientations and labeling of restriction sites, hybridization probes, and hybridizing fragments are as in Figure 2.

(B) Map of the transformed plastid genome produced with plastid transformation vector pΔrpl32A. In this vector, the selectable marker gene aadA replaces the rpl32 gene and is driven by the native promoter upstream of rpl32.

(C) Map of the transformed plastid genome produced with plastid transformation vector pΔrpl32B. In this vector, a chimeric selectable marker gene cassette was used to replace the rpl32 gene. It consists of the promoter of the plastid psbA gene, the coding region of the aadA marker and the 3' UTR derived from the rbcL gene.

(D) RFLP analysis of four plastid transformants. All lines are heteroplasmic and show the 4.7-kb wild type–specific hybridization band. The transplastomes give the expected 5.4-kb band (Δrpl32A) or 6.2-kb band (Δrpl32B). WT, wild type.

(E) Phenotype of a typical (heteroplasmic) Δrpl32 transplastomic plant. The arrow points to a misshapen leaf that lacks almost half of the leaf blade.

(F) Segregation of the rpl32 knockout allele in the progeny as determined by seed germination assays on synthetic medium containing spectinomycin. A single green antibiotic-resistant seedling (that still contains the transplastome) is marked by the arrow. Bar in (E) = 13 cm.
phenotype of the Δrps15 knockout lines, we decided to measure photosynthetic performance and protein complex accumulation in the wild-type and transplastomic plants. Because the demand for de novo synthesis of the proteins of the photosynthetic apparatus decreases with leaf development, we analyzed a developmental series of leaves ranging from young expanding leaves (leaves number 3 and 4, the youngest leaves of sufficient size to be measured fluorometrically) to fully expanded mature leaves (Figure 7).

Although the chlorophyll content in the mutant lines was not significantly different from the wild-type control, the mutants had a clearly lower chlorophyll a:b ratio than the wild type (Figure 7). This points to a reduced accumulation of the (largely plastid-encoded) reaction center proteins in the Δrps15 plants, whereas the nuclear-encoded antenna proteins are unaffected or may even overaccumulate, thus explaining the unchanged total chlorophyll content. Interestingly, the decreased chlorophyll a:b ratio did not ameliorate over the developmental time span examined (Figure 7), suggesting that deficits in photosynthetic complex biogenesis cannot be compensated over time.

The maximum quantum efficiency of photosystem II (PSII), FV/FP, was also significantly reduced in the Δrps15 plants and, similar to the chlorophyll a:b ratio, this reduction was largely independent of leaf age and developmental stage (Figure 7). When we measured the contents of the photosynthetic protein complexes by difference absorption spectroscopy (Schöttler et al., 2007a), the amounts of PSII and the cytochrome b6f complex were found to be significantly reduced in the Δrps15

Figure 4. Targeted Inactivation of the rps3 Gene Encoding Plastid Ribosomal Protein S3.

(A) Physical map of the region in the tobacco plastid genome containing the rps3 gene. Transcriptional orientations and labeling of restriction sites, hybridization probes, and hybridizing fragments are as in Figure 2.

(B) Map of the transformed plastid genome produced with plastid transformation vector pΔrps3. The selectable marker gene aadA replaces the rps3 gene (residing in an operon with many other ribosomal protein genes).

(C) RFLP analysis of two plastid transformants. Both lines are heteroplasmic and show the 1.2-kb wild type–specific hybridization band in addition to the 5.4-kb band diagnostic of the transplastome. The 3.0-kb band corresponds in size to the fragment expected for the co-integrate (resulting from single crossover integration) and was not further characterized. WT, wild type.

(D) Phenotype of a typical (heteroplasmic) Δrps3 transplastomic plant. Arrows point to examples of misshapen leaves that lack parts of the leaf blade.

(E) Segregation of the rps3 knockout allele in the progeny as determined by seed germination assays on synthetic medium containing spectinomycin. Two examples of green antibiotic-resistant seedlings (that still contain the transplastome) are indicated by arrows. Bar in (D) = 13 cm.
lines, whereas the amount of photosystem I (PSI) was much less affected (Figure 7). Because most of the reaction center subunits of the protein complexes involved in photosynthetic electron transport are encoded in the plastid genome, these data tentatively suggested that plastid gene expression is somewhat less efficient in the absence of the ribosomal protein S15. PSII is known to require a particularly high translation capacity due to the constant requirement for repair synthesis of the D1 protein (Takahashi and Badger, 2011). By contrast, PSI is very stable, which may explain why it is less affected in the mutant plants.

Efficient chloroplast protein biosynthesis is particularly important in very young developing leaves (Albrecht et al., 2006; Rogalski et al., 2008a; Rogalski et al., 2008b), most likely because the de novo assembly of the photosynthetic machinery absorbs very high translational capacity. In addition, cold stress has recently been identified as a condition aggravating the phenotype of plants with subtle defects in chloroplast translation (Rogalski et al., 2008b). We therefore tested whether the mild growth phenotype observed in the Δrps15 plants under standard growth conditions is more apparent in very young leaves or becomes more severe under chilling stress. Indeed, very young developing leaves of the mutant plants (of sizes smaller than what can be measured fluorometrically) (Figure 7) were light green compared with leaves of the wild-type plants, suggesting

Figure 5. Targeted Disruption of the rps16 Gene Encoding Plastid Ribosomal Protein S16.

