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Overexpression of phage-type RNA polymerase RpoTp in tobacco demonstrates its role in chloroplast transcription by recognizing a distinct promoter type

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ABSTRACT

Plant cells possess three DNA-containing compartments, the nucleus, the mitochondria and the plastids. Accordingly, plastid gene regulation is fairly complex. Albeit plastids retained their own genome and prokaryotic-type gene expression system by a plastid-encoded RNA polymerase (PEP), they need a second nuclear-encoded plastid transcription activity, NEP. Candidate genes for putative NEP catalytic subunits have been cloned in *Arabidopsis thaliana* (*AtRpoTp*) and *Nicotiana sylvestris* (*NsRpoTp*). To provide evidence for *RpoTp* as a gene encoding a NEP catalytic subunit, we introduced the *AtRpoTp* and *NsRpoTp* cDNAs into the tobacco nucleus under the control of the strong constitutive CaMV 35S promoter. Analysis of transcription from NEP and PEP promoters in these transgenic plants using primer extension assays revealed enhanced transcription from typical type I NEP promoters as P*atpB*-289 in comparison with the wild type. These data provide direct evidence that RpoTp is a catalytic subunit of NEP and involved in recognition of a distinct subset of type I NEP promoters.

INTRODUCTION

Plastids evolved from ancestral cyanobacteria by gradual conversion of the endosymbiont to a plant organelle (1). The plastid genome contains functional *rpo* genes encoding the homologs of the eubacterial RNA polymerase α, β and β′ subunits (2–4), which form the core of the plastid-encoded RNA polymerase (PEP) (5–7). Sigma factors interact with PEP to confer promoter-specific binding and transcription specificity (8,9). Nonetheless, in surprising contrast to their eubacterial ancestors, this RNA polymerase is not sufficient to transcribe the chloroplast genome in higher plants.

The existence of a second, plastid-localized, nuclear-encoded transcription activity [nuclear-encoded plastid RNA polymerase (NEP)] has been established by analyzing mutant and transplastomic plants, respectively, that lack PEP yet transcribe a subset of plastid genes (10–17). It appeared that for tobacco *rpoB* NEP promoter function, a CRT motif is critical *in vitro*. Sequence alignments revealed that most NEP promoters contain such a core sequence (YRTA), similar to plant mitochondrial promoters (type Ia) (18–20). A subclass of NEP promoters shares a GAA-box motif upstream of the YRTA motif, which was shown to be important in transcription from the tobacco P*atpB*-289 NEP promoter (type Ib) (21). The pattern of plastid NEP drug resistance was shown to be different from bacterial-type RNA polymerases and analogous to the phage T7 RNA polymerase, pointing to phage-type RNA polymerases as candidates for NEP activity (22). 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 in dicots (RpoTp) (23–29). An RNA polymerase activity approximately of the size of RpoTp has previously been enriched from spinach chloroplasts (30). More recently, immunoblot analysis and an antibody-linked polymerase assay indicated that the maize RpoTp indeed specifies a chloroplast-localized enzyme (27). Interestingly, a third *RpoT* gene found exclusively in dicots (*RpoTmp*) has been shown to be dually targeted both into mitochondria and plastids (24,31).

Though it is commonly suggested that the phage-type RNA polymerases account for NEP transcription activity, direct evidence for an identity of RpoTp (and/or RpoTmp) with NEP is still lacking (18,19). Also, some other aspects of NEP transcription remain uncertain. Non-consensus NEP promoters, for example, the type II P*clpP*-53 in tobacco, do not contain a YRTA motif (32). Furthermore, some plastid tRNA genes seem to be transcribed from internal promoters (19). Finally, there is no indication regarding why dicots have two plastid-targeted phage-type RNA polymerases and if they fulfill a distinct role in plastid gene expression.

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To investigate the effect of overexpression of the putative NEP catalytic subunit RpoTp on NEP promoter activity, *Nicotiana sylvestris* and *Arabidopsis thaliana RpoTp* cDNAs were cloned into a constitutive expression cassette. Analysis of transcription from NEP and PEP promoters in these mutant plants using primer extension assays revealed that transcription from typical type I NEP promoters, such as P*atpB*-289, is enhanced in comparison with the PEP promoter P*atpB*-255. These data provide direct evidence that RpoTp is a catalytic subunit of the nuclear-encoded plastid transcription machinery NEP.

