*Published online 15 December 2007*

*Nucleic Acids Research, 2008, Vol. 36, No. 3* ***785–792***
*doi:10.1093/nar/gkm1111*

# Impaired function of the phage-type RNA polymerase RpoTp in transcription of chloroplast genes is compensated by a second phage-type RNA polymerase

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Received September 26, 2007; Revised November 12, 2007; Accepted November 28, 2007

## ABSTRACT

**Although chloroplast genomes are small, the transcriptional machinery is very complex in plastids of higher plants. Plastidial genes of higher plants are transcribed by plastid-encoded (PEP) and nuclear-encoded RNA polymerases (NEP). The nuclear genome of *Arabidopsis* contains two candidate genes for NEP, *RpoTp* and *RpoTmp*, both coding for phage-type RNA polymerases. We have analyzed the use of PEP and NEP promoters in transgenic *Arabidopsis* lines with altered RpoTp activities and in *Arabidopsis RpoTp* insertion mutants lacking functional RpoTp. Low or lacking RpoTp activity resulted in an albino phenotype of the seedlings, which normalized later in development. Differences in promoter usage between wild type and plants with altered RpoTp activity were also most obvious early in development. Nearly all NEP promoters were used in plants with low or lacking RpoTp activity, though certain promoters showed reduced or even increased usage. The strong NEP promoter of the essential *ycf1* gene, however, was not used in mutant seedlings lacking RpoTp activity. Our data provide evidence for NEP being represented by two phage-type RNA polymerases (RpoTp and RpoTmp) that have overlapping as well as gene-specific functions in the transcription of plastidial genes.**

## INTRODUCTION

Chloroplasts (plastids) are derived from free-living cyanobacteria that were engulfed as endosymbionts by a eukaryotic host cell, the ancestor of algae and plants. During chloroplast evolution, most genes of the ancestral cyanobacterial genome have been lost or transferred into the nucleus leaving a plastidial genome (plastome) that, in case of higher plants, harbors only about 120 genes for products that function primarily in photosynthesis and gene expression (1). Chloroplast genes coding for components of the plastid translation machinery are essential for tobacco and *Arabidopsis* since lack of plastid protein synthesis leads to embryo lethality (2–4). In striking contrast, lack of the plastid encoded RNA polymerase [PEP, the core subunits of which are coded for the plastid *rpoA*, *rpoB*, *rpoC1* and *C2* genes; (5,6)], although resulting in an albino phenotype and impaired photosynthesis, still allows for heterotrophic growth of tobacco plants (7,8). The comparatively mild effects of the loss of PEP activity are explained by the existence of a second, plastid-localized, nuclear-encoded transcription activity (NEP, nuclear-encoded plastid RNA polymerase) that supplements PEP to fully transcribe the genes encoded in the plastome (6,9). The plastid NEP transcription activity is proposed to be represented by nuclear-encoded, phage-type RNA polymerases (10–13). Genes encoding organellar phage-type RNA polymerases have been found in several higher plant genomes. Aside from mitochondrial targeting (*RpoTm*), it was shown that a second RpoT enzyme is targeted into plastids both in monocots and dicots [RpoTp; (11,12,14–20)]. Moreover, a third RpoT enzyme found exclusively in dicots (RpoTmp) has been shown to be dually targeted both into mitochondria and plastids of *Arabidopsis* and *Nicotiana* (14,18,21).

Promoters recognized by NEP have been characterized by using plants lacking (7,22,23) or with diminished PEP activity (24–30). Most NEP promoters have a core sequence motif (YRTA; type-Ia), similar to promoters of plant mitochondria (31–35). A subclass of NEP promoters shares a GAA-box motif upstream of the YRTA-motif [type-Ib; (36)]. Type-II NEP promoters, represented by dicot *clpP* promoters, lack these motifs and possess crucial sequences located downstream of the transcription initiation site (32,33,37). Furthermore, the existence

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of additional NEP promoters has been reported for the *rrn* operon in spinach, mustard and *Arabidopsis* (Pc promoter), and for the internal promoters of certain tRNAs (6).

The role of RpoTp and RpoTmp in transcription of plastid genes is not understood yet. Recently, the so-called SCABRA mutants lacking RpoTp activity have been reported to show altered steady-state levels of the NEP-transcribed genes *rpoB*, *clpP* and *accD* (38). Evidence for a role of RpoTp in plastid transcription was provided by overexpression of the RpoTp enzyme in transgenic tobacco plants that revealed enhanced transcription from certain type-I NEP promoters but not from the type-II P*clpP*-53 (the number indicates here and for all other promoters the position of the transcription initiation site with respect to the translation initiation site, +1), an indication of the involvement of RpoTp in transcription from type-I NEP promoters (13). Recognition of a type-I promoter by RpoTp was observed in *in vitro* transcription assays (39). Although a role of RpoTmp in transcription of *Arabidopsis* chloroplast genes has been questioned (40) and this enzyme does not recognize NEP promoters *in vitro* (39), several observations suggest a function of RpoTmp as plastidial RNA polymerase. The analysis of an *AthRpoTmp* homolog of spinach was detected in chloroplasts rather than mitochondria (41). T-DNA insertion mutant provided data, which corroborate a function of RpoTmp in plastid rather than mitochondrial transcription and suggest a role in early plant development (42). Analysis of an *rpoTp*/*rpoTmp* double mutant indicated that both RpoTmp and RpoTp might have redundant functions in plant development (38). However, no information is available yet about effects of the mutations on transcription from NEP promoters, i.e. if RpoTp and/or RpoTmp represent the nuclear-encoded plastid RNA polymerase. Moreover, there is no indication regarding why dicots have two plastid targeted phage-type RNA polymerases and if they transcribe the same set of plastid genes. Furthermore, the question remains, which RNA polymerase is responsible for recognition and transcription from 'non-consensus-type' NEP promoters.