(A) Physical map of the region in the tobacco plastid genome containing the rps16 gene. Transcriptional orientations and labeling of restriction sites, hybridization probes, and hybridizing fragments are as in Figure 2.

(B) Map of the transformed plastid genome produced with plastid transformation vector pΔrps16. The aadA cassette disrupts the rps16 gene.

(C) RFLP analysis of three plastid transformants. All lines are heteroplasmic and show the 4.2-kb wild type (WT)-specific hybridization band in addition to the 5.7-kb band diagnostic of the transplastome. Additional larger hybridizing bands are most likely the products of recombination events between homologous expression signals (Rogalski et al., 2006; Rogalski et al., 2008a). They were reproducibly observed in several independent experiments, do not match expected patterns for partial restriction enzyme digestion, and were not further characterized.

(D) Phenotype of a typical (heteroplasmic) Δrps16 transplastomic plant. Arrows point to examples of misshapen leaves that lack parts of their leaf blade.

(E) Segregation of the rps16 knockout allele in the progeny as determined by seed germination assays on synthetic medium containing spectinomycin. Two examples of green antibiotic-resistant seedlings (that still contain the transplastome) are indicated by arrows.

Bar in (D) = 13 cm.
that the synthesis of the photosynthetic apparatus is delayed in Δrps15 plants (Figure 8A). This phenotype became much more pronounced under chilling stress (Figure 8B), suggesting that, in the Δrps15 mutant, chloroplast translational capacity limits the biogenesis of the photosynthetic apparatus in young leaves and under stress conditions.

Plastid Ribosomal Accumulation in Δrps15 Plants

We next wanted to assess the consequences of the lack of the S15 protein at the level of translation and plastid ribosome biogenesis. To this end, we used a microfluidics-based platform for sizing and quantifying the rRNA species. If not assembled in ribosomal subunits, rRNAs are not stable and, therefore, rRNA accumulation can serve as a proxy for ribosomal subunit accumulation (Walter et al., 2010). We first determined the relative abundance of chloroplast to cytosolic ribosomal subunits and calculated the ratios 16S rRNA:18S rRNA and 23S rRNA:18S rRNA. (16S and 23S represent plastid rRNA species, whereas 18S is the rRNA of the small subunit of the cytosolic ribosome.) The data revealed that the Δrps15 plants have reduced amounts of small (30S) subunits of the plastid ribosome, as evidenced by a decreased 16S rRNA:18S rRNA ratio (Figure 9A). Interestingly, the accumulation of large (50S) subunits of the chloroplast ribosome was even slightly higher than in the wild type (evidenced by an increased 23S rRNA:18S rRNA ratio) (Figure 9B), possibly suggesting that the cell senses the chloroplast ribosome deficiency and attempts to compensate for it by an up-regulation of de novo synthesis. Analysis of the 23S rRNA:16S rRNA ratio confirmed that the knockout of the plastid rps15 gene leads to a specific reduction in small ribosomal subunits (Figure 9C), in line with S15 being a component of the 30S subunit of the plastid ribosome.

To examine how chilling stress affects plastid ribosomes in Δrps15 transplastomic plants, we compared the 23S rRNA:18S rRNA and 16S:18S rRNA ratios in young leaves of mutant and wild-type plants grown at 4°C. Consistent with their severe phenotype in the cold (Figure 8), Δrps15 plants showed a drastic reduction in plastid ribosomes. In contrast with growth under unstressed conditions (Figure 9B), now the 50S ribosomal subunit of the plastid ribosome was also strongly affected (evidenced by a severely decreased 23S rRNA:18S rRNA ratio in the mutant compared with the wild type) (Figure 9D), indicating that a secondary loss of plastid ribosomes occurs in Δrps15 plants during chilling stress.

Plastid Translation in Δrps15 Plants

In view of the mutant phenotype (Figures 6 and 8) and the deficiency in plastid ribosomes, it seemed reasonable to assume that Δrps15 transplastomic plants suffer from reduced levels of chloroplast translation. To directly investigate plastid translational activity in the absence of the S15 protein, we conducted polysome...
loading analyses (Figure 10). These assays determine the coverage of mRNAs with translating ribosomes and thus represent a measure of translational activity (Barkan, 1988; Barkan, 1998; Kahlau and Bock, 2008). We comparatively analyzed polysome association of two plastid transcripts: the dicistronic psaA/B mRNA encoding the two reaction center proteins of PSI and the psbD mRNA encoding the reaction center protein D2 of PSII (which is co-transcribed with psbC, the gene encoding the CP43 inner antenna protein of PSI). To explore a possible correlation with the leaf age-dependent phenotype, the analyses were performed in parallel for young leaves and fully expanded leaves (“old” leaves in Figure 10; comparable to leaves 7 and 8 in Figure 7).

Comparison of polysome association in young leaves of the wild-type and mutant plants revealed that plastid translation is indeed less efficient in the Δrps15 mutant. For both transcripts investigated, the maximum in the mRNA distribution is shifted toward lighter fractions in the Suc density gradient in the mutant. For example, the maximum in the psaA/B mRNA distribution is in gradient fractions 8–10 in the wild type, but in fractions 6–8 in the mutant (Figure 10B). Consistent with the observed leaf age-dependent phenotype, older leaves were somewhat less affected than younger leaves.

