

Phage-Type RNA Polymerase RPOTmp Performs Gene-Specific Transcription in Mitochondria of *Arabidopsis thaliana* ^{WJ|OA}

Kristina Kühn,^{a,1} Uwe Richter,^b Etienne H. Meyer,^a Etienne Delannoy,^a Andéol Falcon de Longevialle,^{a,2} Nicholas O'Toole,^c Thomas Börner,^b A. Harvey Millar,^a Ian D. Small,^{a,c} and James Whelan^a

^a Australian Research Council Centre of Excellence in Plant Energy Biology, University of Western Australia, Crawley 6009 WA, Australia

^b Institut für Biologie/Genetik, Humboldt-Universität zu Berlin, 10115 Berlin, Germany

^c Centre for Computational Systems Biology, University of Western Australia, Crawley 6009 WA, Australia

Transcription of mitochondrial genes in animals, fungi, and plants relies on the activity of T3/T7 phage-type RNA polymerases. Two such enzymes, RPOTm and RPOTmp, are present in the mitochondria of eudicotyledonous plants; RPOTmp is additionally found in plastids. We have characterized the transcriptional role of the dual-targeted RNA polymerase in mitochondria of *Arabidopsis thaliana*. Examination of mitochondrial transcripts in *rpoTmp* mutants revealed major differences in transcript abundances between wild-type and *rpoTmp* plants. Decreased levels of specific transcripts were correlated with reduced abundances of the respiratory chain complexes I and IV. Altered transcript levels in *rpoTmp* were found to result from gene-specific transcriptional changes, establishing that RPOTmp functions in distinct transcriptional processes within mitochondria. Decreased transcription of specific genes in *rpoTmp* was not associated with changes in promoter utilization; therefore, RPOTmp function is not promoter specific but gene specific. This implies that additional gene-specific elements direct the transcription of a subset of mitochondrial genes by RPOTmp.

INTRODUCTION

The evolution of mitochondria from the bacterial endosymbiont was accompanied by a loss of the bacterial-type RNA polymerase and replacement by a T3/T7 phage-type RNA polymerase in a common ancestor of almost all extant eukaryotes (reviewed in Tracy and Stern, 1995; Gray and Lang, 1998; Hess and Börner, 1999). In *Saccharomyces cerevisiae*, the nuclear *RPO41* gene encodes a phage-type RNA polymerase operating as the catalytic subunit of the mitochondrial transcription machinery (Greenleaf et al., 1986; Masters et al., 1987). A similar enzyme functions as the core RNA polymerase in human mitochondria (Tiranti et al., 1997; Falkenberg et al., 2002). Genes encoding T3/T7 phage-like RNA polymerases, which are commonly designated *RPOT* genes, have also been identified in the nuclear genomes of various photosynthetic organisms. According to in vitro and in vivo import studies, a small family of three *RPOT* genes in *Arabidopsis thaliana* encodes a mitochondrial (RPOTm)

and a plastidial (RPOTp) RNA polymerase and an enzyme imported into both mitochondria and plastids (RPOTmp) (Hedtkke et al., 1997, 1999b, 2000). Subcellular targeting of *RPOT* gene products to mitochondria, plastids, and both organelles is likewise seen in other eudicotyledonous plants, such as *Nicotiana* species (Kobayashi et al., 2001; Hedtkke et al., 2002). By contrast, cereals harbor only two *RPOT* genes that code for a mitochondrial and a plastidial RNA polymerase, respectively (Chang et al., 1999; Ikeda and Gray, 1999; Emanuel et al., 2004; Kusumi et al., 2004).

Distinct functions of RPOTm and RPOTmp in mitochondria are yet to be assigned. The *RPOTm* and *RPOTmp* genes in *Arabidopsis* have been reported to display overlapping expression patterns in different tissues and at different developmental stages (Emanuel et al., 2006); RPOTm and RPOTmp were therefore proposed to recognize different types of mitochondrial promoters. An alternative picture of RPOTm and RPOTmp functions has been suggested by a study of an *Arabidopsis* line lacking RPOTmp (Baba et al., 2004). Based on a developmental analysis of *RPOT* transcript levels and on the observation that in the mutant, the induction of several plastid genes in dark-grown seedlings upon illumination was delayed, Baba et al. (2004) proposed RPOTmp to be the key RNA polymerase transcribing organellar genes during early seedling development and favored a role of both RPOTm and RPOTp at a later developmental stage. However, plants lacking RPOTmp displayed no changes in the accumulation of several mitochondrial transcripts tested (Baba et al., 2004). RPOTm, but not RPOTmp, has been shown to specifically recognize mitochondrial promoters in vitro (Kühn

¹ Address correspondence to kristina@cyllene.uwa.edu.au.

² Current address: Unité Mixte de Recherche Génomique Végétale (Institut National de la Recherche Agronomique, Centre National de la Recherche Scientifique, Université d'Evry/Val d'Essonne), 91057 Evry, France.

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: Kristina Kühn (kristina@cyllene.uwa.edu.au).

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et al., 2007). The transcriptional role of RPOTmp in mitochondria is thus unclear from the current literature. RPOTmp and RPOTp were suggested to have partially redundant roles in plastids as plants lacking either RPOTmp or RPOTp showed similar morphological and developmental alterations when compared with the wild type; a double mutant disrupted in both of these enzymes was seedling lethal (Hricova et al., 2006).

Recent studies of transgenic lines lacking RPOTmp or RPOTp provided evidence for two distinct plastidial promoters being used by the dual-targeted enzyme (Courtois et al., 2007; Swiatecka-Hagenbruch et al., 2008). Of these promoters, the *rrn16* P_c promoter is active only during germination and early seedling development (Courtois et al., 2007). Its decreasing importance at subsequent stages has been reported to coincide with RPOTmp becoming attached to thylakoids via an intrinsic thylakoid membrane protein (Azevedo et al., 2008). However, the RPOTmp-specific plastidial promoter *PclpP-58* (Swiatecka-Hagenbruch et al., 2008) is active throughout plant development (Zoschke et al., 2007).

Plant mitochondria transcribe their genomes from numerous promoters (Tracy and Stern, 1995). Frequently, multiple promoters are active in the upstream region of one gene (Lupold et al., 1999; Kühn et al., 2005). Using transcriptionally competent mitochondrial extracts, promoter sequences of up to 25 nucleotides comprising the transcription start site have been defined to be required for correct and efficient initiation of transcription in vitro (Rapp and Stern, 1992; Rapp et al., 1993; Caoile and Stern, 1997; Dombrowski et al., 1999). Plant mitochondrial promoter sequences are highly variable, and in contrast with the abundant information on plastid promoters (Hajdukiewicz et al., 1997; Liere et al., 2004; Courtois et al., 2007; Swiatecka-Hagenbruch et al., 2008), there is very little data on the recognition of these promoters by different RNA polymerases. Comparisons of plastidial and mitochondrial promoters in *Arabidopsis* have identified no common motifs that might direct transcription initiation by RPOTmp in both organelles (Kühn et al., 2005; Swiatecka-Hagenbruch et al., 2007).

To define distinct RPOTmp- and RPOTm-dependent transcriptional mechanisms in mitochondria, we studied *Arabidopsis* lines with disrupted *rpoTmp* or *rpoTm* alleles. Disruption of RPOTm was found to be lethal. In plants lacking RPOTmp, the transcription of a subset of mitochondrial genes was decreased, leading to greatly reduced levels of respiratory chain complexes I and IV.

RESULTS

Disruption of RPOTm Is Lethal

We were unable to isolate plants homozygous for a T-DNA insertion in *RPOTm* from two independent T-DNA lines, SALK_005875 (allele *rpoTm-1*) and GABI_350F01 (*rpoTm-2*). Genotyping the offspring of self-pollinated plants heterozygous for an *rpoTm-1* or *rpoTm-2* allele identified fewer heterozygous individuals than expected for an embryo-lethal knockout. In reciprocal backcrosses, we found transmission of *rpoTm* alleles by both male and female gametes to be reduced by two-thirds, which could be due to decreased *rpoTm* gamete fitness, such as

partial gamete lethality, or decreased fitness of heterozygous embryos (see Supplemental Figure 1 online).

Mitochondrial Transcript Abundances Are Altered in *rpoTmp* Mutants

Three independent *rpoTmp* lines lacking a functional *RPOTmp* gene were isolated from T-DNA insertion lines GABI_286E07 (*rpoTmp-1*), SALK_132842 (*rpoTmp-2*), and SALK_086115 (*rpoTmp-3*) (Figure 1A). These T-DNA insertion lines were used previously to study *Arabidopsis* plants lacking RPOTmp (Baba et al., 2004; Courtois et al., 2007), but no molecular defects were detected in mitochondria. We found all three *rpoTmp* mutant

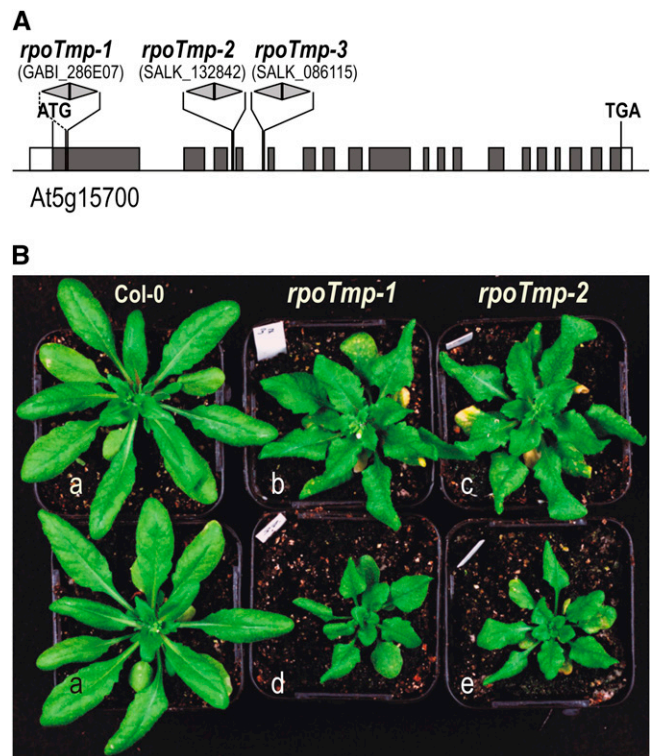


Figure 1. Isolation of *Arabidopsis rpoTmp* Mutants.

(A) *RPOTmp* gene organization and positions of T-DNA insertions in the three *rpoTmp* mutant alleles *rpoTmp-1*, *rpoTmp-2*, and *rpoTmp-3*. *RPOTmp* gene exons are represented as boxes (dark gray, coding sequences; white, untranslated regions). Gray arrowheads indicate T-DNA insertions and point toward the T-DNA left border. Note that double arrowheads represent two T-DNA copies inserted as inverted repeats, with left borders facing outward. Through PCR with primers annealing to the T-DNA left border and the flanking *RPOTmp* sequence, all insertions were found to be present as inverted repeats of the T-DNA. Insertion sites were verified by sequencing of PCR products.

(B) Phenotypes of wild-type plants (Col-0, plants labeled a) and *rpoTmp-1* (b and d) and *rpoTmp-2* mutant plants (c and e) grown in a 16-h photoperiod. A comparison of 4.5-week-old wild-type and mutant plants (a, d, and e) illustrates the developmental delay of *rpoTmp* mutants. Mutants grown for 6 weeks (b and c) are approximately at the same developmental stage and of a similar size as 4.5-week-old wild-type plants.

lines to be delayed in their development from germination onwards (Figure 1B), as reported in the earlier studies. The mutants showed wrinkly rosette leaves, consistent with observations by Baba et al. (2004).

The rosette leaf morphology displayed by *rpoTmp* plants is reminiscent of the leaf phenotype described for mutants impaired in mitochondrial function (Falcon de Longevialle et al., 2007; Reichheld et al., 2007; Van Aken et al., 2007). We therefore investigated the accumulation of mitochondrial transcripts in *rpoTmp* seedlings and rosette leaves using a quantitative RT-PCR (qRT-PCR) screen. This survey, which covered all annotated protein-encoding and rRNA genes of the *Arabidopsis* mitochondrial genome, showed changes in the abundances of numerous mitochondrial transcripts in mutant lines compared with the wild type (Figure 2). To account for the independent transcription of exons of the *trans*-spliced *nad1*, *nad2*, and *nad5* mRNAs, transcripts *nad1e2_e3* (the transcript comprising exons 2 and 3 of *nad1*; other *nad* transcripts are named accordingly), *nad1e4*, *nad1e5*, *nad2e1_e2*, *nad2e3_e4_e5*, *nad5e1_e2*, and *nad5e4_e5* were examined separately in the qRT-PCR screen. In 7-d-old seedlings, a fourfold reduction in transcript levels was measured for *matR*, *nad1e4*, *nad2e1_e2*, *nad6*, *cox1*, and *rps4* in all three mutant lines. Less marked reductions in transcript levels were seen for *nad9* and *ccmC*. Numerous other transcripts were found to be more abundant in mutant lines, with the most elevated (*ccmFc*) being increased threefold. The measured transcript levels were consistent between *rpoTmp-1*, *rpoTmp-2*, and *rpoTmp-3*. This strongly indicates that the altered mitochondrial transcript abundances in mutant plants were due to the absence of a functional *RPOTmp* gene.