To acquire information on their individual role in plastid transcription, we have studied transgenic *Arabidopsis* plants with lowered activity of RpoTp as well as *Arabidopsis* SCABRA mutants lacking functional RpoTp. We report here on differential effects of the altered RpoTp activities on the usage of NEP promoters. Our data provide evidence for the importance of RpoTp for transcription of a subset of NEP promoters and clearly demonstrate that a second phage-type RNA polymerase (RpoTmp) is involved in chloroplast transcription and might play a major role in transcription from P*clpP*-53 (type-II) and P*rrn*-139 (Pc) NEP promoters.

## MATERIALS AND METHODS

### Plant material

*Arabidopsis thaliana* (ecotype Columbia) was grown on MS medium under an 8 h/16 h light/dark cycle at 20°C. After 14 days the seedlings were exposed to a light regime of 16 h/8 h light/dark. *AthRpoTp* insertion mutants *sca3-2* and *sca3-3*, kindly provided by José Luis Micol, were grown as described by Hricová *et al*. (38).

### Plasmid construction

Plasmid pMS1 was constructed by PCR amplification of the Ath*psbA* promoter sequence from *A. thaliana* using (NC_000932) primers 73 TACATTGGTTGACATGG CTAT (1567) and 74 ATCCAGTTACAGAAGCGACC (1383); pMS3 contains a PCR fragment (77 GAAATC CCATATAGCCCG, 71 774 and 78 CCGGTTAGTCCA TAAGGG, 72 897) comprising the promoter region of Ath*clpP*; pMS4 contains a PCR fragment (81 GATTAAA TCCGGGTATTGC, 26 281 and 82 GAACTAAATTA GTAGTGGCAAGTAAAG, 27 626) comprising the promoter region of Ath*rpoB*, cloned into pGEM-T (Promega).

Plasmid pGPTV-sRpoTp was constructed by PCR amplification of a 2.99 kb PCR *AthRpoTp* fragment (At2g24120) from an *Arabidopsis* cDNA library using primer gggtct agaCCTTCC**ATG**GCTTCC (AS 1) and primer GAC**TCA** GTTGAAGAAGTACTGTGATTTGAG (AS 993) and subsequently cloning it XbaI/SmaI into pGPTV (43). A 3.04 kb *AthRpoTmp* (At5g15700) PCR fragment (gggtcta GATTG**ATG**TCCAGTGC, AS 1; CTTTA**TCA**GTTGA AGAAATAAGGTGAATC, AS 1011) was ligated into the XbaI and SmaI sites of the vector pGPTV resulting in pGPTV-sRpoTmp.

### Transformation of *Arabidopsis*

*Arabidopsis thaliana* (ecotype Columbia) plants were transformed by the floral dip method according to Ref. (44) using *Agrobacterium tumefaciens* strain EHA105 transformed with plasmids pGPTV-sRpoTp and pGPTV-sRpoTmp. Plants were selected on plates containing 30 mg/l phosphinotricine (Sigma). Integration of the transgenes was confirmed by PCR and Southern hybridization (data not shown).

### Northern blot analysis

Total leaf RNA was prepared using TRIzol (Invitrogen) following the manufacture's protocol. Five microgram of total RNA was subjected to electrophoresis on 1% agarose-formaldehyde gels, transferred onto nylon membranes and hybridized with random-primed labeled DNA fragments overnight at 55°C. The DNA fragments were prepared by PCR using the primers 13 CATTCAT TGCTGCTCCTCCAGTA (1293) and 14 GAGCCTC AACAGCAGCTAGGTCT (400) for *psbA* and 17 GCTC TGGTGGTTAAGGGTCGAG (54 021) and 18 GCAG GTGCGGGGTCAGT (53 138) for *atpB*.

### Primer extension analysis

Primer extension reactions were carried out with 5 μg (*psbA*), 10 μg (*clpP*), 15 μg (*atpB*, *rrn16*, *ycf1*) and 20 μg (*rpoB*) of total cotyledon and leaf RNA according to standard protocols (45). Briefly, primers PE*AtpsbA*(97), TCCAGT TACAGAAGCGACCCCATAG (5′ position in the *Arabidopsis* plastid genome at 1384), PE*AtclpP*(104), GGTA CTTTTGGAACGCCAATAGGC (5′ position 71 857),

PE4*AtatpB*(5), CAAGCGGATGTGGAATTCAATTTT (5′ position 54 281), PE3*Atrrn16*(6), CGTAGACAAAG CTGATTCGGAATTG (5′ position 100 979/137 670), P3-*ycf1*(213), GGGCCCTATGGAAAATGTGG (5′ position 109 524/129 125) and PE4*rpoB*(2), TACTGAATC ACATGAAATTTTATCCAACTCC (5′ position 26 445) were end-labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase (Fermentas). Primer extensions were performed using Superscript III MMLV reverse transcriptase (Invitrogen) at 50°C and the resulting products analyzed on 5% sequencing gels. To map transcript 5′-ends, sequence ladders were generated using the same primers as listed above (USB Sequenase 2). Quantification of obtained signals was done for three independent experiments with a PhosphoImaging system using the complementary software (BioRad).

### Mapping of transcription initiation sites

To map the transcription initiation site of *rpoB* in *Arabidopsis*, a PCR-based method was employed (46). In a volume of 100 µl, 5 µg of total RNA, 10 U TAP (tobacco acid pyrophosphatase, Epicentre) and 40 U RNasin (Fermentas) were incubated for 1 h at 37°C (+TAP). As a control, 5 µg of RNA was similarly incubated without TAP (−TAP). After precipitation, 150 ng of RNA linker (CGAAUUCCUGUAGAACGAACACUAGAAGA) was added and ligated with 50 U RNA ligase (Epicentre) for 1 h at 37°C. cDNA was synthesized using primer PE1*rpoB* (CCTCTTTTTCATCCCCAAGCATC; 5′ position 26 308) with 200 U Superscript III MMLV reverse transcriptase (Invitrogen) for 1 h at 55°C. Two microliter of cDNA was used in a first PCR using an *rpoB* gene-specific primer (PE1*rpoB*) and an adaptor primer (TGTAGAACGAACACTAGAAGA), followed by a second PCR with 1/50th of the first PCR as a template, using the adaptor primer and a nested *rpoB* gene-specific primer PE4*rpoB*. The products were analyzed on 1.8% agarose gels and bands prominent in the +TAP samples were isolated and sequenced.