Having observed a more severe phenotype of Δrps15 plants under chilling stress, we also examined plastid translational efficiency in the cold. As expected, chilling stress affected plastid translation in both the wild type and the mutant, as evidenced by a shift in the maximum in mRNA distribution across the Suc density gradient. Consistent with the temperature-dependence of the mutant phenotype, the difference in ribosome association between the wild-type and the Δrps15 transplastomic plants was even more pronounced in the cold (Figure 10D).

The Plastid rpl36 Gene Is Nonessential, but Its Knockout Leads to a Severe Mutant Phenotype

Although experimental evidence suggests that the ribosomal protein gene rpl36 may not be essential in E. coli (Baba et al., 2006), the gene is conserved in the plastid genomes of all nongreen lineages (Table 1). To test whether rpl36 encodes an essential component of the plastid ribosome, we constructed a knockout allele by precisely replacing the rpl36 reading frame with the aadA coding region within the rpoA operon (Figures 11A and 11B). Consequently, in the knockout construct, the aadA marker is driven by the endogenous expression signals of rpl36.

Data sets are shown for plants grown under 100 μE m⁻² s⁻¹ for 8 weeks followed by 2 weeks of growth under 350 μE m⁻² s⁻¹. To capture possible changes during development, pairs of leaves (numbered from the top to the bottom of the plant) were analyzed. For each plant line, three different plants were measured, and data were subjected to one-way analysis of variance using a pair-wise multiple comparison procedure (Holm-Sidak method) in SigmaPlot. Highly significant differences are indicated by stars (P < 0.001). Error bars represent the SD. Fv/Fm represents the maximum quantum efficiency of PSII in the dark-adapted state. Photosynthetic complexes were quantified from difference absorbance measurements of cytochrome b559 (PSII), cytochromes b₆ and f, and P700 (PSI) in isolated thylakoids. WT, wild type.
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an extreme reduction in the width of the leaf blade. Because

more slender than the wild-type leaves (Figure 11H), because of

strong alteration in leaf morphology. Mutant leaves were much

larger than the wild-type leaves (Figure 11D and 11E), suggesting that the mutant

rps15 plants grown under cold stress conditions for 65 d

show a much more severe pigment loss than the wild-type leaves.

Bars = 5 cm.

Analysis of transplastomic \( \Delta rpl36 \) plants by DNA gel blotting

suggested homoplasy of all three lines investigated (Figure

11C). During growth in sterile culture on Suc-containing medium,

the \( \Delta rpl36 \) lines displayed a strong phenotype. Mutant leaves

were pale and more elongated than the wild-type leaves. Growth

under extreme low-light conditions resulted in partial greening of

\( \Delta rpl36 \) leaves (Figures 11D and 11E), suggesting that the mutant

plants are highly susceptible to photo-oxidative damage. When

plants were transferred to soil, they grew extremely slowly,

showed severe pigment deficiency (Figure 11F) even under low-

light conditions (50–70 \( \mu \)E m\(^{-2}\) s\(^{-1}\) at 26°C), and did not produce seeds.

In addition, lateral branching, which is not normally seen in the

wild-type tobacco plants, occurred extensively in the mutant,

giving the plants a bushy appearance (Figure 11G) and suggest-

ing that impaired plastid translation reduces apical dominance.

The most dramatic aspect of the mutant phenotype was a

strong alteration in leaf morphology. Mutant leaves were much

more slender than the wild-type leaves (Figure 11H), because of

an extreme reduction in the width of the leaf blade. Because

similar changes in leaf shape are not seen in mutants affected in

photosynthesis (Bock et al., 1994; Hager et al., 2002), this finding

suggests that leaf morphology is specifically influenced by the

translational activity in the plastid.

DISCUSSION

Although knockout of plastid protein biosynthesis is fatal in

tobacco (Ahlert et al., 2003), this does not mean that each

individual component of the chloroplast ribosome is essential.

Previous work has established that the L33 protein of the plastid

ribosome is not required for translation under normal growth con-
ditions but is important under cold stress conditions (Rogalski

et al., 2008b). In this study, we have systematically investi-
gated seven plastid-encoded ribosomal protein genes that were

lost from plastid genomes of nongreen plastid-bearing line-
ages and/or have been suggested to be nonessential in bac-
teria (Table 1).

