Silencing of the Nuclear RPS10 Gene Encoding Mitochondrial Ribosomal Protein Alters Translation in Arabidopsis Mitochondria

Malgorzata Kwasniak, Pawel Majewski, Renata Skibior, Aleksandra Adamowicz, Malgorzata Czarna, Elwira Sliwinska, and Hanna Janska

INTRODUCTION

Bigeneric biogenesis is a characteristic feature of most mitochondrial complexes, such as those of the oxidative phosphorylation system (OXPHOS) and mitoribosomes. The stable stoichiometry of these complexes implies that the synthesis of their subunits must be tightly coordinated both within and between the two genomes. The coordination between the nuclear and mitochondrial genomes could be achieved, in principle, at various levels (i.e., transcriptional, RNA maturation and degradation, translational, and posttranslational).

The regulation of expression of plant mitochondrially encoded genes is not fully understood. Some results suggest a correlation between the number of mitochondrial gene copies and the steady state level of their transcripts (Janska et al., 1998; Hedtke et al., 1999; Kühn et al., 2009; Shedge et al., 2010), but there are also data supporting a lack of such a correlation (Woloszynska et al., 2006; Preuten et al., 2010). Differences in the gene promoter strength were observed between plant mitochondrial genes (Brennicke et al., 1999; Fey and Maréchal-Drouard, 1999)
even for genes coding for components of the same complex (Giegé et al., 2000). However, the transcriptional activity appears to have only a small effect on transcript abundance because it is largely counterbalanced by processes at different steps of RNA maturation and degradation (Giegé et al., 2000; Leino et al., 2005; Holec et al., 2008). Therefore, it is thought that modulation of transcription is not essential for regulation of expression of genes encoded in mitochondria and that posttranscriptional processes are more important. It should be emphasized that hardly anything is known about the regulation of plant mitochondrial gene expression at the translational level (Bonen, 2004). The importance of regulation at the posttranscriptional level was highlighted by the finding of selective proteolysis of proteins synthesized from unedited mRNA (Binder and Brennicke, 2003) and by data demonstrating that the mitochondrial gene expression remained more or less unaffected by sugar starvation at the transcriptional, posttranscriptional, and translational levels, but was modulated at the posttranslational level (Giegé et al., 2005).

Coordination of the expression of some OXPHOS genes between the nuclear and mitochondrial genomes at the transcript level has been reported during, for example, flower development in sunflower (Helianthus annuus; Smart et al., 1994; Ribichich et al., 2001), wheat (Triticum aestivum) leaf development (Topping and Leaver, 1990), and early steps of rice (Oryza sativa) germination (Howell et al., 2009). On the other hand, an evident lack of coordination between mitochondrial and nuclear transcripts of OXPHOS genes has been found in global analyses during sugar starvation (Giegé et al., 2005) and germination in Arabidopsis thaliana (Law et al., 2012). The sugar starvation

Address correspondence to janska@ibmb.uni.wroc.pl.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Hanna Janska (janska@ibmb.uni.wroc.pl).

Online version contains Web-only data.
www.plantcell.org/cgi/doi/10.1105/tpc.113.111294
study has revealed that such coordination is achieved at the posttranslational level, possibly at the level of complex assembly (Giegé et al., 2005). Furthermore, that study suggested that excess unassembled subunits of the complexes were rapidly degraded by ATP-dependent proteases, which had earlier been identified in plant mitochondria (Janska et al., 2010), and their deficit was shown to compromise the assembly/stability of OXPHOS complexes (Kolodziejczak et al., 2007).

Despite the controversy over the transcriptional coordination of mitochondrial and nuclear genes during the biogenesis of mitochondrial complexes, the coordination of expression of nuclear genes themselves is well documented. This regulation operates through the interaction of nuclear transcription factors with specific sequence motifs in promoter regions of nuclear genes encoding mitochondrial proteins. Numerous promoters of nuclear genes encoding OXPHOS components from Arabidopsis and rice contain a sequence known as site II motif (Welchen and Gonzalez, 2006), whose binding by TCP transcription factors ensures coordinated expression (Giraud et al., 2010; Hammani et al., 2011). Furthermore, a global study has shown that expression of mitochondrially encoded genes involved in energy production and gene expression in mitochondria is also highly coregulated at the transcript level, even more tightly than are their nuclear counterparts (Leister et al., 2011).

In plants, all mitochondrial OXPHOS complexes, except complex II, comprise subunits encoded in both the mitochondrial and the nuclear genome. In Arabidopsis, complex I contains the largest proportion of mitochondrially encoded subunits (nine out of the total 30) (Heazlewood et al., 2003), while complex III contains the least (one of 10) (Braun et al., 1994). Mitoribosomes also have a dual genetic origin; however, their exact composition is unknown. In Arabidopsis, the mitochondrial genome encodes four ribosomal proteins of the small subunit (SSU), three of the large subunit (LSU), and all three rRNA species (Bonen, 2004). The full repertoire of nuclear-encoded mitoribosomal proteins remains to be established.

One of the known components of the SSU of Arabidopsis mitoribosomes encoded in the nucleus is the S10 protein. Transgenic lines with a decreased transcript level of ribosomal protein S10 (RPS10) have been generated using the RNA interference method (Majewski et al., 2009). Homozygous Arabidopsis plants presented severe morphological abnormalities and either died during vegetative growth or were rescued by reversion. Among the hemizygous plants, several phenotype categories were identified. These categories were correlated with the timing of the onset of silencing but not with its efficiency. The role of mitochondrial S10 protein in plants is unknown, but in bacteria its homolog, called as NusE, is a multifunctional protein (Kaczanowska and Rydén-Aulin, 2007). NusE is one of the proteins that complete the ribosomal assembly and is involved in transcriptional antitermination and in tethering the ribosome to the RNA polymerase (Baumann et al., 2010).