MATERIALS AND METHODS

Plasmid construction

Plasmid pKL85 was constructed by PCR amplification of the *AtRpoTp* sequence (GenBank accession no. Y08463) (23) from an *A.thaliana* cDNA library (*Arabidopsis* Biological Resource Center, Columbus, OH; CD4-15/16) using primers 703, ccctctagaATGGCTTCCGTCGCGGCG and 704, ccccccgggctcgagttaGTTGAAGAAGTAcTGGG, digested with XbaI and SmaI, and cloned into the XbaI and SacI-blunt sites of pBI121 (33). Lower case letters indicate non-cDNA sequences. Plasmid pKL194 was constructed by PCR amplification of a 300 bp PCR *NsRpoTp* fragment (GenBank accession no. AJ416576) (24) from a *N.sylvestris* cDNA library using primer 56, gggtctagAAAG**aTG**GCTTCCACAGC (AS 1) and primer 57, gggctgcagATTTGATTCTTTAGTCAAAAC (AS 98), and subsequently cloning it XbaI/PstI into pBSC (Stratagene). A triple FLAG-tag was added into the PstI and ApaI sites (primers 60, GGACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAGGGGCC and 61, CCTTGTCATCGTCATCCTTGTAATCGATGTCATGATCTTTATAATCACCGTCATGGTCTTTGTAGTCCTGCA). Finally, a 2.9 kb *NsRpoTp* PCR fragment (primer 58, cccgggcccAAGAGAGTTTTTATTCAAGAC, AS 99; primer 59, cccggtaccTCAGTTAAAGAAGTAGGGC, AS 978) was cloned ApaI/Acc65I (KpnI) into the construct resulting in the complete cDNA, which was cloned XbaI/KpnI into pBI121 resulting in pKL197 or cloned XbaI/KpnI-blunted into the XbaI and SmaI sites of the vector pGPTV (34), resulting in pKL199. Plasmid pKL59 was previously described by Liere and Maliga (22).

Plant transformation

Tobacco plants were transformed using *Agrobacterium* transformation. To introduce the pBI121 derivative pKL85 into *Agrobacterium* LBA4404, triparental mating was carried out with *Escherichia coli* cells containing the pKL85 plasmids, and *E.coli* cells containing helper plasmid pRK2013 resulting in the *Agrobacterium* strain LKL85. *Agrobacterium* strain AGL1 (35) was transformed by electroporation with plasmids pKL197 and pKL199, giving rise to strains AKL197 and AKL199. Co-cultivation of tobacco leaf discs with AKL197, AKL199 and LKL85, and subsequent selection on RMOP medium containing 100 mg/l kanamycin and 500 mg/l carbenicillin (36), resulted in green tobacco calli and shoots, which were transferred individually onto selective (50 mg/l kanamycin) RM medium (37) to form roots. Transgenic plants were verified by PCR, Southern- and northern-blot analyses (data not shown).

Primer extension analysis

Primer extension reactions were carried out with 10 μg of total leaf RNA according to standard protocols (38). Briefly, primers PE*clpP*(206), GGGACTTTTGGAACACCAATAGGCAT (5′ position in the tobacco plastid genome at 74482), PE*accD*(204), GAATATCTTATTTCCTATCAGACTAAGC (5′ position 59771) and PE*atpB*(205), CCCCAGAACCAGAAGTAGTAGGATTGA (5′ position 56744) were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Primer extensions were performed using Superscript III MMLV reverse transcriptase (Gibco BRL) at 55°C and the resulting products analyzed on 5% sequencing gels. The transcription initiation sites were previously mapped using the same primers as listed above (16,17). Quantification of obtained signals was done for three independent experiments with a PhosphorImaging system using the complementary software (Bio-Rad).

Primer extension reactions with RNAs from NEP *in vitro* transcription assays with pKL59 template DNA were carried out as previously described (22).