Similar differences between RNA levels in mutant and wild-type plants were seen when transcripts extracted from rosette leaves of older plants were analyzed (Figure 2). For selected mRNAs, such as *matR*, the differences were even more pronounced at the later developmental stage. The observed differences in transcript abundances between mutant and wild-type plants indicate a developmentally persistent molecular defect in plants lacking a functional *RPOTmp* gene.

No differences were seen when comparing plastidial transcript levels between *rpoTmp* and wild-type seedlings under our growth conditions and at the developmental stages that we studied (see Supplemental Figure 2 online).

Transcript Levels but Not Sizes Differ between *rpoTmp* Mutants and Wild-Type Plants

As a previous study of *rpoTmp* mutants reported no changes in mitochondrial transcript abundances (Baba et al., 2004), we examined the accumulation of selected mitochondrial transcripts through RNA gel blots to validate the qRT-PCR survey. Using probes complementary to transcripts *rps4*, *nad2e1_e2*, *nad6*, *cox1*, *ccmC*, *ccmFc*, and the *nad3-rps12* cotranscript, we confirmed reduced *rps4*, *nad2*, *nad6*, *cox1*, and *ccmC* mRNA abundances as well as the enhanced accumulation of the *ccmFc* and *nad3-rps12* mRNAs in *rpoTmp* mutants (Figure 3). In agreement with qRT-PCR results, the 18S rRNA accumulates to similar levels in mutant and wild-type mitochondria (Figure 3). Additionally, transcripts in mutant and wild-type mitochondria were

examined using probes specific to *nad1e4*, *nad1e5*, and *nad5e2* (see Supplemental Figure 3 online). The RNA gel blot showed the *nad6* mRNA in *rpoTmp* to be even more diminished than determined by qRT-PCR. This may be related to qRT-PCR not being strand specific, as opposed to the strand-specific hybridization technique. The synthesis of antisense transcripts has been documented to occur in *Arabidopsis* mitochondria (Holec et al., 2006). *nad6* transcripts consistently appeared more diminished when examined using strand-specific techniques (RNA gel blot and RNase protection assay) than when non-strand-specific methods were applied (qRT-PCR, run-on transcription assays using double-stranded DNA probes; cf. Figures 2 and 4 with 3 and 7). While transcript levels differed between *rpoTmp* and the wild type for those genes examined by RNA gel blots, no differences were seen in transcript patterns (i.e., no transcripts of unusual size were seen to accumulate in mutants).

Altered Transcript Abundances in *rpoTmp* Coincide with Transcriptional Changes

The observed differences in mitochondrial transcript levels between *rpoTmp* and wild-type plants could be the result of changed rates of either transcript synthesis or/and transcript degradation. To pinpoint the molecular process(es) affected in the mutant, we performed mitochondrial run-on transcription assays with mitochondria prepared from rosettes of *rpoTmp-2* and wild-type plants (Figure 4). We selected genes showing lowered transcript levels (*nad2*, *nad6*, *rps4*, and *cox1*) as well as genes with increased or unaltered transcript accumulation in *rpoTmp* (*rrn26*, *atp6-1*, *ccmFc*, and *rps3*) to be examined in these assays. Figure 4B shows the run-on transcription signals detected in two representative assays with mutant and wild-type mitochondria. We measured signals from three to four independent assays per genotype to quantify and compare the transcription of probed genes between *rpoTmp* and the wild type (Figure 4C). Transcription of *rrn26*, *ccmFc*, and *atp6-1* in the mutant was significantly higher than in the wild type, while transcription of *nad6*, *nad2*, *rps4*, and *cox1* was significantly reduced in the mutant (one-tailed *t* test, $P \leq 0.05$). *rps3* transcription did not differ significantly between mutant and wild-type plants.

The run-on transcription assays and the qRT-PCR assay on *rpoTmp-2* and wild-type rosette leaves (Figure 2) were performed on the same set of plants, allowing the pairing of data from both experiments (Figure 4C). The transcriptional changes in *rpoTmp* generally coincided with the measured differences in transcript accumulation. The reduction in transcription of *rps4* and *nad2e1_e2* in the mutant was not as severe as the measured decrease in transcript accumulation. Although a contribution of altered transcript stabilities to changed transcript abundances in *rpoTmp* cannot be ruled out, it can be inferred from run-on transcription assays that transcriptional changes largely account for the changes in transcript abundances in the mutant.

rpoTmp Has Lower Levels of Respiratory Chain Complexes I and IV

To examine if the observed reduction in mitochondrial transcripts in *rpoTmp* (particularly of the severely diminished *nad6* and *cox1*

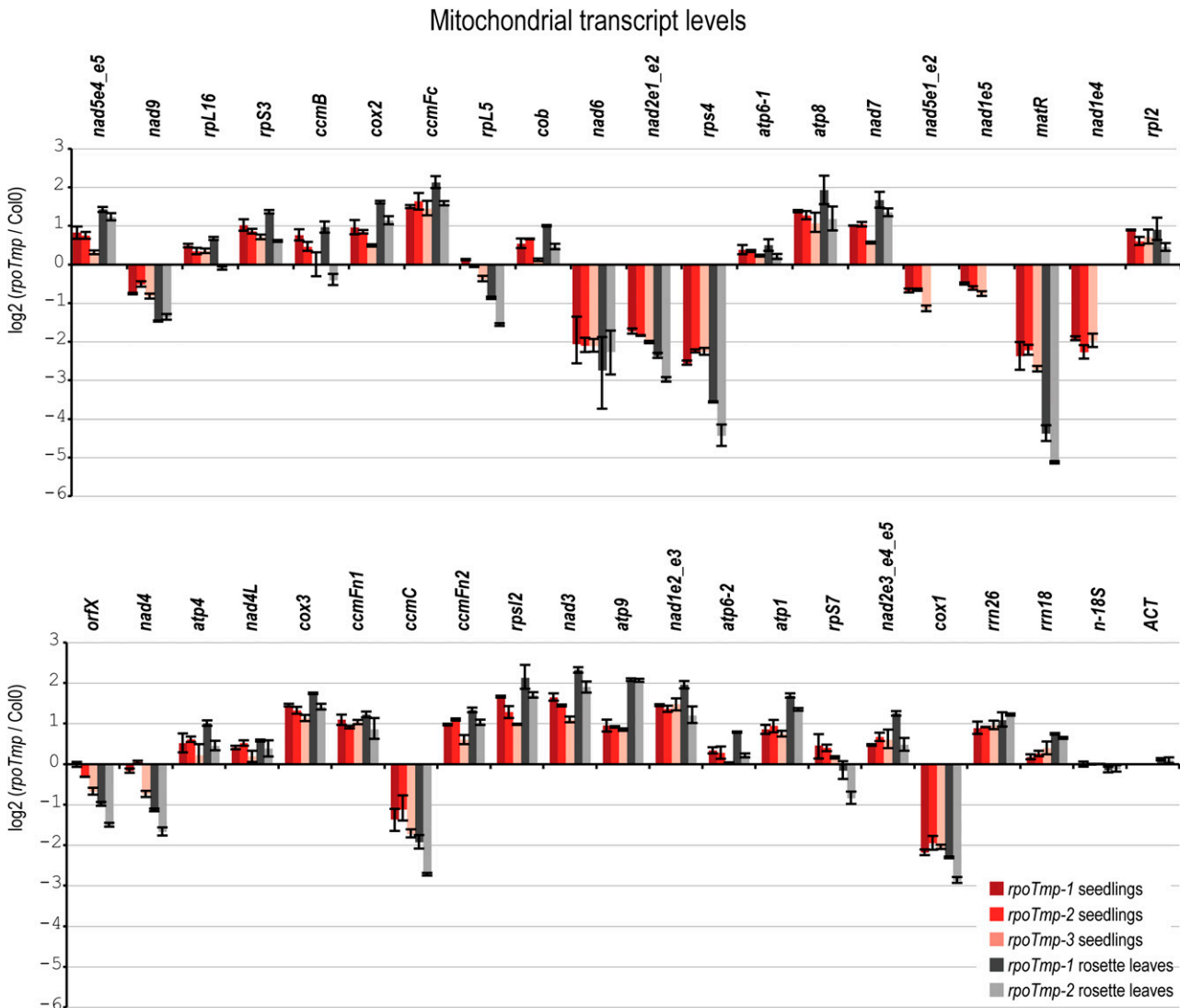


Figure 2. Mitochondrial Steady State Transcript Levels in *rpoTmp* Mutants.

Transcript levels are depicted as the log₂ ratio of transcript levels in mutants compared with levels in wild-type (Columbia-0 [Col-0]) plants. Transcript abundances of all mitochondrial protein-encoding and rRNA genes were determined by qRT-PCR in both mutant and wild-type plants at the seedling and at the rosette stage. Different transcripts were targeted for the *trans*-spliced *nad1*, *nad2*, and *nad5* mRNAs; data points are labeled according to the exons amplified by qRT-PCR (e.g., *nad1e1_e2* for a *cis*-spliced transcript comprising exons 1 and 2 of the *nad2* gene). In the assays done on *nad1*, *nad2*, and *nad5*, the mature mRNA plus the pre-mRNAs that are not yet *trans*-spliced are measured. Because of the low *trans*-splicing efficiencies, the measured transcripts are partly or even mostly pre-mRNAs and reflect the levels of the independently transcribed mRNA parts. Three technical replicates were averaged per genotype; standard errors are indicated. The nuclear *18S* rRNA gene (seedlings) or the nuclear *18S* rRNA and *ACT* genes (rosette leaves) were used for data normalization.

mRNAs) had consequences for the synthesis of proteins encoded by the mitochondrial genome, we analyzed the abundances of different respiratory chain complexes. Mitochondria were isolated from *rpoTmp* and wild-type rosette tissue, and mitochondrial membrane complexes were resolved using blue-native PAGE (BN-PAGE; Figure 5A). To validate the identification of bands in the BN-PAGE profiles, and to compare the abundances of membrane complexes between mutant and wild-type mitochondria, respiratory chain complexes I, III, and IV and

complex V were immunolabeled (Figure 5B). Complex I (NADH: ubiquinone oxidoreductase) and complex IV were barely detectable in mutant mitochondria, whereas complex III (ubiquinol: cytochrome *c* oxidoreductase) and complex V (F₀F₁ ATP synthase) were clearly present (Figure 5A). While complex III and complex V appeared slightly more abundant in the wild type than in mutants, the F1 subcomplex of complex V was equally abundant in mutant and wild-type mitochondria. The unaltered levels of the F1 ATP synthase (which contains the ATP1 subunit

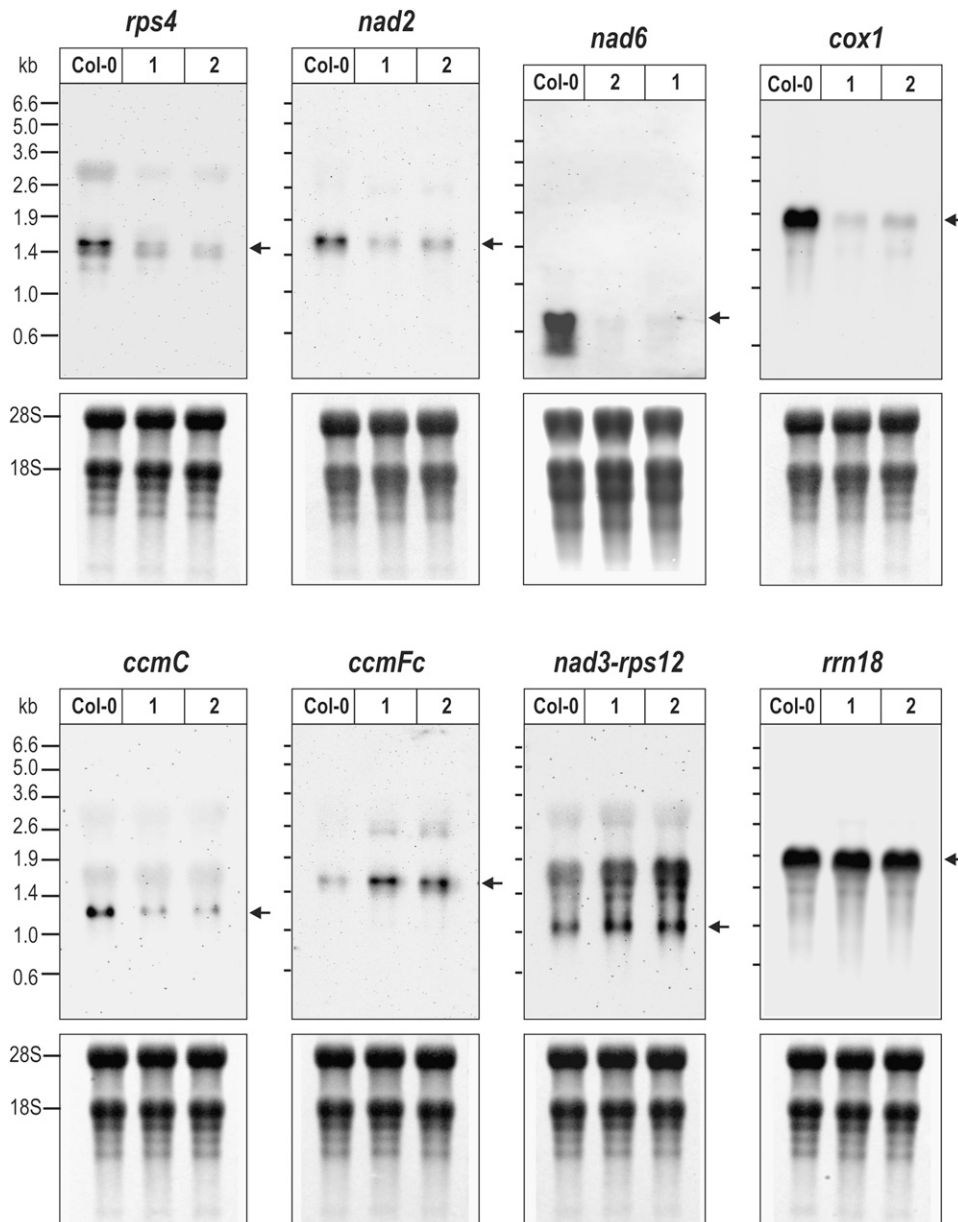


Figure 3. RNA Gel Blot Hybridizations Confirming Mitochondrial Transcript Changes in *rpoTmp*.