In this work, we analyzed the effects of the silencing of the RPS10 gene encoding one of the SSU proteins on the expression of mitochondrial and nuclear genes encoding constituents of OXPHOS complexes and of mitoribosomes. Our findings reveal that alterations in the mitoribosome divergently affect the relative efficiency of translation of different types of mitochondrial mRNA. As a consequence, the amount of mitochondrially encoded ribosomal proteins is higher in rps10 plants than in the wild type, while the opposite is true for mitochondrially encoded OXPHOS subunits. These findings establish that differential regulation of translation in plant mitochondria can be caused by alterations in mitoribosome status. We also confirmed using plants rather than tissue culture the earlier suggestion of Giegé et al. (2005) that the coordination of expression of the mitochondrial and nuclear genomes occurs at the complex assembly level.

RESULTS

Two Phenotypes Are Generated by Different Timing of RPS10 Silencing

Two phenotypes of the rps10 Arabidopsis hemizygous transformants, P2 and P3, differing by the onset of RPS10 silencing were reported earlier (Majewski et al., 2009). In the P2 phenotype, the silencing occurs at the very early stage of vegetative growth and in consequence the decreased level of RPS10 transcripts is observed in all rosette leaves. Since the silencing is associated with leaf morphological abnormalities, all leaves of P2 plants have an altered shape. By contrast, the developmentally late silencing characteristic of the P3 phenotype begins in newly emerging leaves of 7-week-old plants and results in rosettes comprising leaves of different morphology; the older ones are similar to the wild type, while the younger leaves resemble those of P2. An analysis of the unaltered and altered leaves of P3 plants showed a correlation between the reduced RPS10 transcript level and leaf morphology at different developmental stages of Arabidopsis (Figure 1). Unless noted otherwise, all analyses described below were performed using the youngest leaves harvested from 9- to 10-week-old P2, P3, and wild-type plants growing under short-day conditions. At that

Figure 1. Decreased Level of RPS10 Transcript Correlates with Altered Leaf Morphology during Growth of P3 Phenotype of rps10.

The values obtained were averaged for three biological replicates, with error bars representing ±. WT, the wild type.
age, the young leaves of both P2 and P3 plants exhibited abnormal morphology and decreased \textit{RPS10} transcript. It should be emphasized that the efficiency of silencing is similar in the altered leaves of P2 and P3 plants (Majewski et al., 2009).

**Increased but Imbalanced Amounts of Mitoribosomal Subunits Are Present in rps10 Plants**

To check if the \textit{RPS10} silencing affects mitochondrial ribosome biogenesis, the level of mitoribosomal subunits was quantified based on rRNA abundance. As rRNAs are unstable when unassembled, the rRNA level can serve as a proxy for ribosomal subunit accumulation (Walter et al., 2010). We determined the abundance of cytosolic (cyt 18S and cyt 25S), mitochondrial (mt 18S and mt 26S), and chloroplast (chl 16S and chl 23S) rRNAs (Figure 2). Since the S10 protein is a component of the SSU of the mitoribosome, a deficit of mitochondrial SSU and, consequently, also of 18S rRNA was expected in P2 and P3 plants. Surprisingly, we found an increased amount of mitochondrial 18S rRNA in the mutant compared with the wild type (Figure 2A; the ratio mt 18S rRNA/cyt 18S rRNA was 1 for the wild type and −1.5 for both rps10 phenotypes). Moreover, the abundance of the mitochondrial rRNA of LSU, 26S rRNA, was even more increased (Figure 2A; the ratio mt 26S rRNA/cyt 25S rRNA was −3.5). We then calculated the ratio of LSU to SSU for the cytosolic, mitochondrial, and chloroplast ribosomes based on the respective rRNA levels (Figure 2B). A significant increase of that ratio was only found for mitoribosomes in both the P2 and P3 phenotypes (Figure 2B; approximately twofold relative to the −1:1 stoichiometry expected and found for wild-type plants), indicating an imbalance between mitoribosomal subunits. This conclusion is additionally supported by an approximately threefold higher level of a polynucleotide phosphorylase-like protein, an enzyme known to be involved in 18S rRNA degradation (Perrin et al., 2004), in the P2 and P3 phenotypes (3.06 ± 0.12 and 2.78 ± 0.05, respectively) compared with wild-type plants (1.00 ± 0.06).

![Figure 2](image)

**Figure 2.** Accumulation of rRNAs as a Proxy for Corresponding Ribosomal Subunits in P2 and P3 Phenotypes of \textit{rps10} Compared with Wild-Type Plants.

\textbf{(A)} Ratio of mitochondrial to cytosolic rRNAs. WT, the wild type. \textbf{(B)} Ratio of rRNAs of large and SSUs in cytosolic, mitochondrial, and chloroplast ribosomes. The values obtained were averaged for four biological replicates, with error bars representing so. Statistically significant differences from the wild type are indicated by asterisks (Student’s \( t \) test; \( P < 0.05 \)).

To quantitate the abundance of the mitoribosomal subunits in \textit{rps10} in an independent manner, we estimated the amounts of three mitoribosomal proteins, S4 and S10 of SSU and L16 representing LSU, using immunoblotting (Figure 3; see Supplemental Figure 1 online). S4 and L16 are encoded mitochrondially, whereas S10 is a nuclear-encoded protein. Both S4 and L16 were more abundant in \textit{rps10} than in wild-type plants, but the increase was substantially higher for L16 as calculated relative to total mitochondrial protein. This result supports the above conclusion based on rRNA quantity that the LSUs of mitoribosomes are in excess over SSUs in the \textit{rps10} plants. However, the amount of the S10 protein was comparable in the mitochondrial fraction of \textit{rps10} and wild-type plants. This difference in the abundance of the S10 and S4 proteins suggests that in \textit{rps10} plants, a fraction of the mitochondrial SSUs were not fully assembled, lacking at least the S10 protein.