Northern and western blot analysis

Total leaf RNA was prepared using TRIzol (Gibco BRL) following the manufacturer's protocol. Fifty micrograms of total RNA was subjected to electrophoresis on 1% agarose–formaldehyde gels, transferred onto nylon membranes and hybridized with a single-stranded DNA probe overnight at 65°C. The *NsRpoTp* probe was prepared by asymmetric PCR using the C-terminal primer 59 in the presence of [α-^{32}P]dCTP.

For immunoblotting, plastid proteins were isolated as previously described (22). Equal amounts of proteins (10 μg) were separated in 6.5% SDS–polyacrylamide gels and transferred onto nitrocellulose membranes. Immunodetection was carried out using the ECL^{+}-system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Concentrations used for the primary monoclonal antibody Anti-FLAG®M2 (Sigma) were 10 μg/ml and for the secondary anti-mouse antibody 1 to 80 000 dilutions.

RESULTS

Expression of *AtRpoTp* cDNA in tobacco affects transcription from *atpB* type I NEP promoters

To investigate the effect of overexpressing putative *Arabidopsis* NEP catalytic subunits on tobacco NEP promoter activity, we constructed transgenic tobacco plants constitutively expressing a *AtRpoTp* cDNA. Transcript levels from previously characterized NEP and PEP promoters were determined by primer extension analysis mapping transcript 5′ ends in both transgenic and wild-type plants (Fig. 1) (16,17). The *atpB* gene is transcribed from three PEP promoters (P*atpB*-255, P*atpB*-502/488 and P*atpB*-611), active in the tobacco wild-type but not in Δ*rpoB* plants (Fig. 1, *atpB*, lanes 6 and 5; open circles). Additionally, a NEP promoter (P*atpB*-289) is active in both the leaf of wild-type and Δ*rpoB* plants (*atpB*, lanes 6 and 5; filled circles). Analogously, the *clpP* gene is transcribed from a PEP promoter (P*clpP*-95; open circles) and three NEP promoters (P*clpP*-53, P*clpP*-173,

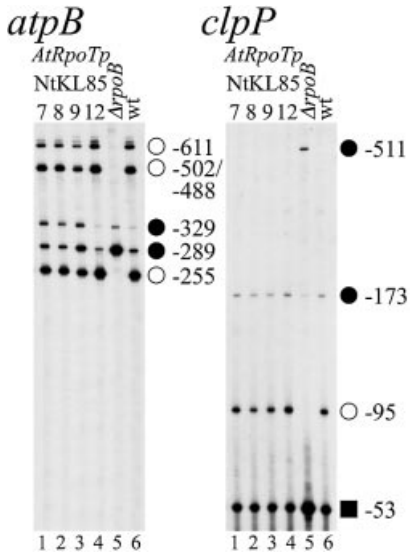

Figure 1. Mapping of transcript 5′ ends in mutant, AtRpoTp expressing (NtKL85) and wild-type tobacco plants. Primer extension data are shown for the *atpB* (left) and *clpP* (right) genes. Mapped type I NEP (filled circles), type II NEP (squares) and PEP (open circles) promoters are indicated by their distance between the transcription initiation site and the translation initiation codon in nucleotides (16,17).

P*clpP*-511; squares and filled circles), which become principal in the Δ*rpoB* plants (*clpP*, lanes 5 and 6).

Overexpression of AtRpoTp showed no influence on transcript 5′ end levels of the type II NEP promoter P*clpP*-53 (Fig. 1, *clpP*). However, the P*clpP*-173 NEP promoter transcripts in the NtKL85-7, -8 and -9 lines accumulated to rather low Δ*rpoB* plant levels (*clpP*, lanes 1–3 and 5), whereas NtKL85-12 showed higher wild-type levels (*clpP*, lanes 4 and 6). Except for the Δ*rpoB* plants (lane 5), no transcripts could be detected for the type I NEP promoter P*clpP*-511. Interestingly, the PEP promoter P*clpP*-95 RNA 5′ ends accumulated to lower levels in the transgenic than in the wild-type plants (Table 1 and Fig. 1, lanes 1–3 and 6).