## RESULTS

### Reduced amounts of *AthRpoTp* transcripts lead to delayed plant development

To investigate the role of RpoTp in plant development, we created *Arabidopsis* plants overexpressing *AthRpoTp*. Plants expressing AthRpoTp with 1 transgene copy (Tp1x, Figure 1a) showed no phenotypical differences compared to the wild type. However, the seeds of plants with five copies of the *AthRpoTp* transgene (Tp5x) were germinating into seedlings with white cotyledons, which eventually greened up to wild-type levels after two weeks (Figure 1b). Interestingly, such a phenotype was not observable in tobacco plants overexpressing either *AthRpoTp* or *NsRpoTp* (13). Northern blot analyses of transgene transcript levels in the *AthRpoTp* mutants revealed that different transgene copy numbers not only resulted in different seedling phenotypes but also in different transcript accumulation of the transgene (Figure 1c). Whereas in Tp1x *AthRpoTp* mRNA accumulated to higher levels

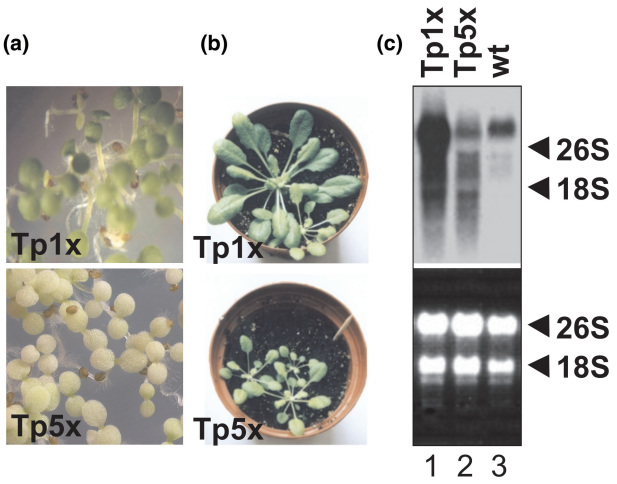


**Figure 1.** Overexpression and partially silencing the native *AthRpoTp* gene by insertion of ectopic *AthRpoTp* gene copies leads to different phenotypes. (**a**) Single copy plants (Tp1x) are not distinguishable from wild-type plants. Multiple copy plants (Tp5x) germinate into white seedlings. The white seedlings will eventually become green reaching wild-type levels after two weeks (**b**). (**c**) In northern blot analyses, 50 µg total RNA of Tp1x (lane 1), Tp5x (lane 2) and wild-type plants (lane 3) was separated in 1% agarose-formaldehyde gels. Ethidium bromide stained gel image is shown as a loading control (bottom panel). The RNA blot was hybridized with a single-stranded full-length *AtRpoTp* antisense DNA probe. Positions of rRNAs are given on the margin. *AtRpoTp* RNA accumulates to highest amounts in overexpressor plants with only one *AtRpoTp* transgene (Tp1x, lane 1). However, in multiple copy plants *AtRpoTp* RNA levels (Tp5x, lane 2) are reduced to lower than wild-type RNA levels (wt, lane 3).

than in the wild type (Figure 1c, lanes 1 and 3), in Tp5x less than wild-type levels of *AthRpoTp* transcripts were observable (lane 2). Both Tp1x and Tp5x *AhtRpoTp* transcripts showed lower molecular degradation products, indicating that the transgene mRNAs were subjected to a faster turnover and might be less stable than the wild-type *AthRpoTp* transcripts. Obviously, high copy numbers of the *AthRpoTp* transgene may lead to delayed greening in early seedling development due to higher *AthRpoTp* transcript turnover and/or gene silencing. We therefore considered Tp5x plants as partially silenced in their *AthRpoTp* gene expression.

To analyze the delayed seedling development in Tp5x plants, we performed northern analysis of the PEP-transcribed *psbA* (Figure 2a) and the *atpB* gene (Figure 2b), which is transcribed by NEP and PEP (30), with total RNA isolated from 6-day-old cotyledons and 3-week-old leaves. In white cotyledons of Tp5x plants, only negligible amounts of *psbA* messages and reduced *atpB* transcript levels were detectable, which returned to wild-type levels in older leaves. To further investigate the effect of reduced *AthRpoTp* expression on *Arabidopsis* PEP and NEP promoter activity in Tp5x plants, we determined transcript levels by primer extension analysis mapping transcript 5′-ends in 4- and 9-day-old cotyledons and 6-week-old leaves from both, transgenic and wild-type plants (Figure 3). Reminiscent to the northern data, the transcript 5′-ends of the P*psbA*-77 PEP promoter (30) accumulated to only ~10–30% of wild-type levels

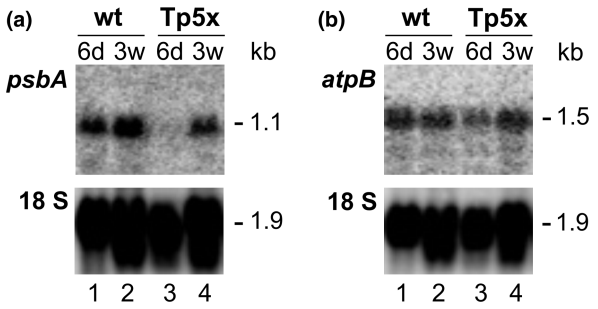


**Figure 2.** Northern blot analysis of *psbA* (**a**) and *atpB* (**b**) transcript levels in wild type and partially silenced Tp5x plants. Five microgram of total RNA of 6-day-old cotyledons of wild type (lanes 1) and Tp5x (lanes 3), and of 3-week-old leaves of wild type (lanes 2) and Tp5x (lanes 4) plants was separated in 1% agarose-formaldehyde gels. The RNA blot was hybridized to the indicated plastid gene sequence (upper panel). The same blot hybridized with an 18S rRNA probe is shown as a loading control (18S, bottom panel). Transcript sizes (kb) are given on the margin.

in 4- and 9-day-old Tp5x cotyledons (see also Figure 4b, lane 6). Similarly, transcript 5′-ends of the type-I NEP promoter of the *rpoB* operon (P*rpoB*-300; 30) were barely detectable in Tp5x cotyledons (see also Figure 4a, lane 6). The *Arabidopsis clpP* gene is transcribed from a weak PEP promoter (P*clpP*-115) and a strong type-II NEP promoter (P*clpP*-58; 30). While P*clpP*-115 followed the same pattern as observed with P*psbA*-77 and P*rpoB*-300 (see also Figure 4d, lane 6), examination of P*clpP*-58 revealed no significant change in its 5′-end accumulation in Tp5x when compared to the wild type (see also Figure 4d, lane 6). Reduced expression of *AthRpoTp* in Tp5x plants may therefore directly affect transcription from type-I, but not from type-II NEP promoters.