**OXPHOS Complexes and Their Subunits Are Less Abundant in \textit{rps10} Mitochondria**

The effect of the increased but altered biogenesis of mitoribosomes on the abundance and activity of OXPHOS complexes was investigated by blue-native gel electrophoresis (BN-PAGE; Figure 4). The intensity of separated bands stained with Coomassie blue (Figures 4A and 4C; protein level) or after specific histochemical staining (Figures 4B and 4C; activity level) was quantified. A significantly lower abundance of OXPHOS complexes was observed in \textit{rps10} compared with the wild type, both as protein amount and activity level. The exception was complex III that was reduced to a much lesser extent, while complex I was the most affected. This suggests an interesting correlation as complex III has only one and complex I the highest number of mitochondrionally encoded subunits among all OXPHOS complexes.

Subsequently, immunoblotting was performed using antibodies against one mitochondrionally encoded and one nuclear-encoded subunit of complexes I, III, IV, and V (Figure 5; see Supplemental Figure 1 online). We consistently observed an approximately similar reduction of the amount of mitochondrionally and nuclear-encoded subunits for complex I (NAD9 and 49 kD; −40% of wild-type level), III (COB and CYTGC; −60% of wild-type level), and IV (COX2 and COXVC; −25% of wild-type level) but not for complex V. There, the level of a nuclear-encoded subunit was markedly less affected (ATP2; −50% of wild-type level) compared with a mitochondrionally encoded one (ATP6; −15% of wild-type level).

The evident imbalance between the above mitochondrially and nuclear-encoded subunits of complex V prompted us to check the overall stoichiometry of the subunits of this complex using two-dimensional BN-PAGE in the P2 phenotype of the \textit{rps10} mutant. In contrast with the imbalance described above, the relative proportion of protein subunits of complex V was...
nearly the same. The ratio of the four analyzed subunits of complex V was as follows: ATP1 and ATP2/ATP γ/FAd was 1/0.19/0.10 in wild-type plants and 1/0.15/0.08 in P2 phenotype of the rps10 mutant.

Mitochondrial DNA Content and Abundance of Mitochondrial mRNAs Are Upregulated in rps10 Plants

Since the biogenesis of OXPHOS complexes and mitoribosomes is bigenomic, we analyzed the consecutive steps of the expression of the mitochondrial and nuclear genomes to understand the effect of RPS10 silencing on the biogenesis of the mitochondrial complexes.

First, we investigated the abundance of mRNAs encoding representative components of mitoribosomes and OXPHOS complexes, both mitochondrially and nuclear encoded, using quantitative RT-PCR (Figure 6). The abundance of mitochondrially encoded transcripts was increased, while that of nuclear-encoded ones was unaltered or slightly decreased for all the complexes surveyed in rps10. The level of mitochondrial transcripts for most of the ribosomal proteins analyzed showed a somewhat stronger upregulation (twofold up to even eightfold) than did transcripts encoding the OXPHOS subunits (1.2-fold up to fourfold). Although a similar trend was observed for both phenotypes, the upregulation of the mitochondrially encoded genes was more pronounced in P3 than in P2. Furthermore, the abundance of the majority of the nuclear-encoded transcripts studied tended to be unchanged in P3, but slightly decreased in P2.

Figure 3. Quantification of Ribosomal Proteins in Mitochondrial Fractions from P2 and P3 Phenotypes of rps10 and Wild-Type Plants.

Protein gel blots of equal amounts (20, 10, and 5 μg) of total mitochondrial proteins were probed with antibodies against S4, S10, and L16. Representative immunoblots are shown in Supplemental Figure 1 online. Intensities of stained bands were analyzed with Image Quant Software and averaged for the three protein loadings. The values obtained were averaged for three independent experiments, with error bars representing SD. Statistically significant differences from the wild type (WT) are indicated by asterisks (Student’s t test; P < 0.05).

Figure 4. Mitochondrial Respiratory Complexes Separated by BN-PAGE from P2 Phenotype of rps10 and Wild-Type Plants.

(A) Coomassie blue staining. WT, the wild type. (B) In-gel activity staining. (C) Quantification of the protein amount (top panel) and activity (bottom panel) of individual OXPHOS complexes in the P2 phenotype of rps10 compared with wild-type plants. Intensities of stained bands were analyzed with Image Quant Software. The values obtained were averaged for three independent experiments, with error bars representing SD. Statistically significant differences from the wild type are indicated by asterisks (Student’s t test; P < 0.05).
We also noticed that the mitochondrial mRNAs encoding subunits of complexes I and IV were more elevated (twofold up to fourfold) than those encoding subunits of complex III and V (2.5-fold or less) in the both phenotypes. This observation is interesting in the light of the earlier finding that RPOTm, one of the RNA polymerases present in Arabidopsis mitochondria, more efficiently transcribes genes encoding subunits of complexes I and IV (Kühn et al., 2009).

