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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

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Abstract Plastids of higher plants operate with at least two distinct DNA-dependent RNA polymerases, which are encoded in the organelle (PEP) and in the nucleus (NEP), respectively. Plastid run-on assays and Northern analyses were employed to analyse gene expression in tobacco mutant plastids lacking the PEP genes *rpoA*, *rpoB* or *rpoC1*. Hybridisation of run-on transcripts to restriction fragments representing the entire tobacco plastid chromosome, as well as to selected plastid gene-specific probes, shows that all parts of the plastid DNA are transcribed in *rpo*-deficient plastids. In comparison to wild-type chloroplasts, which are characterized by preferential transcription of photosynthesis-related genes in the light, mutant plastids exhibit a different transcription pattern with less pronounced differences in the hybridisation intensities between the individual genes. The analysis of steady-state transcript patterns and transcription rates of selected genes in both types of plastids demonstrates that differences in transcription rates are not necessarily paralleled by corresponding changes in transcript levels. The accumulation of large transcripts in the mutant plastids indicates that processing of primary transcripts may be impaired in the absence of PEP. These data suggest that, contrary to the prevailing view, much of the regulation of NEP-driven plastid gene expression in the *rpo*-deficient mutants is not based on differential promoter usage but is exerted at post-transcriptional levels.

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Introduction

The transcriptional apparatus of the chloroplasts of higher plants operates with at least two distinct RNA polymerases, which are encoded in different cellular compartments. The plastid DNA itself harbors the genes for the core subunits of a eubacterial-type RNA polymerase comprised of four polypeptides. The plastid gene products RpoA and RpoB are homologous to the α and β subunits of the *Escherichia coli* RNA polymerase, while the two plastid genes *rpoC1* and *rpoC2* correspond to the N- and C-termini of the *E. coli* β' subunit (Hu and Bogorad 1990; Hu et al. 1991). Together with the *rpoB* gene, the latter two genes form part of one operon. In contrast, *rpoA* is located at the distal end of the S10/SPC/alpha-operon, a large, composite operon that primarily contains genes for ribosomal protein subunits (Herrmann et al. 1992). These gene clusters are co-transcribed into polycistronic primary transcripts that undergo post-transcriptional processing (Ruf and Kössel 1988). Promoter selection by the plastid-encoded polymerase (PEP) is dependent on sigma-like factors (SLFs) (Lerbs et al. 1988; Tiller et al. 1991) that are encoded by nuclear genes (Isono et al. 1997; Tanaka et al. 1997; Kestermann et al. 1998).

Several lines of evidence have suggested the presence of a nucleus-encoded RNA polymerase (NEP) in plastids, in addition to the plastid-encoded enzyme (reviewed by Igloi and Kössel 1992). These include results from biochemical work on plastid RNA polymerase activities (e.g., Greenberg et al. 1984; Lerbs-Mache 1993), and the analysis of ribosome-deficient plastids (Falk et al. 1993; Hess et al. 1993) and plastids lacking one or more of the *rpo* genes (Morden et al. 1991; Allison et al. 1996; Serino and Maliga 1998; DeSantis-Maciossek et al. 1999). Proof of the existence of such an

enzyme has recently been provided by the isolation of a cDNA for a plastid-localized protein that is highly homologous to phage-type and mitochondrial single-subunit-type RNA polymerases (Hedtke et al. 1997).

The discovery of more than one RNA polymerase in plastids is of fundamental significance for phylogenetic as well as ontogenetic aspects of the plant cell (Herrmann 1997). The existence of different enzymes has its counterpart in the promoter design of plastid transcription units. The upstream regions of many, but not all, transcription initiation sites contain sequences that resemble *E. coli* -10 and -35 consensus sequences (Gruissem and Tonkyn 1993), and these are expected to be recognized by the plastid-encoded bacterial-type enzyme. Consequently, they have been designated "PEP promoters". Targeted deletion of plastid *rpo* genes (Allison et al. 1996; Serino and Maliga 1998; DeSantis-Maciossek et al. 1999; Liere and Maliga 1999) and the investigation of transcription initiation in the plastid ribosome-deficient *albostrians* mutant of barley (Hübschmann and Börner 1998) led to the identification of a second promoter type ("NEP promoters") that shares a conserved sequence motif with mitochondrial promoters. Northern analyses, primer extension and in vitro capping experiments with *rpo*-deficient tobacco mutants and wild-type tobacco have, in fact, suggested that transcription of individual plastid genes seems to depend on either the chloroplast-encoded polymerase (class I genes, e.g. for components of photosystems I and II), or on the nucleus-encoded polymerase (class III genes, e.g. *rpoB*, *accD*, *rpl33-rps18*, *ycf2*) (Allison et al. 1996; Hajdukiewicz et al. 1997). A third class comprises operons containing promoters that function with both polymerases (class II genes, e.g. *atpB*, *clpP*, *ndhF*) (Hajdukiewicz et al. 1997; Kapoor et al. 1997). These studies, however, did not allow discrimination between differences in actual transcription rate, post-transcriptional processing and relative stability of primary transcripts.