Our work has identified two additional nonessential protein

components of the plastid ribosome: S15 and L36. In contrast

with the previously analyzed \( rpl33 \) gene (Rogalski et al., 2008b),

knockout of \( rps15 \) and \( rpl36 \) is not phenotypically neutral under

standard growth conditions. \( rps15 \) knockout plants have only an

mild growth phenotype, despite a significant reduction in pho-
tosynthetic complex accumulation, ribosome content, and plas-
tid translational activity (Figures 7, 9, and 10). The phenotypic

effects are most strongly pronounced in young developing

leaves and under chilling stress. This finding is consistent with

the demand for plastid translational capacity being highest in

developing leaves, when the photosynthetic machinery needs to

be synthesized and assembled. By contrast, in mature leaves,

lower levels of plastid translation seem to be sufficient to ensure

maintenance and repair of the photosynthetic apparatus. This is

in agreement with polysome loading in older leaves of the \( \Delta rps15 \)

knockout mutant being less affected than in young leaves (Figure

10B). In the assembly of the \( E. coli \) 30S ribosomal subunit, ribosomal protein S15 binds early to the 16S rRNA and is possibly involved in triggering conformational changes that may facilitate downstream assembly steps (Talkington et al., 2005; Kaczanowska and Ryden-Aulin, 2007; Woodson, 2008; Connolly and Culver, 2009; Sykes et al., 2010). It is, therefore, conceivable that the loss of S15 partially impairs biogenesis of 30S ribosomal particles in plastids, thus explaining the preferential loss of small ribosomal subunits in our transplastomic \( \Delta rps15 \) knockout mutants (Figure 9).

Knockout of \( rpl36 \), a gene not lost from any of the sequenced

plastid genomes in nonphotosynthetic lineages, results in nearly

white plants that show severe morphological aberrations and

hardly grow photo-autotrophically. This indicates that the low

translational efficiency in the absence of the L36 protein is

insufficient to sustain the (presumably relatively low) protein

biosynthesis levels required in the nongreen plastids of the

nonphotosynthetic plants listed in Table 1. L36 is also highly

conserved in bacteria, but is not present in the ribosomes of

archaea and eukaryotes. In \( E. coli \), conflicting data have been

published on the importance of L36 in translation and ribosome

assembly. Whereas one study reported that deletion of the \( rpl36 \)
gene did not cause a mutant phenotype (Ikegami et al., 2005),
another study reported that strains lacking L36 are severely

impaired in growth and concluded that L36 may play a significant

role in organizing the 23S rRNA structure (Maeder and Draper,

2005). Our data obtained in plastids are more consistent with the

latter report, but cannot ultimately explain the reasons for the

conflicting data obtained in \( E. coli \).

Taken together with previous data, we now have a compre-

hensive overview of the essentiality of plastome-encoded ribo-
somal protein genes that were lost in nonphotosynthetic lineages
of parasitic plants and pathogenic protozoa (Table 1). A ribosomal protein gene can be absent from the plastome of a nonphotosynthetic organism for two possible reasons: (1) the gene was lost permanently, because it is functionally dispensable due to the low demand for plastid translational capacity in nongreen plastids, or (2) the gene was transferred from the plastid genome to the nuclear genome. Our data reveal no obvious correlation between the loss of plastid ribosomal protein genes and the dispensability of these genes under heterotrophic conditions. Instead, given that most of these genes are essential in both *E. coli* and plastids (Table 1), they have most likely been transferred to the nuclear genome in the nonphotosynthetic lineages (Timmis et al., 2004; Bock and Timmis, 2008). Because many of these ribosomal protein genes are highly conserved in the plastid genomes of the green lineages (with only occasional examples of gene transfer or gene replacement—see Introduction), this in turn suggests that the loss of photosynthesis and the transition to a heterotrophic lifestyle results in an increased transfer frequency of ribosomal protein genes to the nucleus.

What could be the evolutionary driving force for such an increased gene transfer rate to the nucleus? A reasonable hypothesis could be that the loss of photosynthesis and the concomitantly reduced demand for plastid translational capacity increase the success rate of functional gene transfer to the nucleus. Recent work has shown that the transfer of plastid DNA into the nuclear genome occurs at high frequency (Huang et al., 2003; Stegemann et al., 2003). However, these transferred DNA pieces are usually not expressed in the nucleus, due to the prokaryotic nature of their expression signals (promoter, 5′ untranslated region [UTR]). Their functional activation in the nuclear genome requires rearrangements that change the prokaryotic expression signals into eukaryotic ones (e.g., by promoter capture) (Stegemann and Bock, 2006; Bock and Timmis, 2008). Very likely, whether these transferred DNA pieces regain the full expression level from the nuclear gene copy depends on additional mutations that gradually cause the transferred plastid gene to resemble a typical eukaryotic gene. Thus, it seems conceivable that the success rate of functional gene transfer is correlated to the required expression level of the gene in the nucleus. Genes for which relatively low expression levels suffice have a higher probability of establishing a functional nuclear copy before mutational degeneration of the transferred DNA sets in (Stegemann and Bock, 2006; Sheppard and Timmis, 2009). We therefore, propose that the low demand for plastid translational activity in nonphotosynthetic plastids increases the chances of transferred ribosomal protein genes becoming functional nuclear genes, facilitating their subsequent loss from the plastid genome.