On the contrary, examination of the *atpB* transcription initiation sites revealed a significant change in the RNA 5′ end pattern. Three mutant tobacco lines (NtKL85-7, -8 and -9) expressing AtRpoTp were showing a strong band at position –329 in respect to the translation initiation site (Fig. 1, *atpB*, lanes 1–3). This signal was even more pronounced than in the Δ*rpoB* plants (lane 5 and Table 1), but not visible in the wild type (lane 6). It is possible that this signal is representing the transcript 5′ end of a novel NEP promoter P*atpB*-329. The effects of AtRpoTp overexpression on NEP promoter P*atp*B-289 in these plants were not as prominent as in the Δ*rpoB* line, but transcript levels were still up to two times higher than in the wild type (Table 1). The transcript levels of PEP promoters (e.g. P*atpB*-255) in the same mutant plants were up to 40% decreased (Table 1, NtKL85-7, -8, -9; Fig. 1, *atpB*, lanes 1–3). However, as shown for the *clpP* transcript 5′ ends, this was not true for NtKL85-12. Although NtKL85-12 was proven to be transgenic for the *AtRpoTp* cDNA in Southern blot experiments, northern analysis showed no accumulation of transcripts of the transgene (data not shown). Therefore, we used this plant as a control to prove that the expression of the *AtRpoTp* transgene is causing the effect on accumulation of 5′ transcript ends from the promoters analyzed. The pattern of the *atpB* transcript 5′ ends in NtKL85-12 showed no difference in comparison with the wild type (Table 1, LKL85-12; Fig. 1, *atpB* and *clpP*, lanes 4 and 6). This clearly demonstrates that indeed overexpression of AtRpoTp is responsible for the positive effect on transcription accumulation at positions P*atpB*-329 and P*atpB*-289.

P*atpB*-329 is accurately recognized *in vitro*

Although the *atpB* 5′ end originating at –329 was present in a control using a PEP-deficient tobacco mutant, it was not certain whether this 5′ end was a transcription initiation site promoted by the overexpression of the *AtRpoTp* cDNA or a processing site of a transcript originating further upstream. Therefore, we used an *atpB* promoter fragment with its 5′ end at position –361, just 32 bp upstream of the putative P*atpB*-329 initiation site (Fig. 2B), in a NEP *in vitro* transcription assay (22). Primer extension analysis carried out with derived

Table 1. Comparison of transcript abundance in RpoTp overexpressing NtKL and wild-type plants

	atpB					*accD*	*clpP*			
	P-255 PEP	P-289 NEP-I	P-329 NEP-I	P-502 PEP	P-611 PEP	P-129 NEP-I	P-53 NEP-II	P-95 PEP	P-173 NEP-I	P-511 NEP-I
AtRpoTp										
NtKL85-7	69 ± 2	33 ± 1	212 ± 4	93 ± 5	59 ± 8	–	99 ± 0.5	79 ± 18	11 ± 4	0
NtKL85-8	73 ± 3	26 ± 5	143 ± 30	87 ± 3	56 ± 7	–	100 ± 3	37 ± 3	3 ± 1	0
NtKL85-9	52 ± 6	43 ± 9	209 ± 2	54 ± 17	48 ± 10	–	102 ± 4	43 ± 5	2 ± 1.5	0
NtKL85-12	108 ± 4	12 ± 8	0.2 ± 0.1	104 ± 8	109 ± 8	–	98 ± 2	101 ± 3	99 ± 2	0
Δ*rpoB*	0	**100**	**100**	0	0	–	157 ± 4	0	2 ± 1	**100**
wt	**100**	19 ± 4	0.5 ± 0.4	**100**	**100**	–	**100**	**100**	**100**	0
NsRpoTp										
NtKL197-6	34 ± 5	100 ± 8	253 ± 2	41 ± 3	19 ± 11	8 ± 2	98 ± 0.5	91 ± 3	68 ± 1	22 ± 4
NtKL197-4	31 ± 3	103 ± 6	248 ± 4	36 ± 4	38 ± 9	5 ± 3	97 ± 1	92 ± 2	63 ± 3	27 ± 2
NtKL199-7	68 ± 9	73 ± 10	118 ± 6	62 ± 11	58 ± 5	1 ± 0.4	110 ± 5	100 ± 1	73 ± 4	2 ± 2
Δ*rpoA*	0	**100**	**100**	0	0	**100**	98 ± 2	0	1 ± 0.5	**100**
wt	**100**	50 ± 5	0	**100**	**100**	0	**100**	**100**	**100**	0

Summary of results obtained by three independent primer extension analyses. Percentages (%) with standard error values in respect to wild-type (PEP) and Δ*rpoB* (NEP) plant transcript levels are given. Bold type represents values set to 100% for each promoter. NEP-I, type I NEP promoter; NEP-II, type II NEP promoter.