We have employed run-on transcription assays with plastid lysates (Deng et al. 1987; Mullet and Klein 1987; Krupinska 1992) and Northern analyses to study transcriptional activity per se, and compare transcript patterns in mutant plastids and wild-type chloroplasts. The results demonstrate that transcriptional control in plastids of *rpo* mutants may be substantially more complex than previously suggested, and is not based on differential promoter usage alone.

Materials and methods

Plant material

Wild-type tobacco plants (*Nicotiana tabacum*, cv Havanna) and mutant derivatives in which the *rpoA*, *rpoB* and *rpoC1* genes, encoding three subunits of the plastid encoded DNA-dependent RNA polymerase, had each been disrupted, were used for this study. The generation of the mutants by PEG-based plastid transformation, and their characterization, have been described by DeSantis-Maciossek et al. (1999).

Plastid isolation

Leaf material (50 g) was homogenized in 250 ml of buffer A [0.33 M sorbitol, 25 mM HEPES, 25 mM MES, 4 mM sodium ascorbate, 1.2 mM $MnCl_2$, 0.8 mM $MgCl_2$, 4 mM EDTA, 1 mM KH_2PO_4, 4 mM DTT, 0.2% (w/v) BSA, 0.1% (w/v) PVP-10, pH 6.8]. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, Calif.), and centrifuged at $3000 \times g$ for 60 s. The pellet was then resuspended in a small volume of buffer B (0.33 M sorbitol, 50 mM HEPES-KOH, pH 8.0) and fractionated in 30–80% Percoll gradients, essentially as described by Gruissem et al. (1986). Intact chloroplasts were washed and finally resuspended in buffer B. All steps were performed at 4 °C or on ice. Plastid numbers were determined by microscopy, and the plastid suspensions adjusted to 2×10^6 plastids per µl.

Run-on transcription assays

Run-on transcription assays with 2×10^7 lysed plastids were carried out in a 100-µl volume in the presence of heparin as described (Klein and Mullet 1990; Krupinska 1992). To prepare probes for hybridisation, the mixture was extracted with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1). Unincorporated nucleotides were removed by gel filtration according to Krupinska and Falk (1994). Run-on transcription in the presence or absence of the transcription inhibitor tagetitoxin (Epicentre Technologies, Madison, Wis.), at a final concentration of 20 µM, was performed as described by Krause et al. (1998) with 2×10^6 plastids in a 10-µl volume. Incorporation of [α-^{32}P]UTP into elongating transcripts was determined as described by Hallick et al. (1976) after spotting of aliquots onto DE81 filters (Whatman, Maidstone, UK).

Southern analysis with DNA fragments representing the entire tobacco plastid chromosome

Recombinant pBR322 plasmids containing the *Bam*HI fragments Ba1, Ba2, Ba5, B7, B13, B18, B19, B20, B22, B25, B27, B28 and B29 of tobacco plastid DNA (Sugiura et al. 1986) were digested with *Bam*HI and *Eco*RI. A segment of plastid DNA not represented by these fragments was amplified by PCR using a primer pair corresponding to nucleotide positions 113071–113055 and 109663–109680 of the tobacco plastome, respectively (Wakasugi et al. 1998). The 3.4-kb PCR product was purified and directly digested with *Bam*HI and *Eco*RI. The restriction fragments were fractionated on 1.2% agarose gels and subsequently transferred onto nylon membrane (Hybond N^+, Amersham, Braunschweig, Germany) by capillary blotting using 0.4 M NaOH as a transfer buffer.

Hybridisation of ^{32}P-labeled run-on transcripts to immobilized tobacco plastid DNA fragments was performed according to the membrane manufacturer's protocol. Autoradiography was carried out at – 80 °C with a Trimax Regular 16 intensifying screen (Imation, Rochester, N.Y.) using Hyperfilm MP X-ray film (Amersham). The same filter was used for hybridisation with run-on transcripts of all four samples by stripping and reprobing the filter with the next probe.