Probes specific to the coding sequence of *rps4*, *nad2* (exon 1), *nad6*, *cox1*, *ccmC*, *ccmFc* (exon1), *nad3*, and to the mitochondrial 18S rRNA were hybridized to 15 μ g (*nad6*) or 10 μ g (others) of filter-immobilized total RNA isolated from *rpoTmp-1* (lanes 1), *rpoTmp-2* (2), and wild-type (Col-0) seedlings (top panels). RNA size markers were run alongside samples; marker sizes are indicated on the left of each blot. Signals corresponding in size to expected mature transcripts are indicated by arrows to the right of each panel; sizes have been inferred from transcript 5' and 3' ends defined by Forner et al. (2007). No monocistronic *nad3* or *rps12* mRNAs are detected in *Arabidopsis* mitochondria (Forner et al., 2007). A higher molecular weight RNA visible in the *rps4* blot corresponds to an *rps4-nad2e1_e2* cotranscript and was also seen in the *nad2* blot when overexposed. The larger band seen in the mutants for *ccmFc* is the unspliced transcript. The same filters were stained with methylene blue and are shown as a loading control in the bottom panels.

encoded and synthesized in the mitochondrion) in *rpoTmp* indicate that mitochondrial protein synthesis is not impaired in mutants despite the reduced abundance of the *rps4* mRNA (cf. Figure 3). We additionally compared protein synthesis in mutant and wild-type mitochondria by in organello protein synthesis

assays and found no evidence for impeded translation in mutant mitochondria (see Supplemental Figure 4 online).

In order to quantify the reduction in complex I and complex IV abundances in *rpoTmp*, we measured the electron transport capacities of complex I and complex IV in mitochondrial

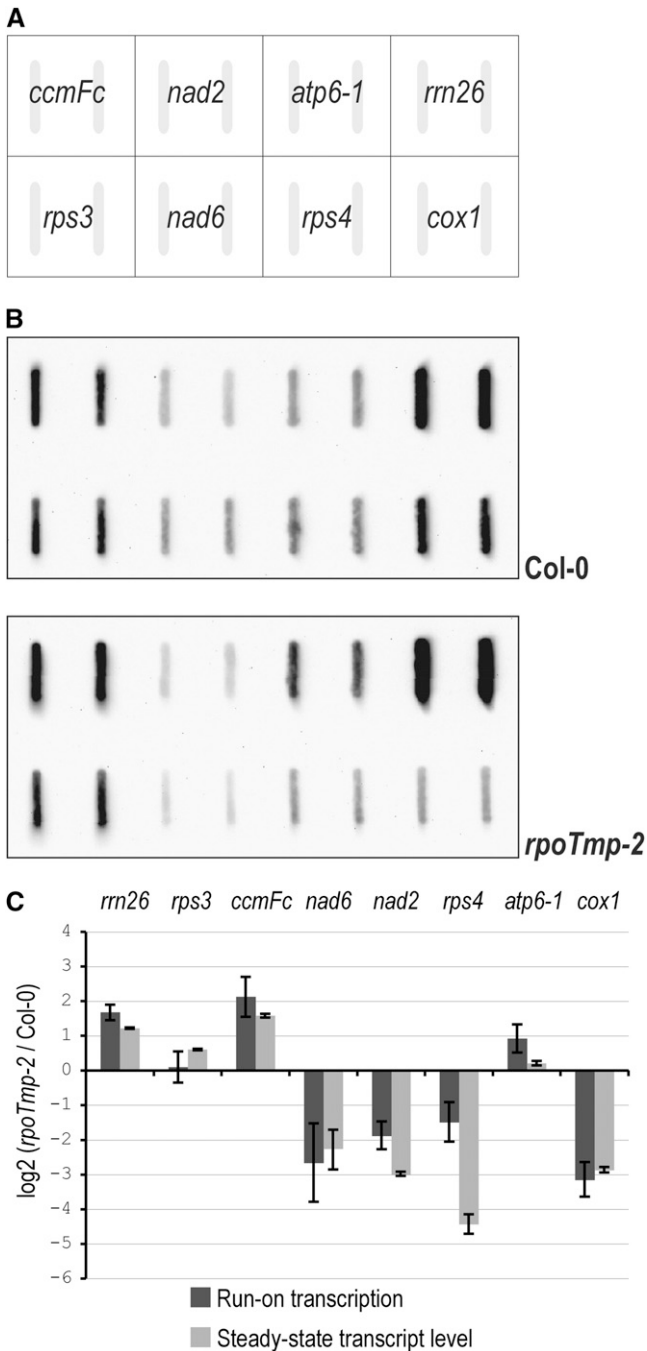


Figure 4. Run-On Transcription Assay of Mitochondrial Transcription in *rpoTnp-2*.

(A) Layout of mitochondrial gene slot blots probed with run-on transcripts; probes were applied to membranes in duplicates.
(B) Filters probed with run-on transcripts derived from mitochondria isolated from wild-type (Col-0) and *rpoTnp-2* rosettes.
(C) Mitochondrial run-on transcription rates determined for *rpoTnp-2* (dark gray) and steady state transcript levels measured for *rpoTnp-2* rosettes (light gray; taken from Figure 2). Transcription rates are depicted as the log₂ ratio of a labeled transcript synthesized by mutant mitochondria compared with labeled transcript synthesized by wild-type

membranes prepared from mutant and wild-type plants (Figure 5C). Mutant membranes retained ~15% of the complex I and complex IV capacities of the wild type.

The changes in the abundances of complex I and complex IV correlate with and are probably due to the highly reduced transcript levels seen for the genes *nad6* and *cox1* (Figure 3), which encode a complex I and a complex IV subunit, respectively.

Identical Promoters Are Active in *rpoTnp* and Wild-Type Plants

Considering the reported specificity of RPOTmp for selected promoters in plastids (Courtois et al., 2007; Swiatecka-Hagenbruch et al., 2008), we compared promoter utilization between *rpoTnp* and wild-type seedlings to test if the reduced transcript levels observed for several genes in mutants were due to the lack of transcription initiation from specific promoters. With the exception of *cox1* (Kühn et al., 2005), none of the genes displaying reduced transcript abundance in *rpoTnp* had been characterized with regard to primary transcript 5' ends and promoters. Two recent studies of mRNA termini in *Arabidopsis* have determined 5' and 3' ends of several mitochondrial transcripts, including transcripts such as *nad2*, *nad6*, *ccmC*, and *ccmFc* (Forner et al., 2007, 2008) that we observed to be changed in abundance in *rpoTnp*. However, those studies were not specifically directed at identifying transcription start sites (TSSs); they mapped major transcript 5' ends and did not distinguish between primary and processed ends. We thus undertook a comprehensive analysis of TSSs and promoters in upstream regions of those genes displaying altered transcript accumulation in mutants.

In plant mitochondria, the promoter sequence comprises the TSS (Binder and Brennicke, 2003); promoters can therefore be identified by TSS mapping. We identified TSSs in *rpoTnp* mutant and wild-type plants using a 5'-rapid amplification of cDNA ends (5'-RACE) technique employed previously for determining primary and processed transcript 5' ends and promoters in *Arabidopsis* mitochondria (Kühn et al., 2005). Figure 6 provides a graphical summary of TSSs and 5' end processing sites determined in our study using total cellular RNA prepared from 7-d-old seedlings. Table 1 lists the mapped TSSs and processing sites and their surrounding sequences. Promoters (P) and their corresponding TSSs are specified with the gene name and the position of the initiating nucleotide with respect to the start of the coding sequence (e.g., *Pcox1*-355). Sites for which transcript 5' end analysis did not support their functioning as TSS and that did not display sequence elements frequently associated with mitochondrial promoters are listed as processing sites. Nearly all of these sites have previously been identified as processing sites resulting from endonucleolytic 5'-terminal processing (Forner

mitochondria over the assay period of 10 min. Transcription rates as shown in the diagram were determined as means from three (*ccmFc*, *nad6*, and *rps4*) or four (all other genes) independent run-on assays; error bars represent standard deviations.

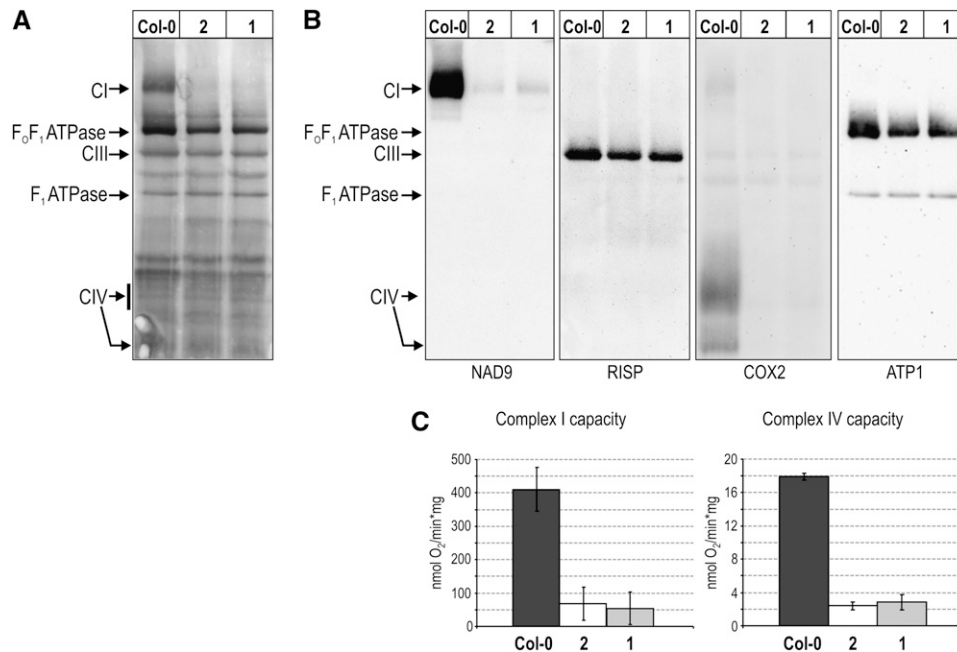


Figure 5. Respiratory Chain Complexes I and IV Are Reduced in *rpoTnp* Mitochondria.

(A) Mitochondria were isolated from *rpoTnp-1* (1), *rpoTnp-2* (2), and wild-type (Col-0) rosettes, and mitochondrial membrane complexes were resolved by BN-PAGE, transferred onto a membrane, and Coomassie stained. Bands corresponding to respiratory chain complexes I (CI), III (CIII), and to the F_0F_1 -ATPase and its F1 subcomplex are indicated. The gel areas in which complex IV (CIV) is migrating are marked.

(B) The membrane shown in **(A)** was probed with antibodies against NAD9, RISP (Rieske iron-sulfur protein), COX2, and ATP1 as marked to label CI, CIII, CIV, F_0F_1 -ATPase, and the F1 subcomplex.

(C) The electron transport capacities of complex I and complex IV in mitochondrial membranes isolated from *rpoTnp-1* (1), *rpoTnp-2* (2), and wild-type (Col-0) rosettes were determined by measuring oxygen reduction in the presence of complex I-specific and complex IV-specific substrates. Three independent mitochondrial preparations per genotype were analyzed to calculate mean values and standard deviations.

et al., 2007). For eight 5' termini mapping to a promoter-like motif, such as *Pnad2e1-114*, 5'-RACE analysis did not distinguish a primary 5' end (see Supplemental Figures 7, 9, 11, 13, and 14 online). Such sites are nonetheless listed as putative promoters because it is sometimes not possible to clearly identify primary transcript ends with the 5'-RACE technique applied here (e.g., when the triphosphates at primary 5' ends are unstable) (Kühn et al., 2005; Forner et al., 2007). These putative promoters have been pointed out in Table 1. Images of all analyzed 5'-RACE products and detailed specifications of identified 5' ends are provided in Supplemental Figures 5 to 14 online.