### Comparison of PEP and NEP initiated transcript 5′-ends in wild-type and *rpoTp* mutants indicates transcriptional compensation during plant development

To confirm the suggested role of RpoTp in plastid transcription, we compared PEP and NEP initiated transcript 5′-ends in wild-type and *rpoTp* mutants. The *rpoTp* mutants *sca3-2* and *sca3-3* displayed pale-green cotyledons and leaves as described by Hricová *et al.* (38). Compared to the wild type, they were retarded both in growth and development, i.e. 4-day-old *rpoTp* seedlings developmentally equal 2-day-old wild-type seedlings. We therefore chose these developmental stages to analyze the accumulation of transcript 5′-ends of plastid genes transcribed from a PEP promoter (*psbA*), a consensus type (type-I) NEP promoter (*rpoB*), by both PEP and type-I NEP promoters (*atpB*, *ycf1*), and by both PEP and 'non-consensus-type' NEP promoters (*clpP*, *rrn16*; 30). To compare the effects of the reduction with the loss of AthRpoTp, we additionally examined the accumulation of these transcript 5′-ends in 4-day-old Tp1x and Tp5x plants (Figures 2 and 3). Unlike Tp5x plants, *rpoTp* mutants showed generally no reduction but rather enhanced accumulation of PEP initiated transcript 5′-ends from P*psbA*-77, P*atpB*-520 (Figure 4b and c, lanes 2 and 3), P*rrn16*-112, and P*ycf1*-34/33 (Figure 5a and c, lanes 2 and 3).

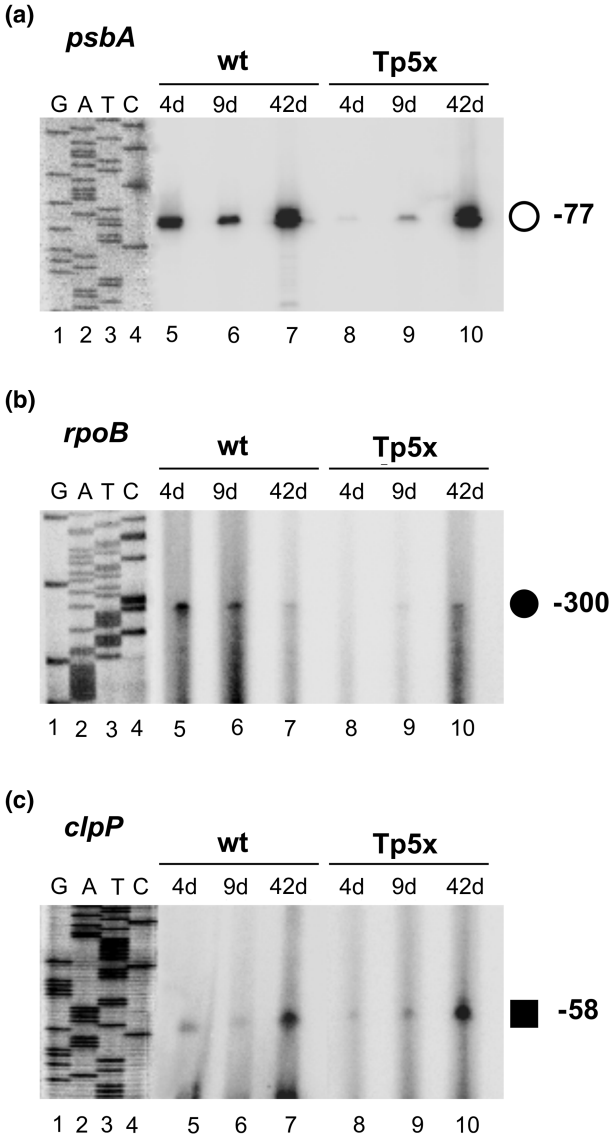


**Figure 3.** Partial silencing of *AthRpoTp* in multiple copy plants negatively affects transcription of the type-I *rpoB* NEP promoter. Less abundant *rpoB* transcript levels most likely lead to white seedlings due to delayed PEP synthesis. However, the type-II *clpP*-58 promoter is not affected. Primer extension data are shown for the *psbA* (**a**), *rpoB* (**b**) and *clpP* genes (**c**). Mapped NEP type-I (filled circle), type-II (filled square) and PEP (open circle) promoters are identified by their distance between the transcription initiation site and the translation initiation codon in nucleotides (30). For reference, the same end-labeled primer was used to generate a DNA sequence ladder.

Interestingly, the very low abundant transcripts from the P*clpP*-115 PEP promoter were absent in 4-day-old *rpoTp* mutants (Figure 4d, lanes 2 and 3), but reappeared in 3-week-old leaves (lane 4). By and large, we observed a similar pattern with all other examined type-I NEP transcripts as well. Similar to Tp5x, transcripts initiated from P*rpoB*-300 (Figure 4a) and the weak P*ycf1*-104 (Figure 5c) type-I NEP promoters showed in comparison to the 2-day-old wild type reduced levels in the 4-day-old *rpoTp* mutants (lanes 1–3), which in 3-week-old leaves of the *rpoTp* mutants returned to levels close to those found