Hybridisation with gene-specific DNA and RNA probes

Gene-specific probes from barley plastid DNA were dotted onto nylon filters (Zeta Probe GT, BioRad Laboratories, Munich, Germany) and hybridised with radiolabeled plastid transcripts synthesized in vitro (Krause et al. 1998). Hybridisation conditions for heterologous probes were as recommended by the manufacturer. Single-stranded RNA probes were generated by in vitro transcription of cloned tobacco plastid gene fragments and dotted onto nylon filters in a series of dilutions (1600 fmol, 400 fmol and 100 fmol) following the instructions of the membrane manufacturer.

Northern analysis

Total RNA from tobacco leaves was isolated using TRIzol Reagent (Life Technologies, Eggenstein, Germany) and fractionated on denaturing 1.2% agarose gels containing 6.7% formaldehyde. The RNA was transferred onto Hybond N^+ membrane (Amersham) by capillary blotting, crosslinked and stained with methylene blue to visualize the ribosomal RNAs. Fragments of tobacco plastid DNA specific for the genes *accD*, *atpB*, *rbcL*, *rpoA* and *rpoB*, which were obtained by PCR, were radioactively labeled using the Random Primed Labeling Kit (Boehringer, Mannheim, Germany) and hybridised to the Northern filters as recommended by the manufacturer.

Results

Inhibition of transcription by tagetitoxin in Δ*rpo* mutants and wild-type plastids

Plastid run-on assays were performed in order to determine the effect of *rpo* gene disruptions on both the transcriptional activity of the plastids and the sensitivity of the transcriptional apparatus to transcriptional inhibitors. All run-on experiments were based on equal numbers of plastids per assay. In plastids of all three mutants tested (Δ*rpoA*, Δ*rpoB*, Δ*rpoC1*), the overall transcriptional activity, as measured by incorporation of radioactive nucleotides into elongating RNA, was only 20 – 30% that in chloroplasts of wild-type plants (WT; Fig. 1). Run-on assays with wild-type and mutant plastids performed in the presence of tagetitoxin, an inhibitor of plastid transcription (Mathews and Durbin 1990), illustrate that sensitivity to this inhibitor is drastically decreased in the white mutant plastids (Fig. 1). While transcription in wild-type chloroplasts was inhibited almost 90% by tagetitoxin , the degree of transcriptional inhibition in *rpo*-deficient plastids compared to the untreated controls was only between 10 and 20% (Fig. 1). The reproducibility of these values was confirmed in four independent measurements using the same chloroplast suspension, and, in the case of wild-type and Δ*rpoC1* plants, with independent sets of plants. In contrast to tagetitoxin, other inhibitors, such as rifampicin, α-amanitin and streptolydigin, did not differentially inhibit transcription in the two plastid types (data not shown).

To compare the transcriptional activities of individual genes in wild-type and mutant plastids, and to estimate the effect upon residual transcription in wild-type plastids exposed to tagetitoxin, hybridisations of run-on transcripts were performed with 10 plastid gene-specific DNA fragments dotted onto nylon filters (Fig. 2). The relative transcription rates of various genes, including those of the *rpoB/C* operon, *rpoA*, *atpB* and the heterogenic *psaA* operon, which contains the two photosystem I genes *psaA* and *psaB*, and the *rps14* gene, were increased in the mutants to different extents in comparison to untreated wild-type plastids. In contrast, transcription of photosystem II genes, especially *psbA*, was drastically reduced, but not entirely eliminated. Relative transcription rates of other genes, like *rrn16* and *trnE*, were not affected by the mutations. No significant difference was noted between the hybridisation patterns of the three mutants (Fig. 2). The hybridisation pattern of