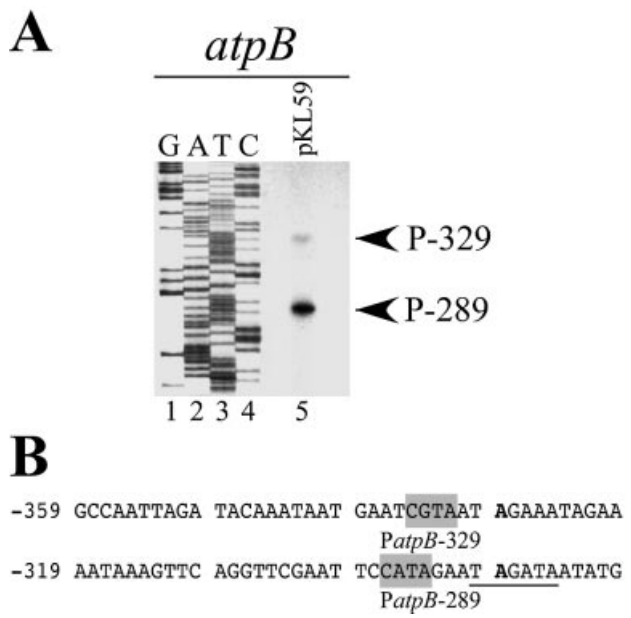

Figure 2. An additional P*atpB*-329 NEP promoter is accurately recognized *in vitro*. (**A**) Primer extension analysis to map the 5′ end of *atpB in vitro* transcripts from Δ*rpoA* plastid extracts. For reference, the same end-labeled primer was used to generate a DNA sequence ladder. (**B**) DNA sequences surrounding the *atpB* NEP transcription initiation sites. Bold nucleotides denote transcription initiation sites (+1). Putative YRTA core motifs are shaded with gray boxes and the –35 PEP promoter motif is underlined.

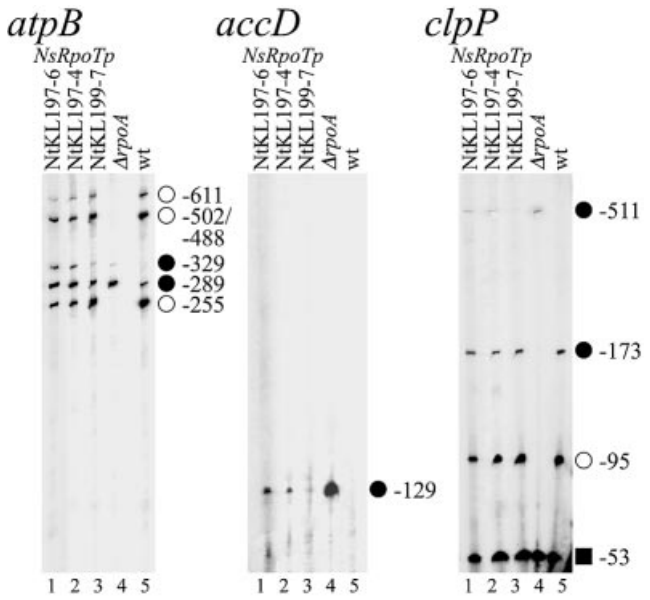

Figure 3. Mapping of transcript 5′ ends in mutant, NsRpoTp expressing (NtKL197 and NtKL199) and wild-type tobacco plants. Primer extension data are shown for the *atpB* (left), *accD* (middle) and *clpP* (right) genes. Type I NEP (filled circles), type II NEP (squares) and PEP (open circles) promoters are indicated.

in vitro transcripts indeed mapped, apart from the the P*atpB*-289 initiation site, an *atpB* RNA 5′ end at position –329 (Fig. 2A). Analysis of the surrounding region revealed a CGTA sequence matching the YRTA consensus motif of NEP promoters (Fig. 2B) (18–20), further substantiating that P*atpB*-329 is a NEP promoter *in vivo* and *in vitro*.