The fact that all of the mitochondrially encoded transcripts tested were elevated in rps10 prompted us to check if that increase could be a consequence of mitochondrial genome amplification. To quantify the mitochondrial genome relative to the nuclear DNA content, we first established the ploidy of the nuclear genome in leaf cells using flow cytometry. 2C, 4C, 8C, and 16C nuclei were detected (see Supplemental Figure 2 online). Surprisingly, the mean C-values in P2 and P3 plants (5.02 ± 0.42 and 4.34 ± 0.47, respectively) were higher than in the wild type (2.92 ± 0.08; P = 0.05, Duncan’s test). This finding implies that the silencing of the RPS10 gene stimulated endoreplication. Then, using quantitative PCR, we estimated DNA level for several mitochondrial genes encoding OXPHOS and ribosomal proteins. These values were corrected for the nuclear genome endopolyploidization to give the absolute number of mitochondrial gene copies per cell. Figure 7 presents copy numbers of individual mitochondrial genes in wild-type and rps10 cells expressed as log2 ratios. All of the mitochondrial genes quantitated gave similar results: They were present in ~4.5- to 6.5-fold more copies in the rps10 cells than in the wild-type ones. Given the above results (Figure 7), we reanalyzed the data presented in Figure 6 to establish the relationship between the copy number of mitochondrial genes and their RNA abundance. For several of the ribosomal proteins, the increase of their mRNA abundance reflected closely the increase in the gene copy number, implying that the accumulation of those transcripts was simply due the elevated mitochondrial DNA level. For the remaining ribosomal proteins, and all of the OXPHOS subunits, the increase in abundance of their transcripts was not as high and more variable. Thus, the level of those mitochondrial transcripts is most probably determined by a combined effect of the elevated cellular mtDNA level and the variable selectivity of RNA polymerases.

Number of Mitochondria Is Unaffected in rps10 Mutants

The elevated number of mitochondrial gene copies per cell could result from an increased DNA content per mitochondrion and/or an increased number of mitochondria per cell. To assess the overall number of mitochondria per cell, we prepared protoplasts from P2, P3, and wild-type plants and counted the mitochondria using MitoTracker Green™ staining and confocal scanning microscopy. The mean number of mitochondria was slightly higher in P2 and P3 compared with wild-type protoplasts.
(see Supplemental Table 1 online), but that difference was not statistically significant. Given the similar numbers of mitochondria in all three types of cells and the markedly different numbers of mitochondrial gene copies per cell, one must conclude that most if not all the rps10 mitochondria contain significantly more mitochondrial DNA copies than do wild-type mitochondria.

The Translation Status of OXPHOS and Mitoribosomal mRNAs Is Differentiated in rps10 Plants

The perturbation of the assembly/stability of mitoribosomes in rps10 seemed likely to affect mitochondrial translation. To check for this, we determined the translational activity of individual mRNAs encoding OXPHOS and mitoribosomal proteins in rps10 and wild-type plants. mRNA attached to ribosomes (polysomal fraction) was separated from unbound mRNA (nonpolysomal fraction) by Suc gradient centrifugation of total RNA extracts from P2, P3, and wild-type leaves, followed by quantitative analysis of specific transcripts by RT-PCR. Two parameters related to translatability of mRNA were analyzed: ribosomal density (the number of ribosomes present on a transcript visualized in the mRNA distribution across the polysome fraction) and ribosomal loading (the proportion of ribosome-bound mRNA expressed as the percentage of total mRNA). Representative data are shown in Figure 8 and Supplemental Figure 3 online.

For the mitochondrially encoded cob, cox2, and atp6 transcripts, the mRNA distribution in the density gradient was shifted toward lighter fractions in both phenotypes of rps10 compared with the wild type (Figure 8A). For the nad9 transcript, the shift was observed only for P3. These shifts indicate that the number of ribosomes present on the examined transcripts is lower in the rps10 mutant, suggesting a lower efficiency of translation. The second parameter describing translatability also implies a decrease in mitochondrial translation for most of the OXPHOS
transcripts, although the decrease in ribosomal loading was considerably variable (Figure 8A; see Supplemental Figure 3A online). The only exception was the cob mRNA, showing a higher proportion in the polysomal fraction. The increased polysome loading with the reduced ribosomal density found for the cob transcript explains why the decrease of the COB protein amount in rps10 was less drastic than that observed for the other mitochondrially encoded OXPHOS subunits (Figure 5). Conversely, enhanced translation was found for the majority of the mitochondrially encoded ribosomal transcripts as judged by both a shift to heavier polysomes in rps10 compared with the wild type (rpl2, rpl5, rps4, and rps7; Figure 8A) and strongly enhanced

Figure 8. Distribution of mRNAs across Polysome Profiles for OXPHOS and Mitoribosomal Mitochondrial and Nuclear Genes.

(A) Transcripts of mitochondrially encoded genes (nad9, cob, cox2, atp6, rpl2, rpl5, rpl16, rps3, rps4, rps7, and rps12).

(B) Transcripts of nuclear-encoded genes (76 kD, CYTC1, COXVC, ATP2-1, RPL29, RPS14, and RPS24). Specific mRNA was analyzed quantitatively over the entire profile (fractions 1 to 10) by real-time PCR. Graphs represent percentage of total mRNA present in each fraction for wild-type (WT), P2, and P3 plants. The distribution of mRNA in the gradients was compared for P2 and P3 phenotypes of rps10 and wild-type plants, and shifts of their maxima to lighter or heavier gradient fractions relative to the wild-type maximum are indicated by arrows.
loading onto polysomes (for all the mitochondrially encoded ribosomal mRNAs; Figure 8A; see Supplemental Figure 3A online).

A similar analysis of the nuclear-encoded transcripts revealed almost unaffected cytoplasmic translation in the P2 phenotype (Figure 8B; see Supplemental Figure 3B online). In P3, a small decrease in ribosomal loading was accompanied by a higher ribosome density on translating mRNAs. Considering the contrasting changes of ribosomal density and loading, we predict no substantial changes in the translation efficiency of the nuclear-encoded OXPHOS and mitoribosomal transcripts in the P3 phenotype either. A clear exception was the COXIVC mRNA showing a significant decrease in polysome loading in both P2 and P3 phenotypes.