An intriguing aspect of the phenotype of our Δrpl36 mutants was the drastic change in two traits that are not typically associated with plastid function and plastid gene expression: apical dominance and leaf shape (Figure 11). Apical dominance is mainly determined by concentration gradients of the phytohormone auxin. Auxin can be synthesized by two biochemical pathways: a Trp-dependent and a Trp-independent pathway (Normany et al., 1993; Cohen et al., 2003; Woodward and Bartel, 2005). Although the Trp-dependent pathway of auxin biosynthesis is reasonably well understood, the biochemistry of the Trp-independent pathway is still a mystery. However, evidence has been obtained that the plastid compartment is crucially involved in the Trp-independent pathway of auxin biosynthesis (Rapparini et al., 1999; Rapparini et al., 2002). Hence, it seems possible that the drastically reduced levels of plastid translation in the Δrpl36 mutants directly or indirectly affect the chloroplast-localized enzymes involved in Trp-independent auxin biosynthesis. This could either occur by a plastome-encoded gene product influencing the activity or turnover of the (nuclear-encoded) auxin

![Figure 9](image-url)
biosynthetic enzymes or, alternatively, by a retrograde signaling pathway emanating from plastid gene expression (Larkin and Ruckle, 2008; Pogson et al., 2008; Kleine et al., 2009) and regulating the expression level(s) of these enzyme(s). Consistent with a possible defect in auxin metabolism or transport in the Δrpl36 mutants, rooting of stem cuttings and regenerated shoots was very inefficient and severely delayed compared with the wild type. Retrograde signaling is also the most likely cause of the leaf shape phenotype in the Δrpl36 mutants. Early genetic work in evening primroses (involving reciprocal crosses between two sexually compatible species, Oenothera odorata and Oenothera berteriana), provided evidence for an influence of the plastid genotype on leaf shape (especially on the width of the leaf blade and the intensity of serration of the leaf margin) (Schwemmle, 1941; Schwemmle, 1943). Because of biparental plastid transmission in evening primroses, hybrid plants with identical nuclear genomes could be produced that had either O. odorata plastids or O. berteriana plastids. Interestingly, the two hybrids differed

Figure 10. Analysis of Translation in Δrps15 Plants.
(A) to (D) Analysis of rRNA distribution in polysome preparations from young leaves (numbers 8, 7, and 6; Figure 9) and old leaves (number 1) of the wild-type (WT) and mutant plants. Polysomes were separated in Suc gradients, and each gradient was fractionated into 10 fractions (numbered from the top to the bottom) as indicated above the panels.
(A) Ethidium bromide-stained agarose gels prior to blotting.
(B) Comparison of polysome association of psaA and psbD transcripts in the wild-type and Δrps15 transplastomic plants. Young and old leaves are compared for both genes.
(C) As a control, polysomes were isolated in the presence of the polysome-dissociating antibiotic puromycin, blotted, and hybridized to the psaA probe (Bottom). For analysis of rRNA distribution, an ethidium bromide-stained agarose gel prior to blotting is also shown (Top).
(D) Analysis of polysome association of psaA and psbD transcripts in leaves of plants grown under cold stress conditions. The gradient fractions containing the bulk of the transcripts are indicated by brackets.
Figure 11. Knockout of the rpl36 Gene Encoding Plastid Ribosomal Protein L36.

(A) Physical map of the region in the tobacco plastid genome containing the rpl36 gene. Transcriptional orientations and labeling of restriction sites, hybridization probes, and hybridizing fragments are as in Figure 2.

(B) Map of the transformed plastid genome produced with plastid transformation vector pΔrpl36. The aadA gene replaces rpl36 in the large operon of ribosomal protein genes.

(C) RFLP analysis of three plastid transformants. All transplastomic lines are homoplasmic and show exclusively the 2.6-kb band diagnostic of the transplastome. WT, wild type.

(D) Phenotype of a Δrpl36 transplastomic plant grown on Suc-containing synthetic medium for 3 months under low-light conditions (5 μE m⁻² s⁻¹). 

(E) Greening of Δrpl36 leaves after plant transfer from medium-light to low-light conditions. The left two leaves are from a plant 4 weeks after transfer from 55 to 5 μE m⁻² s⁻¹, and the right leaf is from a plant continuously grown under 55 μE m⁻² s⁻¹.

(F) Phenotype of a Δrpl36 transplastomic plant growing in soil 2 months after transfer to the greenhouse.

(G) The same plant after 1.3 years. Note the atypical extensive branching.

(H) Severely altered leaf shape in Δrpl36 transplastomic knockout mutants. A wild-type plant (left) is compared with a transplastomic plant at approximately the same developmental stage.

Bars in (D) and (F) = 6 cm; bar in (E) = 3 cm; bar in (G) 13 cm; bar in (H) at left = 13 cm and at right = 6 cm.
markedly in leaf morphology. Plants with O. odorata plastids had narrower leaf blades than leaves with O. berteriana plastids, and, strikingly, the leaf phenotypes of the hybrids very closely resembled the leaf phenotypes of the parent who donated the plastids (Schwemmle, 1941; Schwemmle, 1943). Because none of the known factors determining leaf development is encoded in the plastid genome, it seems reasonable to assume that this is the result of a retrograde signaling pathway from the plastid to the nucleus. The strongly altered leaf shape in the Δrps16 mutants uncovers a role of plastid translation in this process, in that the generation of the retrograde signal that determines leaf shape is dependent on, or at least modulated by, plastid translational activity. Previous work has established that the fidelity of plastid gene expression influences the expression of a subset of genes in the nucleus (Börner et al., 1986; Hess et al., 1994b; Koussovitzky et al., 2007), but until now, no morphological phenotypes have been firmly associated with plastid gene expression-dependent retrograde signaling. Our work identifies leaf shape as a morphological output of retrograde signaling and plastid translational activity as a factor involved in signal generation. More work will be needed to determine the nuclear target genes of this signaling pathway and to pinpoint its components that act downstream of plastid translation.