The majority of 5' ends detected in the wild-type plants were also detected in *rpoTnp* mutants (Figure 6, Table 1); 5'-RACE products amplified from *cox1* transcript 5' termini are shown as examples (Figure 6B). For some promoters, such as *Pnad2e1-114* and *Pnad2e1-297*, 5' ends did not map to exactly the same nucleotides in *rpoTnp* (lowercase positions in Table 1) and the wild type (underlined positions). This could be due to either technical inconsistency of the 5'-RACE technique or genotype-specific differences between initiating nucleotides in *rpoTnp* and the wild type. The first is more likely, as for some TSSs we also saw such differences between two independent 5'-RACE analyses performed on the same gene in the wild type (data not shown).

Selected transcript 5' termini, such as the processed end *nad6-179*, were barely detectable in mutants (Figure 6B). On the other hand, a primary 5' end mapping to *Pnad6-143* downstream of this processing site was more easily detected in *rpoTnp* (Figure 6B). Products mapping to *Pnad6-333* were not detected in the wild type or *rpoTnp-1* but only in *rpoTnp-2*. The discrepancy between the two mutant lines, and also between *rpoTnp-2* and the wild type, could be due to very low abundance of the *Pnad6-333* 5' end. The apparent difference at *Pnad6-333* between *rpoTnp-2* and the wild type was therefore considered insignificant. These occasional inconsistencies are likely to be due to the fact that while the 5'-RACE technique employed here is a powerful tool for identifying primary transcript 5' termini, it is inadequate for quantitative studies and may even fail to amplify selected 5' ends. We therefore attempted to determine the abundances of these 5' termini in mutants and the wild type using quantitative ribonuclease protection assays (RPAs; Figure 7).

Comparison of Transcript 5' End Abundances between *rpoTnp* and Wild-Type Plants

For selected mitochondrial genes, we analyzed the abundance of their different transcript 5' ends through RPAs. Because this

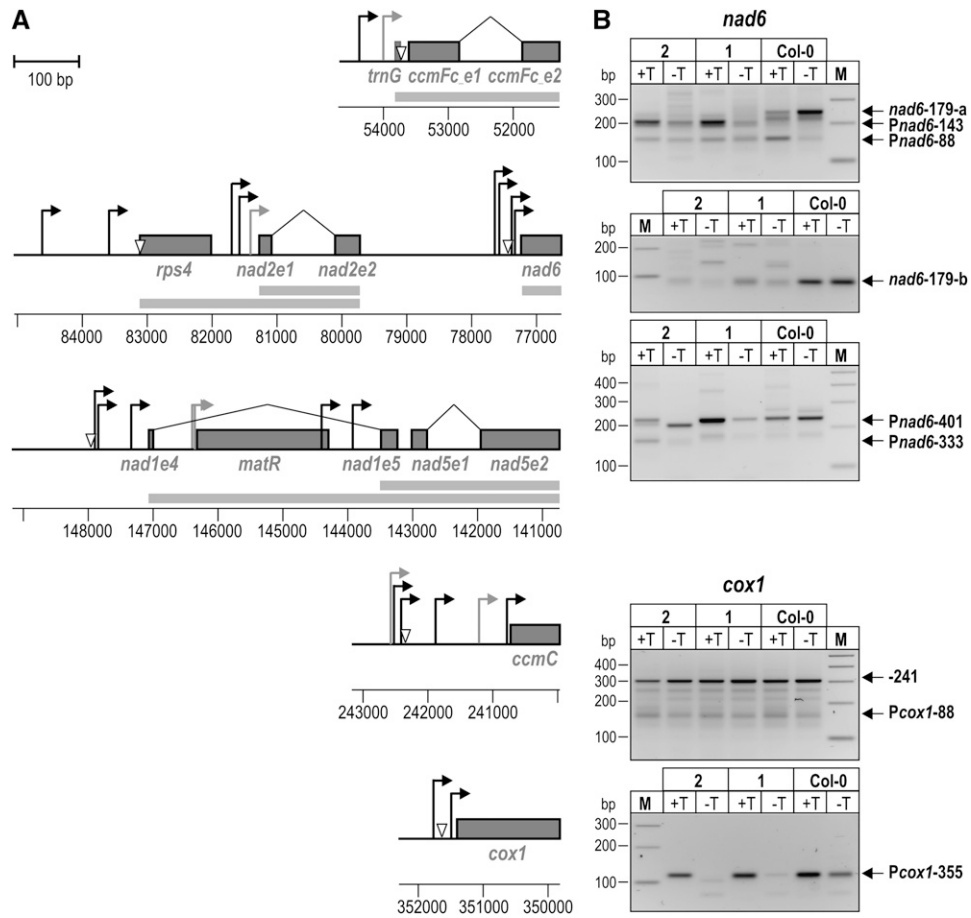


Figure 6. Analysis of TSSs in *rpoTmp* and Wild-Type Seedlings.

(A) Maps of mtDNA regions for which TSSs and transcript 5' end processing sites were analyzed. Protein-coding sequences are displayed as gray boxes and labeled with the corresponding gene names. In the case of *cis*- and *trans*-spliced sequences, exons are labeled with the gene name, followed by the letter e and the exon number. Splicing events are indicated by a bent line between exons. TSSs mapped in 5'-RACE analyses are indicated by bent arrows; open triangles mark 5' end processing sites. Putative promoters, for which primary 5' termini could not be experimentally distinguished, are drawn in gray. Where indicated by experimental results, cotranscription of genes and exons is shown by gray bars below gene maps. Coordinates of the analyzed regions on the mtDNA are indicated.

(B) 5'-RACE analysis of *nad6* and *cox1* transcripts. 5'-RACE was performed on RNA extracted from 7-d-old *rpoTmp-1* (lanes labeled 1), *rpoTmp-2* (2), and wild-type (Col-0) seedlings using a 5'-RACE technique that exploits the difference in 5' end phosphorylation between primary and processed transcript 5' ends (Bensing et al., 1996). Products obtained from TAP-treated (+T) and untreated RNA (-T) were separated on agarose gels alongside a molecular weight marker (M). Products from primary transcript 5' ends are labeled with the name of the corresponding promoter as listed in Table 1; products corresponding to processed ends are labeled accordingly. The product from the processed end *nad6-179* was barely detectable in *rpoTmp-1* and -2 (signal marked *nad6-179-a*) but could be amplified with a reverse primer not detecting any downstream 5' ends (*nad6-179-b*).

technique uses the labeled probe in excess to saturate all target sequences present in one assay, it provides a quantitative detection method for the analysis of transcript 5' ends. Using total cellular RNA prepared from 7-d-old *rpoTmp-1*, *rpoTmp-2*, and wild-type seedlings, we performed RPAs on 5' termini of transcripts reduced in abundance in *rpoTmp* (*nad1e4* and *matR*, *nad2e1_e2*, *nad6*, *cox1*, and *ccmC*) as well as a transcript accumulating more abundantly in mutants (*atp6-1*). *atp6-1* was selected because it represents a gene with known multiple promoters that have been detected previously using the RPA technique (Kühn et al., 2005).

Figure 7 shows the PAGE analyses of protected fragments generated through RPAs performed on mitochondrial transcript 5' ends. Linear diagrams in Figure 7 illustrate the upstream regions, promoters, and processing sites of genes examined by RPAs, as well as riboprobe target sequences. The fragments corresponding to *Pnad6-143* and *Pnad6-88* appeared to migrate in the PAGE as two major bands, with the larger of two transcripts seen for each *Pnad6-143* and *Pnad6-88* at the expected size. The two faster migrating transcripts show the same apparent reduction in size, which would agree with secondary structure formation. However, no equivalent lower signal is seen for the

Table 1. Transcription Initiation Sites and Processing Sites Detected by 5'-RACE

Gene	5' End	Sequence	Col-0		<i>rpoTnp</i>	
			+TAP	-TAP	+TAP	-TAP
<i>cox1</i>	Pcox1-88	CTCTCAAGAACT CGT AGACTATGGA	2/8	0/7	n.a.	n.a.
<i>cox1</i>	<i>cox1</i> -241 ^{a,b}	GAATCCAATGGTAGGCCTGGGCTCT	n.a.	n.a.	n.a.	n.a.
<i>cox1</i>	Pcox1-355 ^a	AATTTATTC AATT ATATaATAATA	n.a.	n.a.	6/8 [#]	n.a.
<i>nad1</i>	Pnad1e4-275	TACAAAAAC ATT CTAGGgAAGGGCT	6/10	1/8	2/2	n.a.
<i>nad1</i>	Pnad1e4-775	CGACTGCTAT CTA AGAGgATAAG	4, 3, 2/13	7, 0, 7/15	8/8	n.a.
<i>nad1</i>	Pnad1e4-838	GGCAATTTAT CGT ATAGaAGAATAG	3, 6/12	0, 0/13	14, 0/14	n.a.
<i>nad1</i>	<i>nad1e4</i> -892	GACCAAGCAAATCTCCTC T CTAGT	-	2/2	-	-
<i>matR</i>	PmatR~3 ^{*,e}	CAATATGAAGAAAGAAaGAAGGGTT				
<i>matR</i>	PmatR~58 ^{*,e}	GCCTTTG ATT AATAAAaGGGGAGC				
<i>nad1</i>	Pnad1e5-427	ACTA ATT ATATATATATaATAAGGT	5/7	0/10	2/8	n.a.
<i>nad1</i>	Pnad1e5-927	CAATATTGTAATCAAGgAAGGTGG	5/18	0/10	2/13	n.a.
<i>ccmC</i>	PccmC-45	CTTTGATTG CGTATT AaGATCCAT	4/16	0/15	1/14	n.a.
<i>ccmC</i>	PccmC-487 ^{c,e}	TATCGATCGG CGTAA GaAAAgATTC	1, 2, 1/15	0, 2, 1/12	1, 1/8	n.a.
<i>ccmC</i>	PccmC-1159	GATTCGTCAT GGT AGAGaGAAAAA	6/6	-	8/8	-
<i>ccmC</i>	<i>ccmC</i> -1634	CCTCGAATCTGTAAAGCT AG CTTGG	1/1	n.a.	9/9	n.a.
<i>ccmC</i>	PccmC-1677	CTACCAAACGTCCTAAT AA AGAGC	8/9	0/7	-	-
<i>ccmC</i>	PccmC-1817	AAGTAATTC ATT TAAAgATA AGT aA	1, 3, 9/13	0, 0, 4/8	2, 2/9	n.a.
<i>ccmC</i>	PccmC-1834 ^e	ACACTACTATATAAGATA AGT AATT	2, 0/2	1,4/6	n.a.	n.a.
<i>rps4</i>	<i>rps4</i> +2 ^b	GAGTCCCAGGGACGCA 4 rGTGGCTG	13/13	n.d	8/8 [#]	n.a.
<i>rps4</i>	Prps4-477	AGAAAGCTCA AGT ATGTaAACAACC	6/7	n.a.	8/8	n.a.
<i>rps4</i>	Prps4-1509	AGATAGGTC AGG TATATaTAGCGAT	4/6	0/8	3/3 [#]	-
<i>nad2</i>	Pnad2e1-114 ^{b,e}	CACTATC ATT A ATT ATATaTTTCAT	6,10, 4/32	5, 3, 2/18	3, 1/7	n.a.
<i>nad2</i>	Pnad2e1-299	CAAGATATTG CGT ATaAATATATAT	2/28	n.a.	1/8	n.a.
<i>nad2</i>	Pnad2e1-413	GAACAAAAGATGTAATGaAAAgATA	2, 3/10	n.a.	4, 3/8	n.a.
<i>nad6</i>	Pnad6-88	GAAGAAAATGAAATAGGA a CAACCG	2,10/16	0,1/7	7/15	n.a.
<i>nad6</i>	Pnad6-143	ATTGAGATT CGTAA GTA ACT CAGT	3/8	n.a.	9/16	n.a.
<i>nad6</i>	<i>nad6</i> -179 ^b	GGGTACAAGATCGAAA g AATGCAT	14/17	12/14	3/7	n.a.
<i>nad6</i>	Pnad6-333	GATG ATT ATGTTGAAAaGATCGAA	-	-	8/8	0/7
<i>nad6</i>	Pnad6-401	CGGGTAC AGT AG TAGG TaAACTTGA	18/19	2/8	8/8	-
<i>ccmFc</i>	<i>ccmFc</i> -124 ^b	AAGTCCCTCCTTCGCT C CGGTGT	n.a.	n.a.	n.a.	n.a.
<i>ccmFc</i>	PccmFc-379 ^e	TTGGATTAT CGT AGAATAAGAGGAG	6/7	6/7	n.a.	n.a.
<i>ccmFc</i>	PccmFc-743	TTTATTATTCT ATT ATAaTAGTCTA	-	-	8/8	-
<i>nad9</i>	<i>nad9</i> -211/210 ^c	GGCGATCCACGAATTTGATCATTCCG	1, 2/8	n.a.	n.a.	n.a.
<i>nad9</i>	Pnad9-1241 ^e	TACGAAATAT AGTAA ATATCGTAAA	3/3	n.a.	n.a.	n.a.
<i>nad9</i>	Pnad9-1371	TTCTCGTAATGTATTTA ACC CATAAA	2/8	n.a.	-	-
<i>nad9</i>	<i>nad9</i> -1400	CAAACAAATGCTATA AG CATAGAA	1/2	n.a.	n.a.	n.a.
<i>nad9</i>	Pnad9~1730 ^e	GATTGATA AAAGG TAAATAGATATGAT	n.a.	n.a.	n.a.	n.a.