**The Pattern of Proteins Synthesized in rps10 Mitochondria Differs from That in the Wild Type**

To provide an independent evidence for aberrant mitochondrial translation in rps10, we conducted in organello protein synthesis using isolated mitochondria from the P2 and P3 rps10 mutants and wild-type plants. Radioactively labeled mitochondrial proteins were separated by SDS-PAGE electrophoresis and visualized by autoradiography (Figure 9). As we expected from the affected association of mRNAs with polysomes, the pattern of labeled proteins synthesized by mitochondria of P2 and P3 plants differed notably from the wild type. The profile of proteins synthesized in mitochondria from wild-type plants resembled that shown by Giegé et al. (2005) and Kühn et al. (2009). By contrast, several of those bands were hardly visible in P2 and P3 mitochondria (Figure 9, open arrowhead), while some bands barely detectable in wild-type mitochondria were very strong in P2 and P3 (Figure 9, closed arrowhead). These results confirm that mitochondrial translation is misregulated in the rps10 mutant with enhanced synthesis of certain proteins and repression of others. Based on the polysome analysis results, we postulate that at least some of the proteins observed almost exclusively in P2 and P3 after the in organello translation are mitoribosomal proteins, while some of those hardly visible are OXPHOS subunits. This prediction is supported by the data presented in Supplemental Figure 4 online. The apparent molecular masses of some proteins synthesized preferentially in mitochondria of rps10 mutants or wild-type plants determined from their electrophoretic mobility matched well the theoretical sizes of several mitochondrially encoded ribosomal or OXPHOS proteins, respectively.

**Mitochondrial ATP-Dependent Proteases Are Upregulated in rps10 Plants**

The data reported here indicate that only the efficiency of synthesis of mitochondrially encoded proteins is significantly altered during biogenesis of OXPHOS complexes in rps10 mutants, but nevertheless at the steady state protein level the ratio of both mitochondrially and nuclear-encoded components is similar, at least for the representative subunits of complexes I, III, and IV. This discrepancy suggests that the excess of synthesized proteins unassembled into the dual-origin complexes should be removed. To test that prediction, we estimated the abundance of mitochondrial ATP-dependent proteases known to digest unassembled/damaged proteins (Janska et al., 2010, 2013). An increased level of transcripts was detected only for the Lon proteases, LON1 and LON4 (Figure 10A), which degrade mainly soluble proteins. Furthermore, despite unaltered transcript levels, the protein abundance of FtsH4 and FtsH10 proteases was also significantly increased in rps10 compared with wild-type mitochondria (Figure 10B). These proteases have been shown to degrade membrane proteins like OXPHOS subunits in yeast.

**DISCUSSION**

In this study, we have shown that a deficit in the ribosomal S10 protein leads to an imbalance between ribosome subunits in *Arabidopsis* mitochondria. Furthermore, a portion of SSUs are incomplete, lacking at least the S10 protein. Next, we found that the expression of mitochondrially encoded components of mitoribosomes and OXPHOS complexes is markedly modulated in response to the ensuing perturbations. As a consequence, the ribosomal proteins are overexpressed, whereas the oxidative phosphorylation system subunits are downregulated. The expression of nuclear-encoded components of mitoribosomes and OXPHOS complexes seems to be less affected. The final coordination of expression of the mitochondrial and nuclear genomes occurs at the level of protein complex assembly, probably with an involvement of mitochondrial ATP-dependent proteases.

To explain how the RPS10 silencing affects mitoribosome biogenesis, we propose the following scenario. The silencing provokes a compensatory response increasing the abundance of...
four biological replicates, with error bars representing SD. WT, the wild type. The mutant does not suffer from an absolute insufficiency of functional mitoribosomes but rather from the excess of LSU and/or the presence of a portion of partially assembled SSU. That such defective complexes are formed is indicated by an excess of another SSU component, S4, over S10. The goal of this study was to evaluate how these changes in the mitoribosome status affect the multiple levels of the bigenomic biogenesis of mitoribosomes themselves and oxidative phosphorylation complexes.

The first step of the feedback response in rps10 occurs at the mitochondrial DNA level. A 4.5- to 6.5-fold increase in the mitochondrial gene copy number was detected in the P2 and P3 phenotypes. This increase was similar for all the genes tested, suggesting amplification of the whole mitochondrial genome. The observed upregulation was not accompanied by a proportional increase in the number/mass of mitochondria but was reflected in enhanced steady state levels of mitochondrial transcripts. The best correlation between the gene copy number and the transcript level was found for several ribosomal proteins. Enhanced transcript levels as a consequence of elevated mitochondrial gene copy numbers have been reported earlier (Hedtke et al., 1999; Kühn et al., 2009; Shedge et al., 2010) and suggested to reflect a feedback response to energy constraints caused by defects in mitochondria (Kühn et al., 2009). In contrast with the upregulated mitochondrial transcripts, the abundance of nuclear-encoded mRNAs of both mitoribosomes and OXPHOS complexes was unchanged or only slightly decreased. Thus, an evident lack of coordination between nuclear and mitochondrial gene expression at the transcript level was detected in the rps10 mutant.

The data regarding polysomes revealed substantial alterations in mitochondrial translation in the rps10 plants. In contrast with the transcript level, where the same trend was noted for both mitoribosome and OXPHOS proteins, a divergent translational response was observed. Generally, within mitochondria, the majority of transcripts of OXPHOS subunits are less actively translated, whereas transcripts of most mitoribosome proteins are preferentially synthesized. This contrasting response was preserved at the protein steady state level, where the OXPHOS subunits were present at reduced levels, while ribosomal proteins overaccumulated. The translation efficiency of nuclear-encoded mRNAs for mitoribosome and OXPHOS components was much less affected. However, there was a significant reduction in the steady state protein level of the nuclear-encoded OXPHOS subunits of complexes I, III, and IV. Moreover, this decrease was comparable to that found for the mitochondrially encoded components of the same complexes. Thus, it seems that the nuclear-encoded OXPHOS proteins, after almost unchanged synthesis, are degraded to bring their abundance down to the level of the mitochondrially encoded components. No similar analysis could be performed for mitoribosomes due to a lack of antibodies against their nuclear-encoded constituents, except for S10.