Expression of *NsRpoTp* cDNA in tobacco affects transcription from type I NEP promoters

Since a positive effect of overexpressing AtRpoTp on tobacco NEP promoter usage was only apparent for *atpB* but not *clpP* promoters, we could not rule out that the heterologous AtRpoTp enzyme was missing distinct factors involved in *clpP* NEP promoter recognition in tobacco (32,39). Therefore, we constructed transgenic tobacco plants constitutively expressing a *N.sylvestris RpoTp* cDNA. Transcript levels from NEP and PEP promoters of the previously analyzed *atpB* and *clpP* genes were determined by primer extension analysis mapping transcript 5′ ends in both transgenic and wild-type plants (Fig. 3, *atpB* and *clpP*). In addition, transcript levels of the *accD* gene were tested, which is transcribed by a sole type I NEP promoter P*accD*-129 usually not detectable in wild-type but in PEP-deficient Δ*rpoA* plants (Fig. 3, *accD*, lane 4) (17).

As shown for the transgenic NtKL85 plants expressing the AtRpoTp enzyme (Fig. 1), primer extension analysis of the tobacco plants expressing NsRpoTp (NtKL197-6, -4 and NtKL199-7; Fig. 3, lanes 1–3) revealed in comparison with the wild type, significant differences in *atpB* RNA 5′ end accumulation (Fig. 3, *atpB*). Though weaker for NtKL199-7, the mutant plants showed in comparison with the PEP-deficient Δ*rpoA* plant (Fig. 3, lane 4), up to two times enhanced transcript levels from P*atpB*-329 (Table 1). While almost reaching Δ*rpoA* levels, transcript abundance from P*atpB*-329 was up to two times higher as found in the wild type (Table 1). However, the usage of PEP promoters (P*atpB*-255, P*atpB*-502/488 and P*atpB*-611) in the transgenic plants was concurrently decreased with their transcript 5′ ends in NtKL197-6 and NtKL197-4 being clearly less abundant than in NtKL199-7 or wild-type plants (Table 1 and Fig. 3, *atpB*, lanes 1–3 and 5; open circles).

In the case of the P*accD*-129 NEP promoter, an even more pronounced effect was detected in the transgenic NtKL197 and NtKL199 plants in comparison with the wild type (Fig. 3, *accD*). Whereas no signal was visible in the wild type (lane 5), a distinct transcript 5′ end of P*accD*-129 was detectable in NtKL197-6 (lane 1) and NtKL197-4 (lane 2); however, this was barely visible in NtKL199-7 (lane 3). These data suggest that indeed, as shown for AtRpoTp, overexpression of NsRpoTp has a positive effect on transcription from type I NEP promoters as P*atpB*-329, P*atpB*-289 and P*accD*-129.

Analysis of *clpP* transcription initiation sites revealed no influence on transcript 5′ end levels of the type II NEP promoter P*clpP*-53 in the NsRpoTp overexpressing tobacco plants (Fig. 3, *clpP*). However, the P*clpP*-173 type I NEP promoter transcripts accumulated in NtKL197-4 and NtKL199-7 to only 70% of the wild-type levels (Table 1 and Fig. 3, *clpP*, lanes 1–3 and 5). On the contrary, the P*clpP*-511 type I NEP promoter transcripts not detectable in the wild type accumulated in the transgenic NtKL plants to up to 27% of the Δ*rpoA* plant levels (Table 1 and Fig. 3, *clpP*), showing an analogous positive effect as on the *atpB* and *accD* type I NEP promoters.

These results strongly suggest that RpoTp is a genuine part of the NEP transcription machinery involved in transcription from type I but not from type II NEP promoters. With one exception, however, RpoTp overexpression rather negatively influenced P*clpP*-173, which is atypical for type I NEP promoters up-regulated in green tissue.