TSSs and processing sites determined for the wild type (Col-0) are underlined in sequences; lowercase positions denote sites mapped for *rpoTnp*. CATA, CGTA, TCTA, ATTA, AGTA, and GGTA motifs frequently found associated with mitochondrial promoter sequences (Fey and Marechal-Drouard, 1999; Kühn et al., 2005) are in bold. The number of clones sequenced for each site is given together with the frequency of the respective transcript 5' end as determined from TAP-treated (+TAP) or untreated (-TAP) seedling RNA. Where more than one nucleotide is marked in one sequence, 5' end frequencies are given in 5'-to-3' order. Mutant transcript 5' ends were mostly analyzed from *rpoTnp-2* 5'-RACE products. In a few cases where products amplified for *rpoTnp-2* were extremely faint but *rpoTnp-1* products formed a clear band, the latter were cloned and sequenced (numbers marked #). As reported by Forner et al. (2007), *matR* 5' ends were poorly defined (sites marked *; see Supplemental Figure 7 online for identified 5' ends). The first codons of the annotated MATR and RPS4 coding sequences are italicized. -, Signal absent in 5'-RACE; n.a., not analyzed.

^aDetermined previously (Kühn et al., 2005).

^bDetermined previously (Forner et al., 2007).

^cDetermined previously (Forner et al., 2008) with minor differences.

^ePutative promoters for which experimental data are insufficient to support their function as TSS.

nad6-179 signal, which argues against secondary structure formation at the 3' end. The additional *nad6* signals could therefore not be assigned to known transcripts and have not been labeled in Figure 7.

Not only the 5' end *nad6*-179, which had barely been detectable by 5'-RACE (Figure 6B) in *rpoTnp* mutants, but also the

termini corresponding to *Pnad6*-143 and *Pnad6*-88 were greatly reduced in abundance in the mutants. We were unable to analyze the abundances of transcripts synthesized from *nad6* promoters located upstream of the *nad6*-179 processing site because this region shows 97% sequence identity to the chloroplast *rbcl* transcript, which interfered strongly with the assay. From the

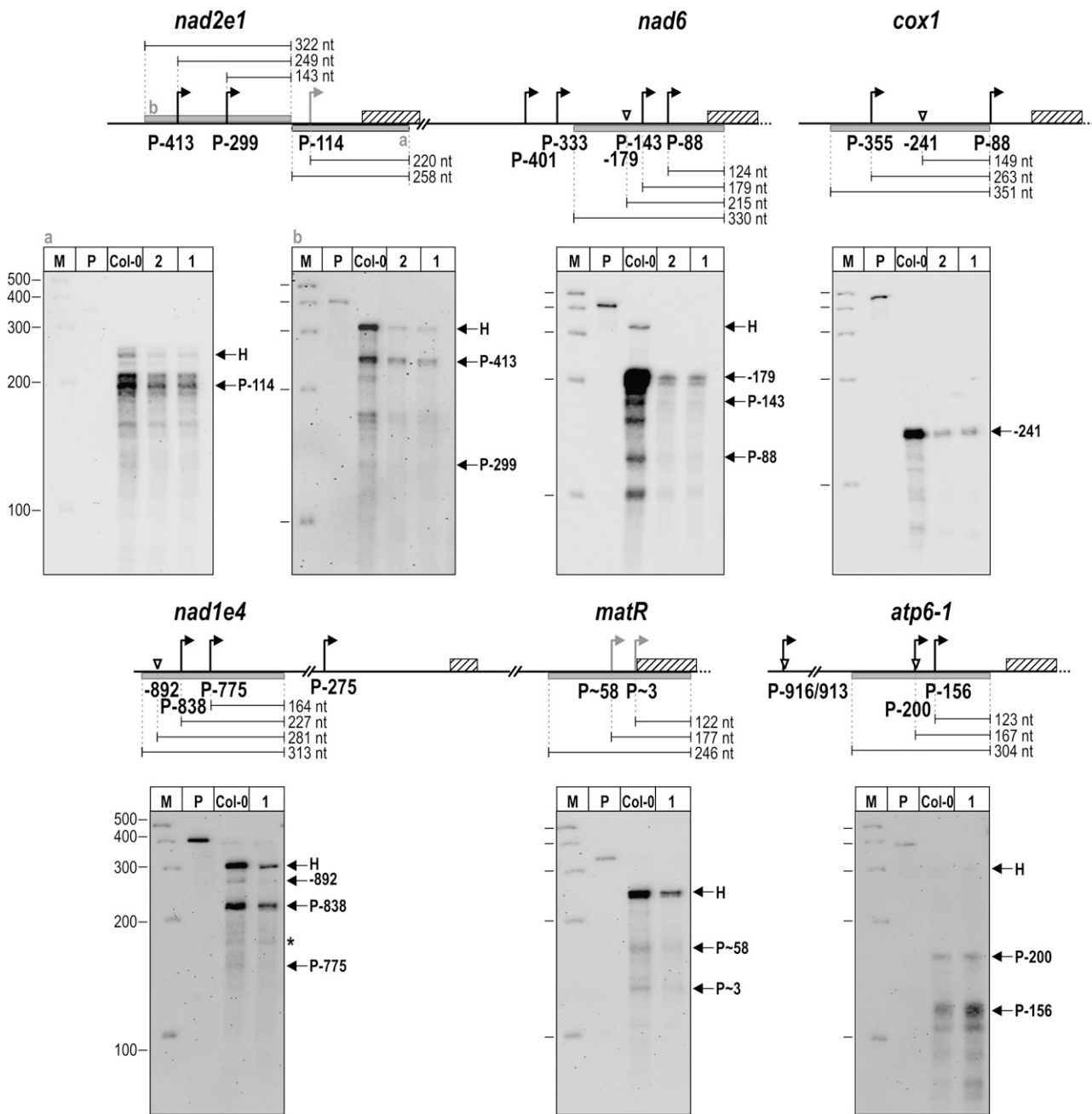


Figure 7. Transcript 5' End Abundances in *rpoTmp* and Wild-Type Seedlings.

RPA was performed on total seedling RNA prepared from *rpoTmp-1* (lanes labeled 1), *rpoTmp-2* (lanes labeled 2), and the wild type (Col-0) using biotinylated riboprobes annealing to the *nad2e1*, *nad6*, *cox1*, *nad1e4*, *matR*, and *atp6-1* 5' regions. Protected RNA fragments were separated on polyacrylamide gels alongside a molecular weight marker (lane M) and the probe (P) used in the respective assay; sizes are given in nucleotides. Specific protected fragments are indicated by arrows and labeled with the name of the promoter or processing site they correspond to. Signals marked H are derived from protected fragments corresponding to the complete homologous segment of a probe. An asterisk in the *nad1e4* panel marks a signal that was considered nonspecific, as it was seen with nonspecific RNA in a control assay (data not shown). Diagrams of the *nad2e1*, *nad6*, *cox1*, *nad1e4*, *matR*, and *atp6-1* 5'-untranslated regions above each gel image illustrate positions of promoters (bent arrows) and processing sites (triangles); coding sequences are shown as hatched bars. Gray bars are drawn below sequences complementary to the riboprobes that were used in RPAs. The sizes of expected protected RNA fragments are given in nucleotides. The two protected fragments derived from the *nad2e1* probe "a" migrated slightly faster than expected, possibly due to secondary structure formation. A fragment that could correspond to *PmatR~3* appears slightly larger than expected.

signal corresponding to the complete protected probe (signal H in the *nad6* panel of Figure 7) and from the abundance of the *nad6-179* 5' end, the formation of which depends on transcription initiation from upstream sites, it can be inferred that transcripts starting at these sites were diminished in *rpoTmp* in comparison to the wild type. Signals corresponding to *Pnad2e1-114*, *Pnad2e1-299*, and *Pnad2e1-413* were similarly reduced in mutants. Likewise, all analyzed *nad1e4* and *matR* 5' termini, primary or processed, were reduced in abundance in *rpoTmp*. By contrast, signals corresponding to *Patp6-1-156* and *Patp6-1-200* were enhanced in mutants.

The fairly prominent signal corresponding to the complete protected *nad1e4* probe (signal H in the *nad1e4* panel of Figure 7) indicates that significant transcription of *nad1e4* and *matR* initiates from TSS upstream of the *nad1e4-892* processing site. We were unable to consistently map these upstream TSSs in two independent 5'-RACE experiments and did not investigate them further; they are therefore not listed in Table 1. However, the signal intensities of the complete protected *nad1e4* probe seen for *rpoTmp* mutants and the wild type indicate that *nad1e4* and *matR* transcription from the undefined upstream TSS is also decreased in *rpoTmp*.

Primary 5' ends corresponding to *Pcox1-355* (Figure 7), *PccmFc-379*, and *PccmFc-743* (data not shown) were not detectable in any genotype using RPAs, probably owing to rapid 5' end processing of these transcripts.

The quantitative analysis of transcript 5' ends showed that all 5' termini of a transcript were diminished in *rpoTmp* when the overall abundance of the transcript was reduced in the mutant. To ensure that this was not a consequence of lowered abundances of subgenomic DNA molecules in *rpoTmp*, we compared copy numbers of mitochondrial genes in mutant and wild-type plants using qPCR. Mitochondrial DNA levels in *rpoTmp-2* were twofold to threefold higher than in the wild type uniformly across all genes tested; no gene-specific variations in gene copy numbers were detected (Figure 8). Nuclear

DNA content in *rpoTmp* is unaltered (E. Cincu, J. Fuchs, and T. Börner, unpublished data; see Supplemental Figure 17 online), permitting the use of a nuclear reference gene in this analysis (Figure 8).

Promoters of Genes with Reduced Transcription in *rpoTmp* Are Not Structurally Distinct

A list of all promoters and putative promoters identified in *Arabidopsis* mitochondria to date is displayed in Figure 9. Sequences spanning 18 nucleotides, from position -14 to $+4$ around the TSS, are displayed. This region has been defined by Dombrowski et al. (1999) to be required for promoter function in dicot plant mitochondria. The promoters and putative promoters of genes transcribed less actively in *rpoTmp* were computationally compared with all other known mitochondrial promoters in *Arabidopsis* to identify hexa-, penta-, or tetranucleotide elements statistically overrepresented in the subset of promoters affected in *rpoTmp* (see Supplemental Table 1 online). No RPO*Tmp*-specific hexanucleotide or pentanucleotide elements were detected. Only the tetranucleotide GAAA was overrepresented among promoters of genes transcribed less actively in the mutant. Since the position of this element in the different promoter sequences is variable, with occurrences upstream of as well as comprising the TSS (Figure 9), this element is unlikely to be significant for RPO*Tmp*-specific promoter function. Among the 25 tested promoters of genes affected in *rpoTmp*, only six displayed a GAAA element. We could find no criteria by which we could identify these promoters as a distinct subset of mitochondrial promoters, other than the fact that the transcripts initiated from them are less abundant in *rpoTmp* plants. A distance tree has been derived of all promoters as listed and aligned in Figure 9, in which *rpoTmp*-affected promoters are interspersed with unaffected promoters throughout most of the tree and do not cluster as an obvious subgroup (see Supplemental Figure 15 online).

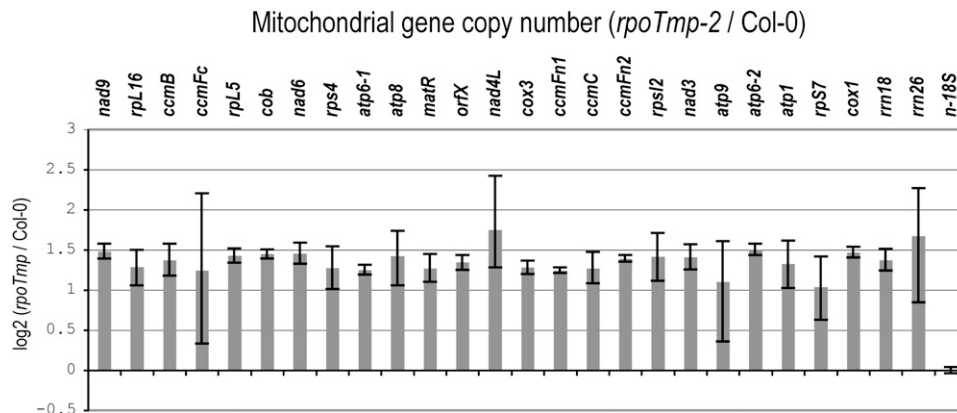


Figure 8. Mitochondrial Gene Copy Numbers in *rpoTmp-2* Seedlings.