The conclusion that the coordination of expression of mitochondrial and nuclear genes coding for subunits of OXPHOS complexes occurs posttranslationally was reached earlier by Giegé et al. (2005) in their study of the biogenesis of Arabidopsis OXPHOS complexes under sugar starvation conditions. They postulated that the coordination takes place at the level of protein complex assembly. Our results further confirmed that suggestion. The nuclear-encoded subunit of complex V was reduced in rps10 to a markedly lesser extent than its mitochondrial encoded one, but the stoichiometry of the complex V subunits was virtually identical to that in wild-type mitochondria. Apart from this similarity, there are also differences in the feedback responses initiated by sugar starvation and by RPS10 silencing. The response to the RPS10 silencing is mainly associated with changes in mitochondrial genome expression, whereas under sugar starvation the biogenesis of OXPHOS complexes was regulated mostly by changes in nuclear genome.
expression. As a consequence, the limiting factor in the biogenesis of OXPHOS complexes in response to sugar deprivation is the availability of nuclear-encoded subunits, while in the case of RPS10 silencing it is the availability of mitochondrially encoded ones. Our results argue that the excess unassembled mitochondrial proteins are degraded by ATP-dependent proteases. We found transcriptional activation of the Lon1 and Lon4 proteases in both phenotypes of rps10 and upregulation of FtsH4 and FtsH10 proteases at the posttranscriptional level. The possibility of translational regulation of FtsH10 expression had been reported earlier (Piechota et al., 2010).

Although the molecular details remain to be determined, our data indicate that the altered translation found in rps10 mitochondria is caused by changes of at least two factors: ribosomal loading and density. The reduced ribosomal densities found for all the mitochondrial OXPHOS transcripts tested explains at least partially the reduced steady state levels of the corresponding OXPHOS subunits. It seems that the diverse decrease in the steady state levels of individual OXPHOS proteins reflects the differences in the ribosomal loading of their mRNAs. Conversely, the highly increased steady state levels of mitochondrially encoded ribosomal proteins result from enhanced ribosomal loading and for majority of proteins also from increased ribosomal density. Taken together, the perturbation in mitochondrial ribosomes resulting from RPS10 silencing leads to extensive and diverse changes in mitochondrial translation reflected in the steady state protein level. The mitoribosomal proteins are over-accumulated, while the OXPHOS subunits are present at reduced levels in rps10. This demonstrates that differential changes of the efficiency of translation in response to compromised ribosomal assembly/stability occur in plant mitochondria. Autoregulation of ribosome biosynthesis by translational control has been reported for a fission yeast mutant depleted of Arg-methylated ribosomal protein S2 (Bachand et al., 2006). This mutant, like the Arabidopsis rps10, shows an imbalanced level of small and large ribosomal subunits. However, in the yeast mutant, an enhanced translation of several mRNAs encoding proteins of SSU was observed with little variation in the overall mRNA abundance. Translational regulation of ribosomal biogenesis has also been documented in bacteria (Nomura, 1999), mammalian cells (Meyuhas and Hornstein, 2000), and chloroplasts (Fleischmann et al., 2011).

Coupling of the rate of synthesis of organellar proteins to the rate of their assembly into complexes is a common feature of several related mechanisms known as control by epistasy of translation (CES) (Choquet and Vallon, 2000). In this context, it is feasible that a CES-like regulation triggered by partially assembled mitoribosome may operate to activate translation of ribosomal proteins in rps10 mitochondria. However, the regulation of translation by the assembly status of the mitoribosome observed in the rps10 mutant is not limited to the components of the mitoribosome itself, as would be postulated by the CES model, but also concerns the OXPHOS subunits, being therefore more general. The effect of the accumulation of free LSU and the presence of a portion of defective SSU on the efficiency of protein synthesis in plant mitochondria could be based on the formation of atypical interactions between those subunits and other components of the translation apparatus. The ribosome filter hypothesis posits that ribosomes are not simply translation machines but also function as regulatory elements that differentially affect or filter the translation of particular mRNAs (Mauro and Edelman, 2007). Our findings support the hypothesis in that the misregulation of the mitoribosome subunits in rps10 differentially affects the translational status of particular mitochondrial transcripts. We further postulate that similar translational regulation could occur in wild-type plants. Variation in the interactions between the ribosome subunits could be triggered by posttranslational modifications of ribosomal proteins. In this respect, it is interesting to note that methylation of the S10 protein in bacteria affects the assembly of ribosomes and protein synthesis (Ren et al., 2010).

Surprisingly, we found that whereas most nuclei in wild-type plants were diploid (mean C-value ~3), the mutants nuclei exhibited a higher ploidy (mean C-value ~4 to 5). However, this change is not dramatic in the light of data showing that the mean C-value of nuclear DNA varies from 4 up to 13 during maturation and aging of Arabidopsis leaves (Zoschke et al., 2007). Recently, it was shown that the mammalian homolog of the S10 protein, like many other ribosomal proteins, has a secondary function as a cell proliferation regulator (Ren et al., 2010; Abbas et al., 2012). Thus, it is highly likely that the plant S10 also has an extraribosomal function connected with cell proliferation and a deficiency of this protein leads to endoreduplication. Further studies will be needed to test this hypothesis.