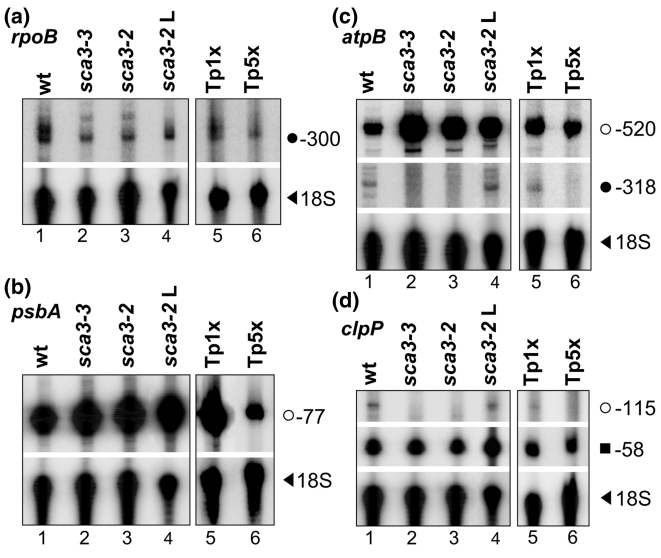


**Figure 4.** Mapping of *rpoB* (**a**), *psbA* (**b**), *atpB* (**c**) and *clpP* (**d**) transcription sites in wild type and in *rpoTp* mutant seedlings. RNA isolated from 2-day-old wild type (lane 1), 4-day-old *RpoTp* T-DNA insertion lines *sca3-3* (lane 2) and *sca3-2* (lane 3), 3-week-old *sca3-2* leaves (lane 4), 4-day-old plants with single (Tp1x, lane 5) and multiple (Tp5x, lane 6) *AthRpoTp* transgene copies were analyzed by primer extension. The lower panels show products generated with a second, cytoplasmatic 18S ribosomal RNA primer in the same primer extension reactions as shown above (18S). Mapped NEP type-I (filled circle), type-II (filled square) and PEP (open circle) promoters are identified by their distance between the transcription initiation site and the translation initiation codon in nucleotides.

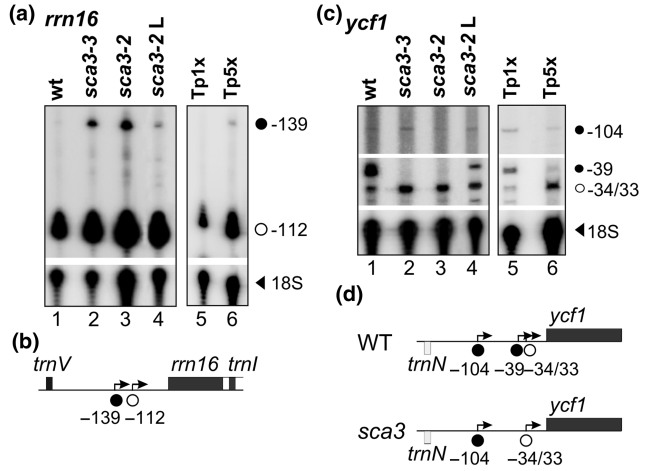


**Figure 5.** Mapping of *rrn16* (**a** and **b**) and *ycf1* (**c** and **d**) transcription sites in wild type and in *rpoTp* mutant seedlings. RNA isolated from 2-day-old wild type (lane 1), 4-day-old *RpoTp* T-DNA insertion lines *sca3-3* (lane 2) and *sca3-2* (lane 3), 3-week-old *sca3-2* leaves (lane 4), 4-day-old plants with single (Tp1x, lane 5) and multiple (Tp5x, lane 6) *AthRpoTp* transgene copies were analyzed by primer extension (a and c). To control for loading and recovery, the primer extension reactions shown above contained a second primer for the cytoplasmatic 18S ribosomal RNA. The generated products are shown in the lower panels (18S). A physical map of the *rrn16* region (b) and the *ycf1* region (d) showing promoter usage in wild type (top) and in the *sca3* mutant (bottom) is shown below. Primary transcripts from NEP and PEP promoters are marked by filled circles and open circles and labeled with their distance between the transcription initiation site and the translation initiation codon in nucleotides.

in the wild type (lanes 1 and 4). In case of P*atpB*-318 (Figure 4c) and P*ycf1*-39 (Figure 5c), no corresponding 5′-ends were observed in the primer extension analyses with RNAs from 4-day-old *rpoTp* mutants (lanes 2 and 3). However, in 3-week-old leaves of *sca3-2* and *sca3-3* RNA from these promoters accumulated close to wild-type levels (lanes 1 and 4).

Analyses of the accumulation of the two NEP promoters P*clpP*-58 (Figure 4d) and P*rrn16*-139 (Figure 5a), however, revealed a different picture. The 5′-ends initiated from both promoters did not decrease in the *rpoTp* mutants (lanes 2 and 3). While accumulation from P*clpP*-58 seemed not to be affected (Figure 4d, lanes 2 and 3), P*rrn16*-139 5′-ends accumulated to much higher levels in *sca3-2* and *sca3-3* (lanes 2 and 3) than in the wild type (lane 1). Interestingly, although to much lower extent, this was also observable in Tp5x plants (lane 6).

We therefore conclude that, to some extent, an additional RNA polymerase is able to compensate for the loss of AthRpoTp, by recognizing and initiating from type-I NEP promoters. AthRpoTmp, as the only known second phage-type RNA polymerase in plastids, is the best candidate to maintain the observed transcript initiation as suggested by Hricová *et al*. (38). Since transcription from non-consensus (type-II) promoters was not negatively, but rather positively affected in the *rpoTp* mutants, one may speculate that AthRpoTmp might be responsible for transcription of these promoters in wild-type plants.