Gene copy numbers are depicted as the log₂ ratio of DNA levels in mutants compared with levels in wild-type plants (Col-0). Gene copy numbers of selected mitochondrial protein-encoding and rRNA genes were determined by qPCR in both mutant and wild-type seedlings employing the PCR protocol and primers used for qRT-PCR. Three technical replicates were averaged per genotype; standard errors are indicated. The nuclear 18S rRNA gene was used for data normalization.

Promoter	Promoter sequence	
<i>Prrn18-156</i>	AATAATAC CGT <u>TATATA</u> AATC	*
<i>Porf291-307</i>	AATAATAC CGT <u>TATATA</u> AATC	*
<i>Patp6-1-200</i>	AATAATAC CGT <u>TATATA</u> AAGA	*
<i>Patp9-295</i>	GTGCTCT CGT <u>TATATA</u> AAGA	*
<i>Patp1-1947</i>	GTGGTAT CGT <u>TATATA</u> AAGA	*
<i>Pcox2-210</i>	TTGGTTT CGT <u>TATATA</u> AAGA	*
PccmC-487	CGATCGG CGTA <u>GAA</u> AAG	
Pcox1-88	TCAAGAACT CGT <u>AGACTA</u>	
Pnad1e4-838	AATTTAT CGT <u>ATAGA</u> AAGA	
Pnad6-143	GAGATT CGT <u>AAGTAA</u> CT	
<i>PccmFc-379</i>	GATTAT CGT <u>AGAATA</u> AAGA	
PccmC-45	TGATT CGT <u>ATTAT</u> AGAT	
Pnad2e1-299	GATATT CGT <u>ATAAA</u> TAT	
<i>Prrn26-893</i>	TCAATTT CATA <u>AGAGA</u> AAG	*
<i>Patp1-1898</i>	TCAATTT CATA <u>AGAGA</u> AAG	*
<i>Patp9-239</i>	TCAATTT CATA <u>AGAGA</u> AAG	*
<i>Patp6-1-156</i>	TCAATCT CATA <u>AGAGA</u> AAG	*
<i>Patp6-2-148</i>	TCAATCT CATA <u>AGAGA</u> AAG	*
<i>Patp8-157</i>	TCAATCT CATA <u>AGAGA</u> AAG	*
<i>Prrn18-69</i>	GGAATT GAATA <u>AGAGA</u> AAG	
<i>Patp8-999</i>	AAATTA AAATA <u>AAAGAG</u> CAA	
<i>Patp8-228/226</i>	ACCATA ACATA <u>TATAT</u> AGAA	
<i>Prrn18-353</i>	TTTTCCAT TCT <u>TATATA</u> AAA	
Pnad1e4-275	AAAAACAT TCT <u>ATAG</u> GAAA	
Pnad1e4-775	CTGCTAT TCTA <u>AGAGA</u> AAGA	
<i>Patp6-1-916/913</i>	CCTTTAT ATT <u>TATATA</u> AATA	
Pcox1-355	TTATTCA ATT <u>TATATA</u> AATA	
<i>Pcox2-481</i>	AATATTC ATT <u>AGATA</u> AATA	
<i>Prps3-1133</i>	AAAAAAT ATT <u>AGTA</u> AATA	
<i>PccmFc-743</i>	ATTATTC ATT <u>ATAAT</u> AG	
Pnad2e1-114	TATC ATT <u>ATATTA</u> TATAT	
Pnad1e5-427	A ATT <u>TATATATATA</u> AATA	
Pnad6-333	G ATT <u>ATGTT</u> GAAA AAGAT	
PccmC-1817	TAATTC ATT <u>ATAAGAT</u> A	
<i>Patp9-652</i>	AGATT GAAGTA <u>AGGAG</u> CA	
<i>Patp6-2-436</i>	TGAATTA AGT <u>ATATA</u> AGAA	
Pnad9-1241	GAAA TAT AGTA <u>AAAT</u> TCG	
Prps4-477	AAGCTCA AGT <u>ATGT</u> AAAC	
<i>Patp6-2-507</i>	AAATTA AGT <u>ATAGTA</u> AATA	
Pnad6-401	GTAC AGT <u>AGTAGG</u> TAAAC	
Pnad9-1730	TTGATA AAAGGTA <u>ATAG</u> AT	
<i>Prrn18-424</i>	AATCCTC GGT <u>ATATA</u> AAAG	
<i>Prps3-1053</i>	TTTATTT GGT <u>AGGTA</u> ACA	
Prps4-1509	TAGGTC AGGTA <u>TATATA</u> TAG	
PccmC-1159	TCGTCAT GGT <u>AGAGA</u> AAGA	
<i>Patp9-487</i>	TCTTATT GGT <u>ATGT</u> GATA	
<i>Pcox2-683</i>	ACGTAA GGT <u>AAAATA</u> AAGA	
<i>Patp8-710</i>	GGAGCTGCCAATAAGCTA	
Pnad1e5-927	ATATTGTA AAATCA <u>AGA</u> AAG	
PccmC-1677	CCAAACGTGCTA ATA AAA	
PccmC-1834	CTACTATATAAG ATA AGT	
Pnad2e1-413	CAAAAGATG TAAT GAAA	
Pnad6-88	GAAA AT GAA TAGGAACA	
Pnad9-1371	GATCCACGAATTT GAT CA	

Figure 9. Promoter Sequences in Arabidopsis Mitochondria.

The list includes promoters identified previously (Kühn et al., 2005) and in this study; sequences have been sorted according to the presence of a

DISCUSSION

Investigations of mitochondrial transcription in higher plants have found this process not to be stringently controlled (Holec et al., 2008). Transcription initiates from multiple sites that are not confined to the upstream regions of annotated mitochondrial genes, continuously giving rise to presumably nonfunctional RNAs and occasionally even detrimental transcripts that are usually efficiently removed by RNA-degrading mechanisms in the organelle (Finnegan and Brown, 1990; Holec et al., 2006). Posttranscriptional processes have been identified as a major determinant of mitochondrial RNA pools, as abundances of individual transcripts do not always reflect transcript synthesis rates (Giegé et al., 2000). Transcriptional mechanisms in mitochondria are thought to provide no significant means of regulating mitochondrial function. It is therefore surprising that in the mitochondria of eudicotyledonous plants, this basic process involves two RNA polymerases, RPOTm and RPOTmp, whose distinct roles have not yet been resolved. While indications of RPOTmp-dependent transcription in plastids have been reported (Courtois et al., 2007; Swiatecka-Hagenbruch et al., 2008), a transcriptional role for RPOTmp in mitochondria has been lacking experimental proof (Baba et al., 2004; Kühn et al., 2007). Our study substantiates and characterizes the function of RPOTmp in mitochondrial RNA synthesis and links this enzyme to a distinct, gene-specific transcriptional mechanism. We demonstrate that while RPOTm is vital for plant development, RPOTmp is required to optimally transcribe a subset of mitochondrial genes that, unexpectedly, are not defined by common promoter sequences.

RPOTmp Is Required for the Transcription of Distinct Mitochondrial Genes

Mitochondrial transcription and steady state transcript abundances were found to be severely altered in the absence of RPOTmp (Figures 2 and 4), establishing a fundamental role for this enzyme in RNA synthesis in mitochondria. The altered transcript levels are unlikely to be the secondary result of an unknown primary defect leading to reduced Complex I and Complex IV levels because the mitochondrial transcriptome profile of *rpoTmp* is very different from that of the *otp43* mutant lacking Complex I due to defective *nad1* splicing (comparison to unpublished data by A. Falcon de Longevialle; see Supplemental Figure 18 online). In particular, *nad6*, *rps4*, and *cox1* transcript levels are not reduced in *otp43*. As the transcript changes observed in *rpoTmp* are gene specific, with distinct transcripts showing reduced levels but others exhibiting unaltered or elevated accumulation, RPOTmp function must be considered gene

CATA, CGTA, TCTA, ATTA, AGTA, or GGTA tetranucleotide (bold black) immediately upstream of the TSS (underlined). The motif TATATA, which is recurring in mitochondrial promoters, is highlighted. The names of promoters of genes showing reduced transcription in *rpoTmp* mutants are printed red. The tetranucleotide GAAA, which is overrepresented in this subset of promoters, is printed green. Promoters displaying a perfect CGTATATAA or CATAAGAA motif are marked with an asterisk.

specific. The differences between *rpoTmp* mutant and wild-type mitochondrial transcript profiles are stable from the seedling to the rosette stage. The requirement for this enzyme corresponds with its expression throughout the *Arabidopsis* life cycle, primarily in tissues with high mitochondrial activity (Emanuel et al., 2006). Remarkably, transcriptional defects incurred by the absence of RPOTmp cannot be compensated for by subsequent mechanisms of gene expression, such as transcript stabilization or enhanced translation. This is manifested by the reduced levels of respiratory chain complexes I and IV in *rpoTmp* mutants (Figure 5) that are probably the result of diminished amounts of the *nad6* and *cox1* transcripts. Complex I function is not essential in *Arabidopsis*, but its absence leads to developmental defects (Falcon de Longevialle et al., 2007). Complex IV, on the other hand, is vital for plant development (Gu et al., 1994). The importance of RPOTmp for the expression of principal mitochondrial components adds substantially to present views of the contribution of transcriptional processes to steady state RNA pools and protein synthesis in plant mitochondria.

Our data are compatible with either a role of RPOTmp in transcribing several mitochondrial genes or, alternatively, a role in stimulating the transcription of these genes by RPOTm. Mitochondrial promoter specificity of recombinant RPOTm but not RPOTmp has been detected in a minimal *in vitro* transcription assay; however, RNA polymerase activity has been demonstrated for both (Kühn et al., 2007). Because of the latter finding, we are in favor of RPOTmp being an RNA polymerase transcribing specific mitochondrial genes, including *nad6* and *cox1* and thus supplementing the transcriptional role performed by RPOTm.

The accumulation of numerous mitochondrial transcripts was enhanced in the absence of RPOTmp. Although stimulation of RPOTm expression and/or activity in *rpoTmp* mutants could account for this, we would mostly attribute it to elevated cellular mitochondrial (mt) DNA levels in mutants. A two- to threefold increase in gene copy number was seen for all mitochondrial genes tested (Figure 8). The increase in abundance of a transcript usually did not exceed the increase in gene copy number per cell (cf. Figures 2 and 8). Interestingly, enhanced mitochondrial transcript levels as a consequence of elevated mitochondrial gene copy numbers have been observed in the barley (*Hordeum vulgare*) mutant *albostrians* with impaired chloroplast function (Hedtke et al., 1999a). Therefore, plants may respond in general to energy constraints caused by defects in the energy-converting organelles by increasing cellular mtDNA levels. Elevated mtDNA levels per nuclear genome could be due to either increased mtDNA copy numbers per mitochondrion or an increased number of mitochondria per cell.

Mitochondrial transcript profiles in *rpoTmp* appear to be the combined result of elevated cellular mtDNA levels and reduced transcription of specific mitochondrial genes, with the extent of reduction in RNA synthesis varying between those genes. Therefore, transcripts with unchanged or mildly elevated abundance in mutants (*rps3* and *atp6-1*) might still be made at lower levels per gene copy in the absence of RPOTmp. The synthesis of those RNAs would mainly be performed by RPOTm and depend on RPOTmp to a minor extent. In the cases of *nad6* and *cox1*, the transcription per gene copy is likely to be even more reduced

than measured, while transcription of *ccmF_c* appears to not require RPOTmp.

The *rps4* gene coding for a ribosomal protein is among the genes showing reduced transcription and steady state transcript levels in the absence of RPOTmp. Mitochondrial protein analyses showed no evidence for mitochondrial translation being inhibited due to the reduced availability of the *rps4* mRNA (Figure 5). Consequently, *rps4* mRNA abundance appears not to be limiting for activity of mitochondrial ribosomes. The functionality of the mitochondrial *rps4* gene in *Arabidopsis* has recently been questioned because the 5'-processed form of the *rps4* mRNA lacks the first nucleotide of the annotated start codon (Forner et al., 2007). The absence of a detectable effect on mitochondrial translation in *rpoTmp* contrasts with the reduced levels of respiratory chain complexes I and IV, which we attribute to the limited availability of *nad6* and *cox1* mRNAs in the mutants. The *nad6* and *cox1* mRNAs were significantly more affected in mutants than the *rps4* mRNA (Figure 3).

An earlier study of RPOTmp-deficient plants found all analyzed mitochondrial transcripts, which included *matR* and *cox1*, unchanged (Baba et al., 2004). Baba et al. studied the effect of RPOTmp loss on the light-induced accumulation of organelle transcripts and conducted their analysis of mitochondrial RNAs on seedlings grown entirely in the dark or seedlings transferred into continuous light for 6 d following the dark growth period. The discrepancies between our mitochondrial transcript analysis in *rpoTmp* mutants and the earlier report could be due to the different growth conditions and/or the analysis of transcripts in seedlings of different ages. RPOTmp may not be detectably active in wild-type mitochondria in the conditions applied in the earlier study. Alternatively, the different technical approach taken by Baba et al., who studied mitochondrial transcript abundances by hybridizing labeled cDNAs to macroarrays, could account for the different findings.