In summary, our work presented here has (1) identified essential and nonessential plastid ribosomal proteins, (2) suggested that the transfer of plastid ribosomal protein genes to the nucleus is greatly accelerated in nonphotosynthetic lineages, and (3) revealed a previously unrecognized role of plastid translational fidelity in two developmental processes: shoot branching and leaf morphogenesis.

METHODS

Plant Material, Growth Conditions, and Phenotypical Assays

To generate leaf material for chloroplast transformation experiments, tobacco plants (Nicotiana tabacum cv Petit Havana) were grown under aseptic conditions on agar-solidified Murashige and Skoog medium containing 30 g/L Suc (Murashige and Skoog, 1962). Transplastomic lines were rooted and propagated on the same medium in the presence of spectinomycin (500 mg/L). For seed production and analysis of plant phenotypes, transplastomic plants were grown in soil under standard greenhouse conditions (relative humidity 55%, day temperature 25°C, night temperature 20°C, diurnal cycle 16 h light and 8 h darkness, light intensity 300–600 μE m⁻² s⁻¹). Inheritance patterns and seedling phenotypes were analyzed by germination of surface-sterilized seeds on Murashige and Skoog medium without or with spectinomycin (500 mg/L).

Growth tests under different light conditions were performed by raising plants in soil at 22°C, diurnal cycle 16 h light and 8 h darkness, light intensities 300–600 μE m⁻² s⁻¹. For cold stress treatments, plants were transferred after 3 weeks of growth under standard conditions to 4°C for ~2 months (at 80 μE m⁻² s⁻¹).

Construction of Plastid Transformation Vectors

Vectors for the targeted knockout of rps15 and rps16, both transcribed as monocistronic mRNAs, were constructed by inserting an aadA cassette conferring spectinomycin resistance (including promoter, 5′ UTR and 3′ UTR) into the coding region of the ribosomal protein genes (insertion mutagenesis). Knockout vectors for all other genes of interest, which are part of operons, were produced by replacing the coding region of the targeted ribosomal protein gene with the aadA coding region, thus employing the endogenous expression signals of the ribosomal protein gene to drive selectable marker gene expression. In the case of rpl32, which is co-transcribed with the sprA gene, both strategies were used in parallel.

An aadA cassette was constructed by digesting plasmid pSK.Kmr (Bateman and Purton, 2000) with Xhol and PstI and inserting the excised aphA-6 cassette into the similarly cut vector pKCZ (Zou et al., 2003), generating plastid pKCZaphA-6. The transgene expression cassette in pKCZ consists of the psbA promoter, the psbA 5′ UTR and the rbcL 3′ UTR from Chlamydomonas reinhardtii. Subsequently, pKCZaphA-6 was cut with PstI, and the recessed ends were converted to blunt ends by a fill-in reaction with the Klenow fragment of DNA polymerase I from Escherichia coli. Following digestion with NcoI, the aadA coding region (Svab and Maliga, 1993) was inserted as an Ncol/XbaI fragment (with the XbaI overhang filled in), generating plasmid pLS1.

For construction of vector pΔrps15, a fragment from the N. tabacum plastid genome (corresponding to nucleotide positions 124,662 to 126,561; Genbank accession number Z00044) was amplified with the primers P5′ndh and P3′ycf1 (all primer sequences are listed in Supplemental Table 2 online). The PCR product was cut with BamHI and HindIII (sites underlined in primer sequences) and inserted into the cloning vector pUC18 digested with the same enzymes. This resulting plasmid clone was partially digested with HindIII to linearize it within the rps15 gene and was ligated to the aadA cassette excised from pLS1 with Smal. A clone harboring the aadA cassette in the desired orientation was selected, and the correctness of all manipulations was confirmed by complete sequencing.

Vector pΔrps16 was constructed by amplifying the rps16-containing region (corresponding to plastome positions 3999 to 6244) with primers P5′rps16 and P3′rps16. The obtained PCR fragment was cloned into pUC18 digested with Smal. The resulting plasmid was linearized with BglII and treated with Klenow DNA polymerase to blunt the overhanging ends. Subsequently, the aadA cassette (obtained by digestion of pLS1 with Smal) was ligated into the blunt-end BglII site, resulting in disruption of the rps16 reading frame. A plasmid clone containing the aadA cassette in the desired orientation was identified and sequenced.

For construction of vector pΔrps23, the genomic region surrounding the rps23 gene (corresponding to nucleotide positions 88,162 to 89,596) was amplified with primers P5′rps23 and P3′rps23. The PCR product was cloned into pUC18 digested with Smal, generating plasmid pSA10. To replace the rps23 coding region with the aadA coding region, a PCR strategy was used. An initial PCR amplification was done with P5′rps2 and P3′rps2 with the wild-type DNA as template. Another PCR was performed with the primers P5′trnl and P3′trn. A third PCR amplified the aadA coding region (adding short overhangs that are complementary to the flanking plastome sequences) using the primers P5′rpl2aadA and P3′trnlaaA and pLS1 as template. In the final PCR reaction, the three PCR products were used as templates for amplification with primers P5′rpl2 and P3′trn. The resulting amplification product was treated with AccI and XcmI and cloned into pSA10 digested with the same enzymes.