## DISCUSSION

Plastid genes in *Arabidopsis* are transcribed by at least three RNA polymerases. PEP uses bacterial-type promoters, which are found upstream of genes encoding components of the photosynthetic apparatus, and often together with NEP promoters upstream of housekeeping genes. NEP activity is required for the expression of most housekeeping genes and most important, for the transcription of the genes for the core subunits of PEP (6). Although most yet described NEP promoters contain an YRTA consensus motif (type-I NEP promoters), several others do not, which in *Arabidopsis* are most prominently represented by P*clpP*-58 (type-II NEP promoter) and the Pc P*rrn16*-139 promoter (30). *In vitro* transcription assays have shown, that for accurate recognition of the majority of promoters by NEP enzymes *in vivo*, additional yet unknown factors are needed (31,39). So far, individual roles of RpoTp and RpoTmp in NEP promoter recognition are unknown. An indication for a role of RpoTp transcription from type-I NEP promoters was provided by overexpression of the RpoTp enzyme in transgenic tobacco plants (13). In this study, we present for the first time evidence that an additional phage-type RNA polymerase other than RpoTp is involved in transcription of plastid genes by recognizing NEP promoters. Obviously, with RpoTm so far shown to be exclusively mitochondrial localized, RpoTmp might represent the additional RNA polymerase activity in plastids.

To investigate the individual role of RpoTp in plastid transcription, we analyzed *Arabidopsis* mutants lacking RpoTp (*sca3-2*, *sca3-3*: *rpoTp*; 38), with partially silenced *AthRpoTp* gene expression (Tp5x) and *Arabidopsis* plants overexpressing the *AthRpoTp* gene (Tp1x). Although we detected an enhanced level of *RpoTp* transcripts in Tp1x overexpressor plants, they did not differ from the wild type. Since we found also indications for a fast degradation of the *RpoTp* transcripts in Tp1x, these plants may not possess markedly higher RpoTp amounts and activity than the wild type. In contrast, *rpoTp* plants displayed a pale-green phenotype throughout their development, whereas Tp5x plants with reduced *AthRpoTp* transcript levels germinated into seedlings with white cotyledons, which eventually greened up after nine days, and subsequently developed normally (Figure 1). The *sca3-2* and *sca3-3 RpoTp* insertion mutants were previously shown to lack functional RpoTp (38). Analysis of the transcript 5′-ends of NEP and PEP promoters in 4-day-old plants showed striking similarities for the effect on NEP but not on PEP promoter usage between Tp5x and the *rpoTp* mutants. It seems likely that reduction of *AtRpoTp* transcript levels leads to lower AthRpoTp enzyme amounts, which may be responsible for the observed negative effect on transcription of the *rpoB* operon. This possibly results in decreased amounts of PEP, which in turn results in delayed accumulation of PEP transcripts. After 9 days AtRpoTp amounts may build up to levels that allow for sufficient expression of PEP-transcribed photosynthesis genes such as *psbA* and finally facilitating greening up of the cotyledons. In *rpoTp* plants, however, transcript accumulation from P*rpoB*-300 is not as strongly affected as in Tp5x plants and levels of the PEP P*psbA*-77 5′-ends seem not to be affected (Figure 4). In contrast to partially silenced Tp5x plants, *rpoTp* plants completely lack AthRpoTp. Therefore, different control mechanisms of maintaining plastid gene expression in interaction with the nucleus may take place, e.g. by stronger expression of RpoTmp, the second phage-type RNA polymerase in plastids, or of transcription factors responsible for specific promoter recognition, or by increasing the transcript stability of plastid genes transcribed by PEP.

Accumulation of 5′-ends from NEP promoters in 4-day-old *rpoTp* plants showed remarkable differences to the wild type (Figures 4 and 5). While transcript 5′-ends of some type-I NEP promoters such as P*rpoB*-300 and P*ycf1*-104 showed reduced levels, no accumulation could be observed for P*atpB*-318 and P*ycf1*-39. However, in all cases, accumulation of these promoters returned to wild-type levels in 3-week-old *rpoTp* plants. With AthRpoTmp being the second phage-type RNA polymerase in plastids, we propose that AthRpoTmp is able to take over the transcription from type-I NEP promoters.

The T7 RNA polymerase is a single-polypeptide polymerase and does not need any transcription factor (47) whereas mitochondrial phage-type RNA polymerases of animals and yeasts need two transcription factors for promoter recognition and to optimal transcriptional efficiency (48). Such general transcription factors are not known yet in plants but predicted to exist from data obtained by *in vitro* transcription assays with the Arabidopsis phage-type RNA polymerases (39). Moreover, our data indicate the existence of more promoter-specific factors acting only at certain developmental stages. The differential recognition of NEP promoters in mutants and wild type, in particular the inability to use P*ycf1*-39 and P*atpB*-318 only in very young seedlings of the mutants, points to missing interaction between activator(s) and RpoTmp. Interestingly, accumulation of 5′-ends from so-called non-consensus type NEP promoters (type-II) such as P*clpP*-58 and the P*rrn16*-139 (Pc promoter) was not affected and even enhanced, respectively, in 4-day-old *rpoTp* plants. We therefore conclude that AthRpoTmp may have a special function in maintaining transcription from this type of promoters in wild-type plants. AthRpoTp, in turn, seems to be important for recognition and transcription from type-I NEP promoters, as suggested by Liere *et al.* (13). Beside a division of labor between RpoTp and RpoTmp with regards to promoter usage, the two polymerases may be of variant importance in different tissues. It was recently reported that RpoTp shows maximal expression in green tissues whereas the highest RpoTmp promoter activity was observed in meristematic and young cells containing non-green plastids (49).

In conclusion, we have analyzed the usage of PEP promoters (recognized by the bacterial-type plastid-encoded RNA polymerase) and NEP promoters (recognized by phage-type nuclear-encoded RNA polymerases) in *Arabidopsis* plants with altered and lacking RpoTp activity. Low or lacking activity of RpoTp led to lower levels of transcripts originating from NEP promoters with the consensus YRTA motif. The strong NEP promoter that drives transcription of the essential *ycf1* gene in wild-type chloroplasts was even not used at all in very young mutant seedlings without functional RpoTp. This data demonstrates on one hand the importance of RpoTp for chloroplast transcription. On the other hand, most NEP promoters were active even in the absence of RpoTp activity, thus providing clear evidence for the participation of another phage-type RNA polymerase (RpoTmp) in the transcription of plastid genes from NEP promoters. Interestingly, usage of the non-consensus promoters of the *clpP* gene and the *rrn* operon was not affected and even enhanced, respectively, by the lack of RpoTp activity. Our data indicate that RpoTp and RpoTmp together form the NEP activity and play overlapping as well as specific roles in the transcription of plastid genes. During the reviewing process of this manuscript, Courtois *et al.* (50) published data, which are in agreement with our results. Further studies including also plants with altered RpoTmp activity have to show if the two phage-type polymerases play different roles in different tissues and at certain stages of plant development.