RPOTmp Activity Is Gene Specific Rather Than Promoter Specific

Although RPOTmp, unlike RPOTm, was not shown to specifically initiate transcription from mitochondrial promoters *in vitro* (Kühn et al., 2007), our data suggest that RPOTm and RPOTmp transcribe from overlapping sets of promoters. Mitochondrial transcription in RPOTmp-deficient plants initiates essentially from the same promoters as in wild-type plants. All 5' ends of a transcript reduced in abundance in mutants appear diminished in comparison to the wild type; hence, transcription from all promoters of a gene transcribed less actively in *rpoTmp* appears to be reduced. RPOTmp activity is therefore gene specific rather than promoter specific.

For eight 5' ends considered in this study as primary 5' ends because they map to promoter-like sequences, experimental evidence for the termini being primary rather than processed is insufficient (see Supplemental Figures 7, 9, 11, 13, and 14 online). The experimental identification of a primary transcript 5' ends depends on the presence of its 5'-triphosphate. Considering the poor stability of triphosphates at several known primary transcript 5' ends in mitochondria (Kühn et al., 2005), the lack of distinguishable 5' triphosphates at 5' termini does not

preclude them from mapping to promoters. For 19 out of 27 promoters characterized in this study, the analysis of 5'-RACE products was nevertheless sufficient to validate them as TSSs.

The neighboring genomic positions of genes showing reduced transcription in *rpoTmp* raise the question if some of these genes, such as *rps4*, *nade1_e2*, and *nad6*, could be transcriptionally linked. According to the results of our transcript analyses, cotranscription can only partly account for reduced transcript levels of adjacent genes. While *nad2e1_e2* appears to be partly cotranscribed with *rps4*, promoters are active in the *rps4-nad2e1_e2* intergenic region from which transcription also seemed reduced in *rpoTmp* (Figure 7). We have found no indication of cotranscription of *nad2e1_e2* and *nad6*, either because 5' end processing of the *nad6* mRNA is extremely efficient or because there is no cotranscription. Transcription from the promoters *Pnad6-88* and *Pnad6-143* immediately upstream of the *nad6* coding region is also reduced (Figure 7). While *matR* is mostly, if not exclusively, cotranscribed with *nad1e4*, *nad1e5* is at least partly transcribed from promoters immediately upstream of *nad1e5*; a transcript whose 5' end maps to one of these promoters is also reduced (Figure 6; see Supplemental Figures 3 and 8 online). Altogether, we found no evidence that reduced transcript levels of clustered genes in mutants were entirely due to the reduction in a polycistronic message spanning all genes; rather, transcription from all promoters in the examined region appeared to be reduced.

The analysis of promoters and putative promoters of genes transcribed less actively in *rpoTmp* and their comparison to promoters of other mitochondrial genes did not identify RPOTmp-specific promoter sequence motifs. We noticed that none of the genes possessing in their upstream region a perfect CATAGAGAA or CGTATATAA promoter motif showed reduced mitochondrial transcript levels in *rpoTmp*, which can be interpreted as a link between these promoters and RPOTm function. However, because several genes exhibiting unaltered or elevated RNA levels in *rpoTmp* have none of the cited motifs in their upstream region, these promoters could only represent a minor portion of promoters used by RPOTm. *ccmFc* does not seem to require RPOTmp for its transcription, yet its promoter sequences are not noticeably different from those of genes with decreased RNA abundances in *rpoTmp*.

Promoter utilization in *rpoTmp* mutants indicates that there are no RPOTmp-specific promoter sequences in *Arabidopsis* mitochondria. Instead, additional gene-specific elements distinct from the known promoters are likely to direct the transcription of specific mitochondrial genes by RPOTmp. These elements may act through RPOTmp-specific *trans*-factors. The identification of the proposed factors may be aided by the knowledge of genes transcribed by RPOTmp. The known promoters, rather than functioning as elements controlling transcription through recruitment of a specific RNA polymerase, may simply function as sites at which the double helix is easily opened to initiate transcription. Although mitochondrial promoter selection has been reported to be controlled by nucleus-encoded factors in alloplasmic lines of *Nicotiana tabacum* and maize (*Zea mays*; Newton et al., 1995; Edqvist and Bergman, 2002), these factors and their possible roles in regulating mitochondrial function remain to be identified. Due to frequent rearrangements of

mitochondrial sequences, the mtDNAs of dicotyledonous plant species show occasional substitutions of promoters between different mitochondrial genes (Unsold et al., 1997; Kubo et al., 2000; Handa, 2003; Forner et al., 2008). Given that promoters seem to be interchangeable between genes and that RPOTmp function is gene specific rather than promoter specific, we speculate that the transcription of mitochondrial genes may partly be controlled through elements within the transcribed regions rather than upstream of the TSS.

From comparing analyses of mitochondrial transcripts (this study) and of plastidial transcripts (Baba et al., 2004; Courtois et al., 2007) in mutants lacking RPOTmp, the dual-targeted RNA polymerase is likely to have dissimilar roles in the two organelles. The presence of three transcriptases in plastids of eudicotyledonous plants (Liere and Börner, 2007) complicates individual functional studies of RPOTmp in this organelle. In *Arabidopsis* plastids, the *rrn16* Pc promoter is highly RPOTmp specific and nearly inactive in the absence of RPOTmp (Courtois et al., 2007). In addition to the Pc promoter, RPOTmp is capable of initiating transcription from several other plastidial promoters (Swiatecka-Hagenbruch et al., 2008). RPOTmp in plastids has been reported to be immobilized and inactivated following germination (Azevedo et al., 2008). This is in agreement with the developmental variation of the Pc promoter activity in *Arabidopsis* and with our plastidial transcript screen, which showed no differences between wild-type and mutant transcript levels in plastids of 7-d-old plantlets (see Supplemental Figure 2 online). The apparent developmentally limited importance of RPOTmp in plastids contrasts with the requirement of this enzyme for the expression of mitochondrial genes in seedlings as well as in adult plants.

Roles of RPOTmp- and RPOTm-Dependent Transcriptional Mechanisms

Our study confirms that RPOTm functions as the basic RNA polymerase in mitochondria. It is unconditionally required for the transcription of most, if not all, mitochondrial genes. RPOTmp fine-tunes this basic level of transcription by enhancing the levels of specific mitochondrial RNAs. RPOTmp-dependent RNA synthesis may present a transcriptional mechanism allowing mitochondria to independently control the expression of a subset of mitochondrial genes. A challenge for future research into RPOTmp function lies in exploring the conditions where such a mechanism might operate. It could have evolved as a control on the abundances of respiratory chain complexes I and IV, thereby enabling mitochondria to fine-tune the electron flow through these complexes in response to the developmental or metabolic requirements of the organelle and the cell.

METHODS

Plant Material and Culture Conditions

The T-DNA insertion lines SALK_132842 (*rpoTmp-2*) and SALK_086115 (*rpoTmp-3*) were obtained from the ABRC seed stock center; line GABI_286E07 (*rpoTmp-1*) was acquired from GABI-Kat (Rosso et al., 2003). The *Arabidopsis thaliana* (ecotype Col) T-DNA mutants and wild-type plants were germinated on half-strength Gamborg B5 agar

containing 1% sucrose at 22°C and a light intensity of 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ in a 16-h photoperiod. Seedlings for DNA and RNA isolation were harvested after 7 d; plants were further grown on soil (two parts Scotts Shamrock growing medium and one part vermiculite) under identical conditions. For the isolation of RNA and mitochondria from rosettes, mutant and wild-type plants were germinated on soil at 22°C and a light intensity of 80 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ in a 16-h photoperiod. After 10 d, plants were grown further in an 8-h photoperiod; plant tissue was harvested from 7-week-old (wild type) and 9-week-old plants (mutants).

DNA and RNA Isolation and RT-PCR Analysis

Genomic DNA isolated from seedlings as described by Edwards et al. (1991) was used for PCR analysis of *rpoTmp* alleles with *Taq* DNA Polymerase (Qiagen; see Supplemental Figure 16 online for primer information and PCR results). RNA was isolated from *rpoTmp-1*, *rpoTmp-2*, *rpoTmp-3*, and Col-0 seedlings grown on agar in a 16-h photoperiod for 7 d and from rosette leaves of plants grown on soil in an 8-h photoperiod for 9 weeks (*rpoTmp-1* and *rpoTmp-2*) or 7 weeks (Col-0); this difference in age was necessary to obtain plants at a similar developmental stage. The plant RNeasy extraction kit (Qiagen) was used for RNA isolation according to the manufacturer's instructions. The RNA was then subjected to two consecutive treatments with TURBO DNase (Ambion) and confirmed by PCR to be free of detectable amounts of DNA. Three micrograms of RNA were reverse-transcribed with Superscript III (Invitrogen) according to the manufacturer's protocol using random hexamers (Invitrogen). One microliter of the 25-fold diluted reverse transcription reaction was used for PCR with *Taq* DNA Polymerase (Qiagen; see Supplemental Figure 3 online for primer information and RT-PCR results).

Quantitative RT-PCR Analysis

RNA was isolated from seedlings and rosette leaves and reverse-transcribed as detailed above. Quantitative RT-PCR was performed in 384-well plates using the LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics) and a LightCycler 480 Roche real-time PCR system as described previously (Falcon de Longevialle et al., 2008); primer sequences are listed in Supplemental Table 2 online. Primer pairs specific for the nuclear *18S* rRNA gene and the nuclear *ACT2* and *ACT8* genes were used to measure the accumulation of nucleus-encoded transcripts. The specificity of each primer pair was tested by sequencing the PCR product and subsequently by melting curve analysis. For each primer pair and each comparison between mutant and wild type, a standard curve based on serial dilutions of the wild-type cDNA was included. The accumulation of each transcript was analyzed in triplicate. Values displayed in Figure 2 represent means of triplicates; the transcript analysis was done on mutant lines *rpoTmp-1*, *rpoTmp-2*, and *rpoTmp-3* representing three independent biological replicates. The raw data were analyzed using the LightCycler 480 software release 1.5 (Roche Diagnostics) and crossing points determined by second derivative maximum analysis. The accumulation of mitochondrial transcripts was normalized by setting the average ratio of nuclear transcripts to 1.

RNA Gel Blot Analysis

Total RNA extracted from *Arabidopsis* seedlings was resolved on 1.2% agarose-formaldehyde gels and transferred onto Hybond N⁺ nylon membranes (GE Healthcare). Hybridizations with biotinylated oligonucleotides or in vitro-biotinylated complementary RNA (cRNA) probes were performed as detailed by Falcon de Longevialle et al. (2008). The Pierce chemiluminescent nucleic acid detection module kit (Thermo Scientific) was used for cRNA probe detection; an ImageQuant-RT ECL system (GE Healthcare) was employed for chemiluminescence imaging.

To generate cRNA probe templates for *nad2*, *nad6*, *cox1*, and *ccmFc*, fragments of mitochondrial genes were amplified from total *Arabidopsis* DNA using Phusion Hot Start high-fidelity DNA polymerase (Finnzymes) with primer pairs *rps4-t7-R* (5'-taatacagctactataggATTCTCTGTGCTCAGCGAAGGA-3') and *rps4-t7-F* (5'-CAAGCAAGGCAGCCGATAAGTC-3'), *nad2-t7-R* (5'-taatacagctactataggCTTCCGTGGAAAATT-CAGACT-3') and *nad2-t7-F* (5'-TGCGGCACCTCTCCTAACTATTG-3'), *nad6-t7-R* (5'-taatacagctactataggAGCATTTCTCGGAATACATCCTG-3') and *nad6-t7-F* (5'-CGTTTTGTTCCATCCAGTCT-), *cox1-t7-R* (5'-taatacagctactataggATACCGAATCCAGCAGAATGAG-3') and *cox1-t7-F* (5'-GTAGGTAGCGGCACTGGGTG-3'), *ccmC-t7-R* (5'-taatacagctactataggAAATAGAAGCCGGTTCGACAGG-3') and *ccmC-t7-F* (5'-TTGTTGTTCTTAACAGCGATGG), *nad3-t7-R* (5'-taatacagctactataggACAGATCAATCTTGTGGGAGGTAC-3') and *nad3-t7-F* (5'-CGCATAAGGTCTGCAAGCCT-3'), and *ccmFc-t7-R* (5'-taatacagctactataggCGCTTCGCTGACCTATCGC-3') and *ccmFc-t7-F* (5'-ACTATTGAAATGGTTCGTACGTAGAGATG-3'). Lowercase nucleotides in primer sequences represent the T7 promoter sequence. The template for transcribing the *rrn18* probe was amplified with primers *rrn18-F* (5'-CAGCTCGTGTGCTGAGATG-3') and *rrn18-R* (5'-CCCACCTTCCCTCCAGTATATCA-3'), ligated in the appropriate orientation into pDrive (Qiagen), and reamplified with *rrn18-R* and the M13R universal primer to generate a template with an attached T7 promoter. The PCR products were used as templates for synthesizing biotinylated cRNA probes using biotin-CTP (Invitrogen) and the Ambion MAXIScript kit (Applied Biosystems) according to the manufacturer's instructions.