METHODS

Plant Material and Growth Conditions

Wild-type and mutant Arabidopsis thaliana plants were of the Columbia-0 ecotype. The transgenic lines of rps10 were generated using the RNA interference method (Majewski et al., 2009). Seeds of wild-type plants were germinated on half-strength Murashige and Skoog, supplemented with kanamycin (50 mg L$^{-1}$) for the rps10 mutant. Seedlings were grown for 3 d at 4°C in the dark followed by 11 d in an 8-h-light/16-h-dark (short day) photoperiod at 22°C with light intensity of 100 μmol m$^{-2}$ s$^{-1}$. After that time, the wild-type and the kanamycin-resistant rps10 plants were transferred to the soil and grown in a growth chamber (short day, 22°C, 100 μmol m$^{-2}$ s$^{-1}$).

Nucleic Acid Isolation and cDNA Synthesis

Nucleic acids were extracted from fresh plant leaves and stored at ~20°C. Genomic DNA was isolated using a GeneMATRIX Plant and Fungi DNA purification kit (EURx). Total RNA was isolated using a GeneMATRIX Universal RNA purification kit (EURx). The reverse transcription reaction was performed using up to 2 μg of total RNA and a reverse transcription kit (Applied Biosystems). The resulting cDNA was used as a template for quantitative real-time PCR.

Protoplast Isolation, Confocal Imaging, and Determination of the Number of Mitochondria per Cell

Protoplasts were isolated from leaves using the method described by Yoo et al. (2007). Isolated protoplasts were pelleted at 1000 g for 1 min at room temperature and resuspended in 50 μL MMg solution (0.4 M mannitol, 15 mM MgCl$_2$, and 4 mM MES, pH 5.7) containing 200 nM MitoTracker Green FM (Molecular Probes), prewarmed at 37°C. Images of a half-
protoplast were acquired as a z-series with 0.39-μm intervals using a Zeiss LSM510 confocal laser scanning microscope equipped with a ×60 water immersion objective. The MitoTracker Green FM stain was visualized with a 488-nm argon laser and a BP505-530 filter. The number of mitochondria was estimated using an approach similar to that of Preuten et al. (2010). Twenty randomly chosen protoplasts from each of three separate isolations were analyzed for P2 and P3 phenotypes of rps10 and for wild-type plants. The number of mitochondria per protoplast was determined using Imaris Software 7.6.1 (Bitplane).

Flow Cytometric Analysis of Nuclear DNA Content

For flow cytometry, samples were prepared as described previously (Sliwinska et al., 2012). Single leaves were chopped in 1 mL of nuclei isolation buffer supplemented with propidium iodide (50 μg mL⁻¹) and RNase A (50 μg mL⁻¹) and analyzed using a CyFlow SL Green flow cytometer (Partec) equipped with a high-grade solid-state laser with green light emission at 532 nm, long-pass filter RG 590 E, DM 560 A, and side and forward scatters. For each sample, the DNA content of 3000 to 7000 nuclei was measured. Analyses were performed on at least six replicates using a logarithmic amplification. Histograms were evaluated using the FloMax program (Partec), and the percentage of nuclei with particular DNA contents and the mean C-value (Lemontey et al., 2000) were calculated before applying a one-way analysis of variance and the Duncan’s test.

Isolation of Polyosomal RNA

Polyosomes were fractionated from homogenized leaf tissue as described previously by Kahlaü and Bock (2008) with slight modifications. Leaves (200 mg) were ground in 1 mL of freshly prepared extraction buffer (200 mM Tris–HCl, pH 9.0, 200 mM KCl, 35 mM MgCl₂, 25 mM EGA, 200 mM Suc, 100 mM β-mercaptoethanol, 1% [v/v] Triton X-100, 2% [v/v] polyoxyethylene-10-tridecyl ether, 6% [w/v] digitonin, 1 mg/mL heparin, 100 μg/mL chloramphenicol, and 25 μg/mL cycloheximide) and centrifuged at 4°C and 13,200 g for 5 min to remove cell debris. The cleared extract was supplemented with 1/20 volume of 10% (w/v) sodium deoxycholate and fractionated in a Suc density gradient (56:40:30:15% [w/v] Suc in 40 mM Tris–HCl, pH 8.5, 20 mM KCl, 10 mM MgCl₂, 100 μg/mL chloramphenicol, and 500 μg/mL heparin) by ultracentrifugation (4°C, 200,000 g, 80 min). Ten aliquots of ~410 μL each obtained by fractionation were subjected to RNA extraction using phenol/chloroform/isoamyl alcohol (25:24:1). Control gradients supplemented with puromycin were used to identify fractions containing polysomes (fractions 6 to 10) and free mRNA and ribosomes (fractions 1 to 5). RNA isolated from all of these fractions was subjected to 2.5 M LiCl treatment to remove the heparin.

Real-Time PCR Analysis

The real-time PCR analyses were performed using DNA or cDNA on a LightCycler 2.0 instrument (Roche Applied Science). The Real-Time 2x PCR Master Mix SYBR version B (A&A Biotechnology) was used. Reactions were performed in a total volume of 15 μL with a final concentration of 0.5 μM primers. Relative quantification analysis using the second derivative maximum method of the LightCycler 4.0 software was used. The wild-type plants served as the calibrator, and the rps10 gene (At3g18780) was used as a reference. The values of amplification efficiency of the analyzed amplicons were calculated on a standard curve. The standard curves were generated using six serial twofold dilutions of the cDNA or DNA samples obtained from wild-type plants. The amplification protocol consisted of the following: denaturation, 95°C for 1 min; amplification, 45 cycles at 95°C for 10 s, 50 to 65°C (the annealing temperature was specific for used primers) for 10 s, 72°C for 20 s with single data acquisition; cooling, 40°C for 30 s. The specificity of the amplification products was verified by analysis of the melting curve. The primers used are listed in Supplemental Table 2 online.