## ACKNOWLEDGEMENTS

We are grateful to Conny Stock for excellent technical assistance and to José Luis Micol, who kindly provided seeds of the *sca3-2* and *sca3-3* mutants. This work was funded by Deutsche Forschungsgemeinschaft (SFB 429 to K.L. and T.B.). Funding to pay the Open Access

publication charges for this article was provided by Deutsche Forschungsgemeinschaft, SFB429.

*Conflict of interest statement*. None declared.

## REFERENCES


1. Martin,W. (2003) Gene transfer from organelles to the nucleus: frequent and in big chunks. *Proc. Natl Acad. Sci. USA*, **100**, 8612–8614.
2. Ahlert,D., Ruf,S. and Bock,R. (2003) Plastid protein synthesis is required for plant development in tobacco. *Proc. Natl Acad. Sci. USA*, **100**, 15730–15735.
3. Rogalski,M., Ruf,S. and Bock,R. (2006) Tobacco plastid ribosomal protein S18 is essential for cell survival. *Nucleic Acids Res.*, **34**, 4537–4545.
4. Legen,J., Wanner,G., Herrmann,R.G., Small,I. and Schmitz-Linneweber,C. (2007) Plastid tRNA genes *trnC*-GCA and *trnN*-GUU are essential for plant cell development. *Plant J.*, **51**, 751–762.
5. Shiina,T., Tsunoyama,Y., Nakahira,Y. and Khan,M.S. (2005) Plastid RNA polymerases, promoters, and transcription regulators in higher plants. *Int. Rev. Cytol.*, **244**, 1–68.
6. Liere,K. and Börner,T. (2007) Transcription of plastid genes. In Grasser,K. D. (ed.), *Regulation of Transcription in Plants*, Blackwell Publishing, Oxford, pp. 184–224.
7. Allison,L.A., Simon,L.D. and Maliga,P. (1996) Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants. *EMBO J.*, **15**, 2802–2809.
8. Krause,K., Maier,R.M., Kofer,W., Krupinska,K. and Herrmann,R.G. (2000) Disruption of plastid-encoded RNA polymerase genes in tobacco: expression of only a distinct set of genes is not based on selective transcription of the plastid chromosome. *Mol. Gen. Genet.*, **263**, 1022–1030.
9. Hess,W.R. and Börner,T. (1999) Organellar RNA polymerases of higher plants. *Int. Rev. Cytol.*, **190**, 1–59.
10. Lerbs-Mache,S. (1993) The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: single-subunit enzyme or catalytic core of multimeric enzyme complexes? *Proc. Natl Acad. Sci. USA*, **90**, 5509–5513.
11. Hedtke,B., Börner,T. and Weihe,A. (1997) Mitochondrial and chloroplast phage-type RNA polymerases in *Arabidopsis*. *Science*, **277**, 809–811.
12. Chang,C.-C., Sheen,J., Bligny,M., Niwa,Y., Lerbs-Mache,S. and Stern,D.B. (1999) Functional analysis of two maize cDNAs encoding T7-like RNA polymerases. *Plant Cell*, **11**, 911–926.
13. Liere,K., Kaden,D., Maliga,P. and Börner,T. (2004) Overexpression of phage-type RNA polymerase RpoTp in tobacco demonstrates its role in chloroplast transcription by recognizing a distinct promoter type. *Nucleic Acids Res.*, **32**, 1159–1165.
14. Hedtke,B., Legen,J., Weihe,A., Herrmann,R.G. and Börner,T. (2002) Six active phage-type RNA polymerase genes in *Nicotiana tabacum*. *Plant J.*, **30**, 625–637.
15. Weihe,A., Hedtke,B. and Börner,T. (1997) Cloning and characterization of a cDNA encoding a bacteriophage-type RNA polymerase from the higher plant *Chenopodium album*. *Nucleic Acids Res.*, **25**, 2319–2325.
16. Young,D.A., Allen,R.L., Harvey,A.J. and Lonsdale,D.M. (1998) Characterization of a gene encoding a single-subunit bacteriophage-type RNA polymerase from maize which is alternatively spliced. *Mol. Gen. Genet.*, **260**, 30–37.
17. Ikeda,T.M. and Gray,M.W. (1999) Identification and characterization of T7/T3 bacteriophage-like RNA polymerase sequences in wheat. *Plant Mol. Biol.*, **40**, 567–578.
18. Kobayashi,Y., Dokiya,Y. and Sugita,M. (2001) Dual targeting of phage-type RNA polymerase to both mitochondria and plastids is due to alternative translation initiation in single transcripts. *Biochem. Biophys. Res. Commun.*, **289**, 1106–1113.
19. Emanuel,C., Weihe,A., Graner,A., Hess,W.R. and Börner,T. (2004) Chloroplast development affects expression of phage-type RNA polymerases in barley leaves. *Plant J.*, **38**, 460–472.
20. Kusumi,K., Yara,A., Mitsui,N., Tozawa,Y. and Iba,K. (2004) Characterization of a rice nuclear-encoded plastid RNA polymerase gene *OsRpoTp*. *Plant Cell Physiol.*, **45**, 1194–1201.
21. Hedtke,B., Börner,T. and Weihe,A. (2000) One RNA polymerase serving two genomes. *EMBO Rep.*, **1**, 435–440.
22. Hajdukiewicz,P.T.J., Allison,L.A. and Maliga,P. (1997) The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. *EMBO J.*, **16**, 4041–4048.
23. Hübschmann,T. and Börner,T. (1998) Characterisation of transcript initiation sites in ribosome-deficient barley plastids. *Plant Mol. Biol.*, **36**, 493–496.
24. Silhavy,D. and Maliga,P. (1998) Plastid promoter utilization in a rice embryonic cell culture. *Curr. Genet.*, **34**, 67–70.
25. Silhavy,D. and Maliga,P. (1998) Mapping of the promoters for the nucleus-encoded plastid RNA polymerase (NEP) in the *iojap* maize mutant. *Curr. Genet.*, **33**, 340–344.
26. Miyagi,T., Kapoor,S., Sugita,M. and Sugiura,M. (1998) Transcript analysis of the tobacco plastid operon *rps2/atpI/H/F/A* reveals the existence of a non-consensus type II (NCII) promoter upstream of the *atpI* coding sequence. *Mol. Gen. Genet.*, **257**, 299–307.
27. Kapoor,S., Suzuki,J.Y. and Sugiura,M. (1997) Identification and functional significance of a new class of non-consensus-type plastid promoters. *Plant J.*, **11**, 327–337.
28. Vera,A., Hirose,T. and Sugiura,M. (1996) A ribosomal protein gene (*rpl32*) from tobacco chloroplast DNA is transcribed from alternative promoters – similarities in promoter region organization in plastid housekeeping genes. *Mol. Gen. Genet.*, **251**, 518–525.
29. Vera,A. and Sugiura,M. (1995) Chloroplast rRNA transcription from structurally different tandem promoters: an additional novel-type promoter. *Curr. Genet.*, **27**, 280–284.
30. Swiatecka-Hagenbruch,M., Liere,K. and Börner,T. (2007) High diversity of plastidial promoters in *Arabidopsis thaliana*. *Mol. Genet. Genomics*, **277**, 725–734.
31. Liere,K. and Maliga,P. (1999) *In vitro* characterization of the tobacco *rpoB* promoter reveals a core sequence motif conserved between phage-type plastid and plant mitochondrial promoters. *EMBO J.*, **18**, 249–257.
32. Weihe,A. and Börner,T. (1999) Transcription and the architecture of promoters in chloroplasts. *Trends Plant Sci.*, **4**, 169–170.
33. Liere,K. and Maliga,P. (2001) Plastid RNA polymerases. In Andersson,B. and Aro,E.-M. (eds), *Regulation of Photosynthesis*, Kluwer Academic Publishers, Netherlands, Dordrecht, pp. 29–49.
34. Kühn,K., Weihe,A. and Börner,T. (2005) Multiple promoters are a common feature of mitochondrial genes in *Arabidopsis*. *Nucleic Acids Res.*, **33**, 337–346.
35. Binder,S. and Brennicke,A. (2003) Gene expression in plant mitochondria: transcriptional and post-transcriptional control. *Philos. Trans. R Soc. Lond. B Biol. Sci.*, **358**, 181–189.
36. Kapoor,S. and Sugiura,M. (1999) Identification of two essential sequence elements in the nonconsensus Type II P*atpB*-290 plastid promoter by using plastid transcription extracts from cultured tobacco BY-2 cells. *Plant Cell*, **11**, 1799–1810.
37. Sriraman,P., Silhavy,D. and Maliga,P. (1998) The phage-type P*clpP*-53 plastid promoter comprises sequences downstream of the transcription initiation site. *Nucleic Acids Res.*, **26**, 4874–4879.
38. Hricová,A., Quesada,V. and Micol,J.L. (2006) The SCABRA3 nuclear gene encodes the plastid RpoTp RNA polymerase, which is required for chloroplast biogenesis and mesophyll cell proliferation in *Arabidopsis*. *Plant Physiol.*, **141**, 942–956.
39. Kühn,K., Bohne,A.-V., Liere,K., Weihe,A. and Börner,T. (2007) *Arabidopsis* phage-type RNA polymerases: accurate *in vitro* transcription of organellar genes. *Plant Cell*, **19**, 959–971.
40. Kabeya,Y. and Sato,N. (2005) Unique translation initiation at the second AUG codon determines mitochondrial localization of the phage-type RNA polymerases in the moss *Physcomitrella patens*. *Plant Physiol.*, **138**, 369–382.
41. Azevedo,J., Courtois,F. and Lerbs-Mache,S. (2006) Sub-plastidial localization of two different phage-type RNA polymerases in spinach chloroplasts. *Nucleic Acids Res.*, **34**, 436–444.
42. Baba,K., Schmidt,J., Espinosa-Ruiz,A., Villarejo,A., Shiina,T., Gardestrom,P., Sane,A.P. and Bhalerao,R.P. (2004) Organellar gene transcription and early seedling development are affected in the *RpoT;2* mutant of *Arabidopsis*. *Plant J.*, **38**, 38–48.
43. Becker,D., Kemper,E., Schell,J. and Masterson,R. (1992) New plant binary vectors with selectable markers located proximal to the left T-DNA border. *Plant Mol. Biol.*, **20**, 1195–1197.