5'-RACE Analysis of Mitochondrial Transcripts

Primary ends of mitochondrial transcripts carry 5' triphosphates that have to be converted to monophosphates by tobacco acid pyrophosphatase (TAP) in order to be detected by 5'-RACE; amplification of processed ends requires no such modification. 5'-RACE will yield products from TAP-treated RNA for both primary and processed transcripts, whereas without exposure to TAP, products resulting from primary transcript termini will be significantly reduced or absent. The comparison of 5'-RACE products obtained from TAP-treated and untreated RNA will thus identify primary transcripts after sequencing of cloned 5'-RACE products (Table 1).

RNA was subjected to TAP treatment and 5'-RACE as described previously (Kühn et al., 2005). Five micrograms of RNA were incubated with 10 units of TAP (Epicentre Technologies), extracted, and subsequently ligated to an RNA oligonucleotide 5'-adapter using T4 RNA ligase (Epicentre Technologies). Transcripts were then reverse transcribed using gene-specific primers (see Supplemental Table 3 online) and Superscript III (Invitrogen). Adapter primer P1a (5'-CGAATTCCTGTAGAACAACAC-TAGAAG-3') served as forward primer in PCR reactions; a list of all gene-specific reverse primers is provided in Supplemental Table 3 online. Sequencing of cloned 5'-RACE products was performed by Macrogen.

Ribonuclease Protection Assay

To generate cRNA probe templates, fragments of mitochondrial genes were amplified from total *Arabidopsis* DNA using *Taq* DNA polymerase (Qiagen) with primer pairs *RPA-nad1e4-F* (5'-AGATTCGGCAGACGAA-GACCAAG-3') and *RPA-nad1e4-R* (5'-AGCTCGCAGAGCATAAGT-CAGC-3'), *RPA-nad2e1-F1* (5'-CATTTTTTTATTGAGCCGAATCACT-3') and *RPA-nad2e1-R1* (5'-CATGAATGAGCAAAATGGAGGT-3'), *RPA-nad2e1-F* (5'-CCTAGTCCGGGTGGGTGG-3') and *P5-nad2e1* (5'-GAA-AAGAAAGGGCGGAATAGCA-3'), *RPA-nad6-F* (5'-CTCGCTACCATAC-CACAAGGC-3') and *P4-nad6* (5'-GACCAAAGCAGGGCTCGAC-3'), *RPA-cox1-F1* (5'-CGAGGAAGAAAGTTTTGATCTGTG-3') and *P4-cox1-b* (5'-GAGTTCTTGAGAGTCCCGTG-3'), *RPA-matR-F* (5'- CCAAAA-GACTGAACTGAGGGAAG-3') and *P3-matR* (5'-CGAAATGCGATGTG-TCTGG-3'), and *RPA-atp6-1-F* (5'-CACCGCACGAGTCAGACCT-3') and

RPA-atp6-1-R (5'-TCCAGACAGCTTCACTCCGTC-3') and ligated into pDrive (Qiagen) in the appropriate orientation. Biotinylated cRNA probes were made from these plasmids through *in vitro* transcription in the presence of biotin-CTP (Invitrogen) using the Ambion MAXIscript kit (Applied Biosystems) according to the manufacturer's instructions. Transcripts were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1), ethanol-precipitated from the aqueous phase, and dissolved in ultrapure water.

Between 20 and 40 μ g of seedling RNA and 5 ng of biotinylated cRNA probe were subjected to ribonuclease protection using the Ambion RPA III ribonuclease protection assay kit (Applied Biosystems) according to the manufacturer's protocol, except that hybridizations were performed overnight at 55 to 65°C. Protected transcripts were separated in 5% urea-polyacrylamide gels and transferred onto Hybond N⁺ nylon membranes (GE Healthcare). Signal detection was performed as described for RNA gel blots.

Isolation of Mitochondria from Arabidopsis Plants

The aerial parts of plants were ground at 4°C in extraction buffer (0.3 M sucrose, 5 mM tetrasodium pyrophosphate, 10 mM KH₂PO₄, pH 7.5, 2 mM EDTA, 1% [w/v] PVP40, 1% [w/v] BSA, 5 mM cysteine, and 1 mM DTT) using a Polytron (30 mL of buffer for 10 g of plants, speed 4). The homogenate was centrifuged 5 min at 3000g and the supernatant centrifuged 10 min at 20,000g. The pellet was resuspended in wash buffer (0.3 M sucrose, 1 mM EGTA, and 10 mM MOPS/KOH, pH 7.2) and subjected to the same low-speed (3000g) and high-speed (20,000g) centrifugations. The pellet was then resuspended in a small volume of wash buffer and loaded on top of a Percoll step gradient (from bottom to top: 1 volume 50%, 5 volumes 25%, and 1 volume 18% Percoll in wash buffer, equivalent to 20 g of plant loaded per gradient). The gradient was centrifuged at 40,000g for 45 min. The mitochondrial fraction located at the interface between the 50 and 25% layers was collected and washed three times in wash buffer before being used in run-on transcription assays or stored as a mitochondrial suspension at -80°C.

Run-On Transcription Assay

Previously described protocols (Giegé et al., 2000; Zoschke et al., 2007) were adapted for use with biotin-CTP. Intact mitochondria (the equivalent of 400 μ g of mitochondrial protein) were disrupted in 100 μ L of reaction mix containing 20 mM Tris, pH 7.6, 40 mM KCl, 10 mM MgCl₂, 1 mM DTT, 0.5 mM of each ATP, GTP, and UTP, 0.3 mM CTP, 1 mM biotin-CTP [Invitrogen], and 40 units ribonuclease inhibitor [Invitrogen] and incubated at 25°C for 10 min. The reactions were terminated by adding 100 μ L of stop solution (50 μ g/mL sodium lauroylsarcosine, 50 mM Tris, pH 8.0, and 25 mM EDTA), and transcripts were extracted twice with phenol/chloroform 5:1, pH ~4.5 (Sigma-Aldrich), once with chloroform/isoamyl alcohol 24:1, ethanol-precipitated, and resuspended in 100 μ L of ultrapure water. Transcripts were hybridized overnight in 250 mM NaH₂PO₄, pH 7.2, 7% (w/v) SDS, and 2.5 mM EDTA at 58°C to DNA probes (500 ng) dot-blotted in duplicates onto Hybond N⁺ nylon membranes (GE Healthcare). A slot blotting apparatus (MilliBlot-S; Millipore) was used for filter preparation. The experiment was repeated four times per genotype with independently labeled RNA obtained from two separate mitochondrial preparations per genotype. Signal detection was performed as described for RNA gel blots. Signals were analyzed using the ImageQuantTL 1D gel analysis software (Amersham Biosciences). The background signal measured for the dot-blotted nonspecific pDrive plasmid sequence was subtracted from each gene-specific signal.

To generate DNA probes, mitochondrial DNA fragments were amplified with Taq DNA polymerase (Qiagen) using gene-specific primers (see Supplemental Table 4 online) and ligated into pDrive (Qiagen). From these plasmids, DNA probes were amplified using M13 primers; the vector fragment used as nonspecific probe was amplified from self-ligated pDrive.

BN-PAGE and Immunodetections

BN-PAGE was performed according to the method described by Jänsch et al. (1996). Mitochondrial proteins (100 μ g) were solubilized with dodecylmaltoside (1% [w/v] final) in ACA buffer (750 mM amino caproic acid, 0.5 mM EDTA, and 50 mM Tris-HCl, pH 7.0) and incubated 20 min at 4°C. The samples were centrifuged 10 min at 20,000g, and Serva Blue G (0.2% [v/v] final) was added to the supernatant. The samples were loaded onto a 4.5 to 16% gradient gel. After migration, the gel was transferred on a polyvinylidene difluoride membrane (ImmobilonP; Millipore) in Cathode buffer (50 mM Tricine and 15 mM Bis-Tris-HCl, pH 7.0) for 16 h at 4°C. The membrane was stained with a Coomassie Brilliant Blue solution (40% [v/v] methanol, 7% [v/v] acetic acid, and 0.02% [w/v] Coomassie R250) and scanned. After destaining in 100% (v/v) methanol, the membrane was probed with antibodies against ATP1 (gift from T. Elthon, University of Nebraska, Lincoln, NE), COXII (AS04 053A; Agrisera), the Rieske iron-sulfur protein (D. Price, Australian National University, Canberra, Australia), or NAD9 (G. Bonnard, Institut de Biologie Moléculaire des Plantes, Strasbourg, France). A secondary antibody linked to horseradish peroxidase (Sigma-Aldrich) was used, and signals were detected by chemiluminescence (ECL plus; GE Healthcare) and recorded using an ImageQuant-RT ECL system (GE Healthcare). Between immunodetections, antibodies were stripped off the membrane by incubating the membrane at 50°C for 30 min in a solution containing 2% (w/v) SDS and 1% (v/v) β -mercaptoethanol.

Activity Measurements

The activity of complex I and complex IV were measured using a Clark-type oxygen electrode (Hansatech Instrument). A membrane suspension (100 μ g proteins) was incubated in 1 mL of respiration buffer (0.3 M sucrose, 5 mM KH₂PO₄, 10 mM TES, 10 mM NaCl, 2 mM MgSO₄, and 0.1% [w/v] BSA, pH 6.8), and specific substrates were added: 0.5 mM deaminoNADH for complex I or ascorbate (10 mM) and cytochrome c (25 μ M) in the presence of Triton X-100 (0.05% [w/v]) for complex IV. Oxygen consumption rate was monitored and used to calculate specific activities. The specificity of the reactions was controlled by addition of the specific inhibitors rotenone (5 mM) or KCN (1 mM) for complex I and complex IV, respectively.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *RPO1Tm*, At5g15700 (*RPO2*); *RPO2m*, At1g68990 (*RPO1M*); ArthMr003 (*18S*, *rrn18*), ArthMp035 (*atp6-1*), ArthMp079 (*ccmC*, *ccb256*), ArthMp017 (*ccmFc*, *ccb452*), ArthMp109 (*cox1*), ArthMp045 (*matR*), ArthMp044 (*nad1*), ArthMp026 (*nad2*), ArthMp086 (*nad3*), ArthMp006 (*nad5*), ArthMp024 (*nad6*), ArthMp007 (*nad9*), ArthMp085 (*rps12*), and ArthMp027 (*rps4*). T-DNA insertion mutants used were SALK_005875 (allele *rpoTm-1*), GK-350F01 (*rpoTm-2*), GK-286E07 (*rpoTm-1*), SALK_132842 (*rpoTm-2*), and SALK_086115, (*rpoTm-3*).

Supplemental Data

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

Supplemental Figure 1. Lethality of Loss of RPO1M.

Supplemental Figure 2. Plastidial Steady State Transcript Levels in *rpoTm* Mutants.

Supplemental Figure 3. RNA Gel Blot Hybridizations with Probes Specific to Exons *nad1e4*, *nad1e5*, and *nad5e2*.

Supplemental Figure 4. In Organello Protein Synthesis by Mitochondria Isolated from *rpoTm* and Wild-Type Plants.

Supplemental Figure 5. 5'-RACE Analysis of *cox1* Transcripts.

Supplemental Figure 6. 5'-RACE Analysis of *nad1e4* Transcripts.

Supplemental Figure 7. 5'-RACE Analysis of *matR* Transcripts.

Supplemental Figure 8. 5'-RACE Analysis of *nad1e5* Transcripts.

Supplemental Figure 9. 5'-RACE Analysis of *ccmC* Transcripts.

Supplemental Figure 10. 5'-RACE Analysis of *rps4* Transcripts.

Supplemental Figure 11. 5'-RACE Analysis of *nad2e1_e2* Transcripts.

Supplemental Figure 12. 5'-RACE Analysis of *nad6* Transcripts.

Supplemental Figure 13. 5'-RACE Analysis of *ccmFc* Transcripts.

Supplemental Figure 14. 5'-RACE Analysis of *nad9* Transcripts.

Supplemental Figure 15. Neighbor Joining Distance Tree of Mitochondrial Promoter Sequences in *Arabidopsis*.

Supplemental Figure 16. Identification of *rpoTmp* Mutant Lines.

Supplemental Figure 17. Nuclear Genome Ploidy Levels.

Supplemental Figure 18. Mitochondrial Steady State Transcript Levels in the *Arabidopsis otp43* Mutant.

Supplemental Table 1. Sequence Motifs Significantly Overrepresented Among Promoters of Genes Showing Reduced Transcription in *rpoTmp*.

Supplemental Table 2. Primers Used for Quantitative RT-PCR Analysis.

Supplemental Table 3. Primers Used for 5'-RACE.

Supplemental Table 4. Gene-Specific Primers Used to Amplify DNA Probes for the Detection of Mitochondrial Run-On Transcripts.

Supplemental Data Set. Promoter Sequence Alignment File Used to Calculate the Distance Tree of Mitochondrial Promoters Shown in Supplemental Figure 15.

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