Isolation of Mitochondria and Gel Electrophoresis of Proteins

Isolation of mitochondria for SDS-PAGE and BN-PAGE experiments was performed as described by Urantowka et al. (2005), whereas functional mitochondria for in organello protein synthesis were isolated as described by Lister et al. (2007). SDS-PAGE electrophoresis as well as protein and catalytic staining of BN-PAGE gels were performed as described by Kolodziejczak et al. (2007).

Protein Gel Blot Analysis

Equal amounts of mitochondrial proteins (20, 10, and 5 μg) were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Blots were probed with antibodies against the following OXPHOS subunits: NAD9 (obtained from P. Giege, France), 49 kD (obtained from L. Sweetlove, UK), COB (obtained from L. Sweetlove), CYT1 (obtained from P. Giege), COX2 (purchased from Agrisera), COXVC (obtained from P. Giege), ATP6 (obtained from Christiane D. Chase, USA), and ATP2 (obtained from C. Knopp, Sweden). Furthermore, blots were also probed with antibodies purchased from Agrisera, against Arabidopsis mitoribosomal proteins, anti-S10, anti-S4, and anti-L16, as well as against mitochondrial proteases, anti-FtsH4 and anti-FtsH10. Anti-rabbit antibodies conjugated with horseradish peroxidase were used as secondary antibodies and visualized with enhanced chemiluminescent ECL reagent. Chemiluminescence was recorded using a chemiluminescence imager (BioDoc-It Imaging System; UVP). Bands were quantified using QuantiOne software (Bio-Rad).

In Organello Protein Synthesis

Mitochondrial proteins (150 μg) were resuspended in a solution containing 5 mM KH₂PO₄, pH 7.0, 400 mM mannitol, 60 mM KCl, 50 mM HEPES, 10 mM MgCl₂, 10 mM malic acid, 1 mM pyruvate, 2 mM GTP, 2 mM DTT, 4 mM ADP, 0.1% (w/v) BSA, 25 μL unlabeled 19-amino acid solution, and 30 μCi [35S]Met (>1000 Ci/mmol). Reactions were performed in 100 μL for 30 and 60 min at 25°C on an orbital shaker and stopped by addition of puromycin (50 μg/mL) and 350 μL mitochondria wash buffer containing 10 mM unlabeled Met. In control experiments, 25 mM Na-acetate was used instead of malic acid and pyruvate. Radio-labeled proteins were separated by SDS-PAGE and visualized by autoradiography.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: AtMg00090 (rps3), AtMg00290 (rps4), AtMg01270 (rps7), At3g23300 (RPS10), AtMg00980 (rps12), At2g34520 (RPS14), At1g28060 (RPS24), AtMg00560 (rps2), AtMg00210 (rps13), AtMg00080 (rps16), At1g07830 (RPL29), AtMg00285 (nad2), AtMg00580 (nad4), AtMg00270 (nad6), AtMg00070 (nad9), At3g37510 (76 kD), At3g12260 (B14 SU), At5g11770 (PSST SU), AtMg00220 (cob), At5g0810 (CYTc1), At1g15120 (11 kD SU), At1g51190 (MPP α), AtMg01360 (cox1), AtMg00160 (cox2), AtMg00730 (cox3), At1g0230 (COXIVb), At2g7380 (COXIVc), At5g28060 (COXIVb), AtMg01170 (atp6), AtMg00480 (atp8), AtMg01080 (atp9), At5g08670 (ATP2-1), At5g08680 (ATP2-3), At2g33040 (ATP7), At5g28680 (LON1), At3g05790 (LON4), At5g23140 (CLPP2), At5g53350 (CLPX), At2g29080 (FTSH3), At2g26140 (FTSH4), At1g07510 (FTSH10), and At3g18780 (ACT2).
Supplemental Data

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

Supplemental Figure 1. Representative Immunoblots of Total Mitochondrial Protein Isolated from Wild-Type, P2, and P3 Phenotypes of rps10 Plants.

Supplemental Figure 2. Selected DNA Histograms of Nuclear Preparations from Wild-Type Plants and P2 and P3 Phenotypes of rps10 and Wild-Type Plants.

Supplemental Figure 3. Ribosome Loading for Mitochondrially and Nuclear-Encoded Gene Transcripts in P2 and P3 Phenotypes of rps10 and Wild-Type Plants.

Supplemental Figure 4. Identification of Mitochondrially Encoded Proteins Whose Expression Is Affected by the Mitoribosome Defect.

Supplemental Table 1. Number of Mitochondria per Cell.

Supplemental Table 2. Primers Used for qRT-PCR.

ACKNOWLEDGMENTS

We thank Christine Chase, Carina Knorpp, Philippe Giegé, and Lee Sweetlove for supplying the antibodies for OXPHOS complexes. This work was supported by Grant N N301 784940 from the Ministry of Science and Higher Education, Poland.

AUTHOR CONTRIBUTIONS

H.J. designed the study. M.K., P.M., A.A., M.C., and E.S. performed experiments. H.J., M.K., P.M., R.S., A.A., M.C., and E.S. analyzed data. H.J. designed the study. M.K., P.M., R.S., A.A., M.C., and E.S. performed experiments. H.J. wrote the article with significant input from M.K.

Received March 7, 2013; revised May 3, 2013; accepted May 10, 2013; published May 30, 2013.

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Silencing of the Nuclear RPS10 Gene Encoding Mitochondrial Ribosomal Protein Alters Translation in Arabidopsis Mitochondria
Malgorzata Kwasniak, Pawel Majewski, Renata Skibior, Aleksandra Adamowicz, Malgorzata Czarna, Elwira Sliwinska and Hanna Janska
Plant Cell 2013;25;1855-1867; originally published online May 30, 2013;
DOI 10.1105/tpc.113.111294

This information is current as of June 20, 2017