44. Clough,S.J. and Bent,A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.*, **16**, 735–743.
45. Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
46. Bensing,B.A., Meyer,B.J. and Dunny,G.M. (1996) Sensitive detection of bacterial transcription initiation sites and differentiation from RNA processing sites in the pheromone-induced plasmid transfer system of *Enterococcus faecalis*. *Proc. Natl Acad. Sci. USA*, **93**, 7794–7799.
47. Cheetham,G.M. and Steitz,T.A. (2000) Insights into transcription: structure and function of single-subunit DNA-dependent RNA polymerases. *Curr. Opin. Struct. Biol.*, **10**, 117–123.
48. Cotney,J. and Shadel,G.S. (2006) Evidence for an early gene duplication event in the evolution of the mitochondrial transcription factor B family and maintenance of rRNA methyltransferase activity in human mtTFB1 and mtTFB2. *J. Mol. Evol.*, **63**, 707–717.
49. Emanuel,C., von Groll,U., Müller,M., Börner,T. and Weihe,A. (2006) Development- and tissue-specific expression of the *RpoT* gene family of *Arabidopsis* encoding mitochondrial and plastid RNA polymerases. *Planta*, **223**, 998–1009.
50. Courtois,F., Merendino,L., Demarsy,E., Mache,R. and Lerbs-Mache,S. (2007) Phage-type RNA polymerase RPOTmp transcribes the *rrn* operon from the PC promoter at early developmental stages in *Arabidopsis*. *Plant Physiol.*, **145**, 712–721.