Changes in RNA levels of NEP-dependent genes correlate with NsRpoTp expression levels

Interestingly, the primer extension results obtained with NtKL199-7 (approximately two transgenes) were less evident than with NtKL197-4 and NtKL197-6 (approximately five transgenes), suggesting differences in the expression of NsRpoTp in these plants. Northern blot analysis of *NsRpoTp* transcript levels revealed that indeed the transgene-RNA in NtKL199-7 accumulated to lower levels than in NtKL197-4 and NtKL197-6 (Fig. 4). To investigate to what extent the transgene-RNA translates into NsRpoTp protein, we took advantage of a FLAG-tag engineered into the NtKL transgene constructs and performed western blot analyses of plastid extracts from mutant and wild-type plants. Using monoclonal Anti-FLAG antibodies a strong signal with an apparent molecular mass of 120 kDa was detected in both mutant and wild-type plants. However, we detected a second, weaker signal with the expected molecular mass of ~110 kDa which was absent in the wild type. Therefore, we concluded that this signal represents the FLAG-tagged NsRpoTp protein expressed and imported into plastids in the mutant NtKL lines. The results shown in Figure 4 indicate that NsRpoTp expression is highest in the NtKL197-4 and -6 lines, which reflects the effect observed on transcription from type I NEP promoters in the mutant plants (Fig. 3).

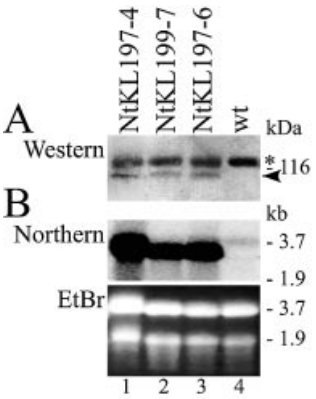

Figure 4. Expression analysis of NsRpoTp in mutant NtKL plants. (**A**) Western blot analysis of 10 µg of plastidial proteins separated in 6.5% SDS–polyacrylamide gels. Monoclonal Anti-FLAG antibody detected 120 (asterisk) and 110 kDa (arrowhead) proteins. The 120 kDa protein is probably non-specific because it was also detected in wild-type extracts. (**B**) Northern blot analysis of 50 µg of total RNA separated in 1% agarose–formaldehyde gels. Ethidium bromide stained gel image (EtBr) is shown as a loading control (bottom). The RNA blot was hybridized with a single-stranded full-length NsRpoTp antisense DNA probe. Note the weak 3.7 kb signal detected in the wild-type (lane 4) which most likely represents endogenous *NtRpoTp* transcripts.

DISCUSSION

We report here the first functional analysis of a nucleus-encoded, phage-type RNA polymerase (RpoTp) in plastids. Through analysis of transcript 5′ ends from various NEP and PEP promoters of the *atpB*, *clpP* and *accD* genes in transgenic plants overexpressing *A.thaliana* and *N.sylvestris RpoTp* cDNAs, we have provided evidence that RpoTp is a catalytic subunit of the nuclear-encoded plastid transcription machinery NEP involved in recognition of a distinct subset of type I NEP promoters.

It has been shown that an RpoTp antibody from maize repressed a biochemically purified NEP transcription activity from the unique *rrn16* PC promoter in spinach *in vitro* (NEP-1) (40). Further biochemical analyses in the same study revealed a second NEP transcription activity, NEP-2, as well as recognizing the PC promoter *in vitro*. However, the maize RpoTp antibody did not inhibit NEP-2 transcription. Although both enzymes seemed to recognize the T7 promoter *in vitro*, it remained uncertain whether NEP-1 or NEP-2 are phage-type RNA polymerases or even that they share the same core enzyme (i.e. RpoTp). To specifically elucidate the role of RpoTp in plastid NEP transcription in tobacco, we constructed transgenic plants overexpressing the *Arabidopsis* (*AtRpoTp*) and tobacco (*NsRpoTp*) cDNAs for plastid phage-type RNA polymerases and examined the RNA levels of 5′ ends from known NEP and PEP promoters by primer extension analysis.