Vector pΔrpl36 was constructed by amplifying the rpl36 genomic region (corresponding to nucleotide positions 80,921 to 83,276) with the primers P5′rpl36 and P3′rpl36. The PCR fragment was cloned into the Smal site of pUC18, resulting in vector pSA8. To replace rpl36 with aadA, the aadA coding region was amplified from pLS1 plasmid DNA with primers carrying overhangs complementary to the plastome sequences. The obtained PCR fragment was cloned into pUC18 digested with Smal, generating plasmid pSA10. The resulting product was then digested with BsrGI and Smal and ligated into the similarly cut pSA8, generating transformation vector pΔrpl36.
To generate vectors for targeted inactivation of the rpl32 gene (pJpred2A and pJpred2B), the rpl32 genomic region (corresponding to nucleotide positions 114,021 to 116,232) was amplified with primers P5’rpl32 and P3’rpl32. The introduced BamHI and Psfl restriction sites are underlined in the primer sequences. The obtained PCR fragment was digested with BamHI and Psfl and cloned into the similarly cut vector pUC18, producing plasmid pSA11. Vector pJpred2A, in which the coding region of rpl32 is replaced by the aadA coding region, was produced by a PCR strategy. First, the coding region of aadA was amplified from pLS1 using the primers P5’aadA and P3’aadA. In a second PCR, the region upstream of rpl32 was amplified with primers P5’ndhF and P3’ndhF using pSA11 as template. The two PCR products were then combined by performing an amplification reaction with primers P5’ndhF and P5’aadA. This resulting fragment was digested with BamHI and BsrBI and was inserted into the similarly cut plasmid pSA11, generating plastid transformation vector pJpred2A. Vector pJpred2B, in which an aadA cassette disrupts the rpl32 gene, was constructed by partial digestion of pSA11 with SspI (to linearize the vector in the rpl32 gene) followed by insertion of an aadA cassette (obtained as described above for vector pJpred15).

The knockout vectors for rpl22 and rps3 are both based on a cloned PCR fragment generated with primers P5’rpl22 and P3’rpl22. The amplified region of the tobacco plastid genome (corresponding to positions 64,183 to 67,454) contains both genes and was cloned into a Smal-digested pUC18 vector. The resulting plasmid clone was named pTF11. The rpl22 gene overlaps with rps3 by 16 nucleotides. To sustain transformation of rps3 upon knockout of rpl22, the psbE/psbF spacer region (5’ GAGGCCCTA 3’) was inserted downstream of the aadA marker gene to create (in combination with the TAG stop codon of the aadA) a perfect Shine-Dalgarno sequence upstream of rpl22. As a control, the rps3 knockout was constructed including the same spacer sequence. In addition, the stop codon of rpl22 was mutated from TAA to TAG to provide a consensus Shine-Dalgarno sequence for the downstream aadA gene. To construct a knockout allele for rpl22, its coding region was replaced with the aadA coding region. For this end, the sequence downstream of rpl22 was amplified by PCR with tobacco DNA as template and the primers P5’Clal and P3’Clal. The sequence upstream of rpl22 was amplified with the primers P5’NcoI and P3’NcoI. A third PCR was used to amplify the aadA coding region using pLS1 as template was performed with the primers P5’Rpl22aadA and P3’Rpl22aadA. The fourth PCR used the products of the first three PCRs as templates and the primers P5’Clal and P3’NcoI. The product was treated with Ncol and Clal and cloned into pTF11 digested with the same enzymes, generating plastid transformation vector pJpred22.

To exchange the rps3 coding region with aadA, an analogous strategy was pursued. The two flanking regions were amplified from tobacco DNA with the primer pairs P5’Eagl/P3’Eagl and P5’BamHI/P3’BamHI. A third PCR was performed to amplify the aadA coding region with the primers P5’Rps3aadA and P3’Rps3aadA using plasmid pLS1 as template. The final PCR combined the three PCR products by amplification with the primers P5’BamHI and P3’Eagl. The obtained PCR product was cut with Eagl and AflII and ligated into the similarly digested plasmid pTF11, generating plastid transformation vector pJpred3.

Plastid Transformation and Selection of Transplantable Lines

Young leaves from separately grown tobacco plants were bombardarded with plasmid-coated 0.6 μm gold particles using a helium-driven Biolistic gun (PDS1000He; BioRad). Primary spectinomycin-resistant lines were selected on plant regeneration medium containing 500 mg/L spectinomycin (Svab and Maliga, 1993). Spontaneous spectinomycin-resistant plants were eliminated by double selection tests on medium containing both spectinomycin and streptomycin (500 mg/L each) (Svab and Maliga, 1993; Bock, 2001). Several independent transplantable lines were generated for each construct and were subjected to three to four additional rounds of regeneration on spectinomycin-containing plant regeneration medium to enrich the transplastome and select for homoplasmy.