Analysis of the *atpB* RNA 5′ ends revealed in RpoTp overexpressing plants, i.e. NtKL85-7, -8, -9, NtKL197-4, -6 and to some lesser extent NtKL199-7 (Figs 1 and 3), a novel type I NEP promoter P*atpB*-329 not detectable in wild-type plants. A second *atpB* type I NEP promoter, P*atpB*-289, also showed, in comparison with the wild-type, up to 2-fold higher transcript levels (Table 1). This was also true for P*accD*-129. This type I NEP promoter is usually not detectable in green wild-type tissue but is prominent in PEP-deficient tobacco mutants (Fig. 3) (16,17). However, we observed a signal at this position in green tissue of NtKL197-4, -6 and NtKL199-7 (Table 1), indicating that the improved amounts of RpoTp in these mutants yielded stronger transcription, hence, detectable amounts of *accD* RNA 5′ ends. When we looked at another, even in PEP-deficient plants, weak type I NEP promoter, P*clpP*-511, detectable transcript amounts were only found in NsRpoTp- but not in AtRpoTp-expressing mutants (Table 1). It might well be that AtRpoTp, which is expressed in tobacco and interacts with the heterologous transcription factor(s) is not as efficiently recognizing this type I NEP promoter as the homologous enzyme.

Interestingly, a further *clpP* type I NEP promoter, P*clpP*-173, is negatively affected by the expression of both AtRpoTp and NsRpoTp. Since P*clpP*-173 is unusually highly expressed in green wild-type tissue, it is conceivable that its expression is regulated by distinct transcription factors. This is echoed in the PEP-deficient plants, which in fact show in comparison with the wild type a similar effect on P*clpP*-173 transcription (Figs 1 and 3 and Table 1) (16,17). It is possible that by overexpression or up-regulation of RpoTp these probably limited factors become largely depleted, resulting in generally less transcript levels from this promoter. On the other hand, it is also possible that up-regulation of P*clpP*-173 in green wild-type tissue is due to a distinct regulatory network, which becomes unbalanced by RpoTp overexpression.

Surprisingly, PEP transcription from P*atpB*-255, P*atpB*-502/488, P*atpB*-611 and P*clpP*-95 was decreased in the mutant plants (Figs 1 and 3 and Table 1). In the case of P*atpB*-255 the explanation is fairly easy. The promoters P*atpB*-255 (PEP) and P*atpB*-289 (NEP) are both in close proximity. The transcription initiation site of P*atpB*-289 is located within the –35 consensus box of P*atpB*-255 (Fig. 2B). Therefore, in the case of higher RpoTp amounts, the binding equilibrium in this region is rather in favor of the NEP promoter than the PEP promoter. However, a similar, but not as eminent effect appears to affect the other *atpB* and *clpP* PEP promoters. Since other tested PEP promoters from *rbcL* and *psbA* were not affected in the mutant plants (data not shown), it seems that the higher transcription rates of *clpP* and *atpB* by the overexpressed RpoTp are paralleled by decreased transcription from the *clpP* and *atpB* PEP promoters.

We have observed that the amount of transcript 5′ ends from the type II P*clpP*-53 promoter was not affected by RpoTp overexpression (Figs 1 and 3 and Table 1). This was not necessarily expected, even though P*clpP*-53 is remarkable in both promoter architecture and expression characteristics (high transcript levels in wild-type and PEP-deficient plants). Therefore, we conclude that in tobacco, RpoTp is most likely involved in transcription from type I NEP promoters, but not from the type II P*clpP*-53. The question remains, which RNA polymerase is responsible for recognition and transcription from this 'non-consensus' promoter, active in dicot but not monocot plants (32). A second phage-type RNA polymerase, RpoTmp, is present in plastids of dicot plants (24,31). Furthermore, the existence of additional nuclear-encoded transcription activities was reported for the spinach (NEP-2) (40) and *Arabidopsis* (39) *rrn* operon, and for the internal promoters of certain tRNAs (19,41,42). One may speculate that one of these plastid transcription activities is responsible for recognition and transcription from P*clpP*-53. Further experiments, for example, overexpression of RpoTmp in tobacco, should provide more clues to understand P*clpP*-53 transcription in dicot plants.

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