Isolation of Nucleic Acids and Hybridization Procedures

Total plant DNA was extracted from plants grown under spectinomycin selection in vitro by a cetyltrimethylammoniumbromide-based method (Doyle and Doyle, 1990). For RFLP analysis, DNA samples were treated with restriction enzymes and separated on 0.8 to 1.2% agarose gels and blotted onto Hybond N nylon membranes (GE Healthcare). For hybridization, [α-32P]dCTP-labeled probes were produced by random priming (Multiprime DNA labeling kit; GE Healthcare). Restriction fragments (generated as indicated in the corresponding figures) were used as hybridization probes. The probe for analysis of the rps3 knockout was produced by PCR amplification with the primers P5’rpl16 and P3’rpl16. Hybridizations were performed at 65°C using standard protocols. Total plant RNA was isolated with the NucleoSpin RNA Plant Kit (Macherey-Nagel) or, alternatively, by a guanidine isothiocyanate/pheno filtr-based method (peqGOLD TriFast; Peqlab). RNA samples were denatured, separated in denaturing formaldehyde-containing agarose gels (1 to 1.2%) and blotted onto Hybond N nylon membranes (GE Healthcare).

rRNA and Polysome Analysis

Polysomes were purified as described previously (Rogalski et al., 2008a), except that gradients were fractionated into 10 fractions to obtain higher resolution. RNA pellets were dissolved in 30 μL of water, 5 μL of which was heat denatured and loaded onto a denaturing formaldehyde-containing 1.2% agarose gel. Specific probes for detection of psaA and psbD transcripts were generated by PCR using the following primers: P5’psaA, P3’psaa, P3’psbD, and P3’psbD. For rRNA quantitation, RNA samples were analyzed in an Agilent 2100 Bioanalyzer with the Agilent RNA 6000 nano Kit according to the instructions of the manufacturer (Agilent Technologies). rRNA ratios were determined as described previously (Walter et al., 2010). To follow ribosome accumulation during leaf development, three biological replicates and two technical replicates were measured for each plant line. In the cold stress assays, two technical replicates were measured.

Ribosome Isolation and Mass Spectrometry

Ribosomes were isolated according to the polysome purification protocol (Rogalski et al., 2008a), except that the preparations were not loaded onto Suc gradients but layered onto a 1 M Suc cushion containing 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM magnesium acetate, and 7 mM β-mercaptoethanol. The ribosomes were pelleted by centrifugation at 86,000 g for 17 h, and the pellet was resuspended in 40 mM Tris-HCl, 20 mM KCl, 10 mM MgCl₂ (pH adjusted to 8.5), followed by precipitation with three volumes of 90% acetone, 10% methanol, 10 mM DTT overnight at −20°C. Subsequently, the ribosomes were collected by centrifugation at 14,000 g for 15 min, washed twice with acetone, and air-dried for 10 min. The pellet was resuspended in 500 nmol/L Tris/HCl, pH 8.6, 10 μM MgCl₂, 10 μM DTT, 10 μM DTT overnight at −20°C. Chlorophyll contents were determined in 80% (v/v) acetone (Porra et al., 1999). Chlorophyll fluorescence was recorded with a pulse-amplitude modulated fluorimeter (Dual-PAM-100; Heinz Walz) on intact plants (grown under 350 μE m⁻² s⁻¹) at room temperature after dark adaptation.
for 20 min. The contents of PSII, the cytochrome b,f complex, and PSI were determined by difference absorption spectroscopy as described previously (Schöttler et al., 2007a; Schöttler et al., 2007b). Significance analyses were performed using a one-way analysis of variance with a pair-wise multiple comparison procedure (Holm-Sidak method) in SigmaPlot.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank database under the following accession numbers:

Cuscuta reflexa plastid genome, NC_0019616; Epifagus virginiana plastid genome, NC_001568; Euglena longa plastid genome, NC_002652; Eimeria tenella apicoplast genome, NC_004823; Thalassia parva apicoplast genome, NC_007758; Toxoplasma gondii apicoplast genome, NC_001799; Rhizanthella gardneri plastid genome, NC_014874; N. tabacum chloroplast genome, NC_01879; N. tabacum plastid ribosomal gene rpl22, GenID:800419; N. tabacum plastid ribosomal gene rpl23, GenID:800421; N. tabacum chloroplast ribosomal gene rps15, GenID:800849; N. tabacum plastid ribosomal gene rps16, GenID:800493; N. tabacum plastid chloroplast photosystem gene psbD, GenID:800525; N. tabacum chloroplast photosystem gene psaA, GenID:800453; C. reinhardtii chloroplast photosystem gene psbA, GenID:2716987; E. coli gene aadA, GenID:1446561; E. coli knockout strain for rpl32 from the Keio collection: CGSC#: 9028, JW 1075-1.

Supplemental Data

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

Supplemental Table 1. Comparative Mass Spectrometric Analysis of Plastid Ribosomal Proteins in the Wild Type and the Δpsa15 Knockout Mutant.

Supplemental Table 2. Sequences of Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

T.T.F., L.B.S., S.A., M.A.S., and R.B. designed the research; T.T.F., S.A., S.H., and M.A.S. performed research; T.T.F., L.B.S., S.H., M.A.S., and R.B. analyzed data; R.B. wrote the article with significant input from T.T.F., L.B.S., and M.A.S.

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Nonessential Plastid-Encoded Ribosomal Proteins in Tobacco: A Developmental Role for Plastid Translation and Implications for Reductive Genome Evolution

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