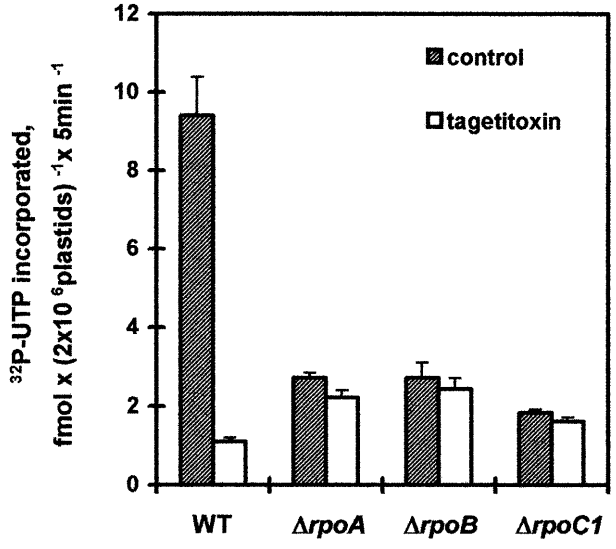

Fig. 1 Inhibition of plastid gene expression by tagetitoxin. Run-on transcription assays were carried out as described in Materials and methods. Incorporation of [α-^{32}P]UTP under standard conditions (Klein and Mullet 1990; Krupinska 1992) was determined by liquid scintillation counting of aliquots spotted onto DE81 filters (Hallick et al. 1976) and compared to incorporation of radiolabeled nucleotides into run-on transcripts synthesized in the presence of 20 μM tagetitoxin. The amount of UTP incorporated into RNA during a 5-min incubation using 2×10^6 plastids is given as a measure for total transcriptional activity. WT, wild-type; Δ*rpoA*, Δ*rpoB* and Δ*rpoC1*, mutants deficient in the products of the plastid genes *rpoA*, *B* and *C1*, respectively

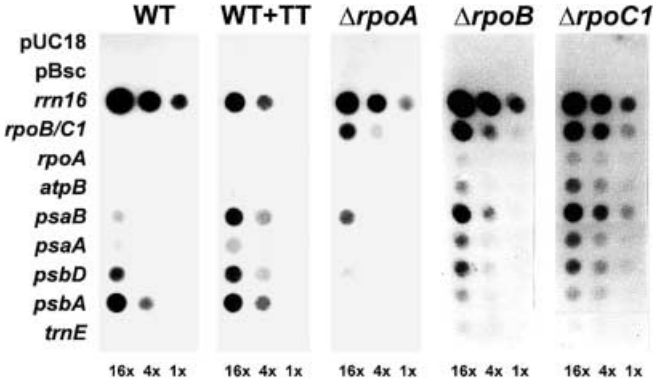

Fig. 2 Dot-blot analysis of run-on transcripts derived from untreated and tagetitoxin-treated wild-type tobacco chloroplasts and from Δ*rpo* plastids. α-^{32}P-labeled run-on transcripts derived from untreated (WT) and tagetitoxin-treated (WT + TT) wild-type chloroplasts and from *rpo*-deficient plastids (Δ*rpoA*, Δ*rpoB* and Δ*rpoC1*) were hybridised to identical dot blots containing heterologous DNA fragments specific for 10 different chloroplast genes, and plasmids pUC18 and pBluescript as controls. The designations of the gene probes are given on the *left*. Each probe was dotted onto a nylon filter in three dilutions (as indicated at the *bottom*): 328 fmol (16×), 82 fmol (4×) and 20.5 fmol (1×). Run-on transcripts derived from 2×10^7 plastids were prepared as described in Materials and methods and hybridised to the filters under standard conditions

wild-type plastids treated with tagetitoxin resembles that of untreated wild-type plastids rather than that of Δ*rpo* mutants, with the exception of *psaB* levels. These latter are reminiscent of those in the NEP-derived plastid run-on transcription pattern in Δ*rpo* mutants (Fig. 2; see below). The strong hybridisation signals obtained with the *psbA* and *psbD* probes and the low transcription rate of the *rpoB*/*C* operon are characteristic of the PEP activity that predominates in wild-type plastids.

Transcriptional analysis of the entire plastid chromosome

To analyse the relative transcription rates of all the various transcription units of the tobacco plastid chromosome in wild-type and *rpo*-deficient plastids, run-on transcripts were hybridised to plastid DNA fragments representing the complete plastid chromosome. Recombinant pBR322 plasmids containing 13 *Bam*HI fragments of the tobacco plastome (Sugiura et al. 1986) were digested with *Bam*HI and *Eco*RI to ensure that most plastid operons were represented by at least one operon-specific fragment. The inserts represent the entire plastid chromosome with the exception of a 1.2-kb interval that was specifically amplified by PCR (Fig. 3A). The resulting DNA subfragments were separated by agarose gel electrophoresis (Fig. 3B) and blotted onto a nylon filter. The same filter was subsequently used for hybridisation with run-on transcripts derived from all four plastid types (i.e. from wild-type, Δ*rpoA*, Δ*rpoB* and Δ*rpoC1* material).

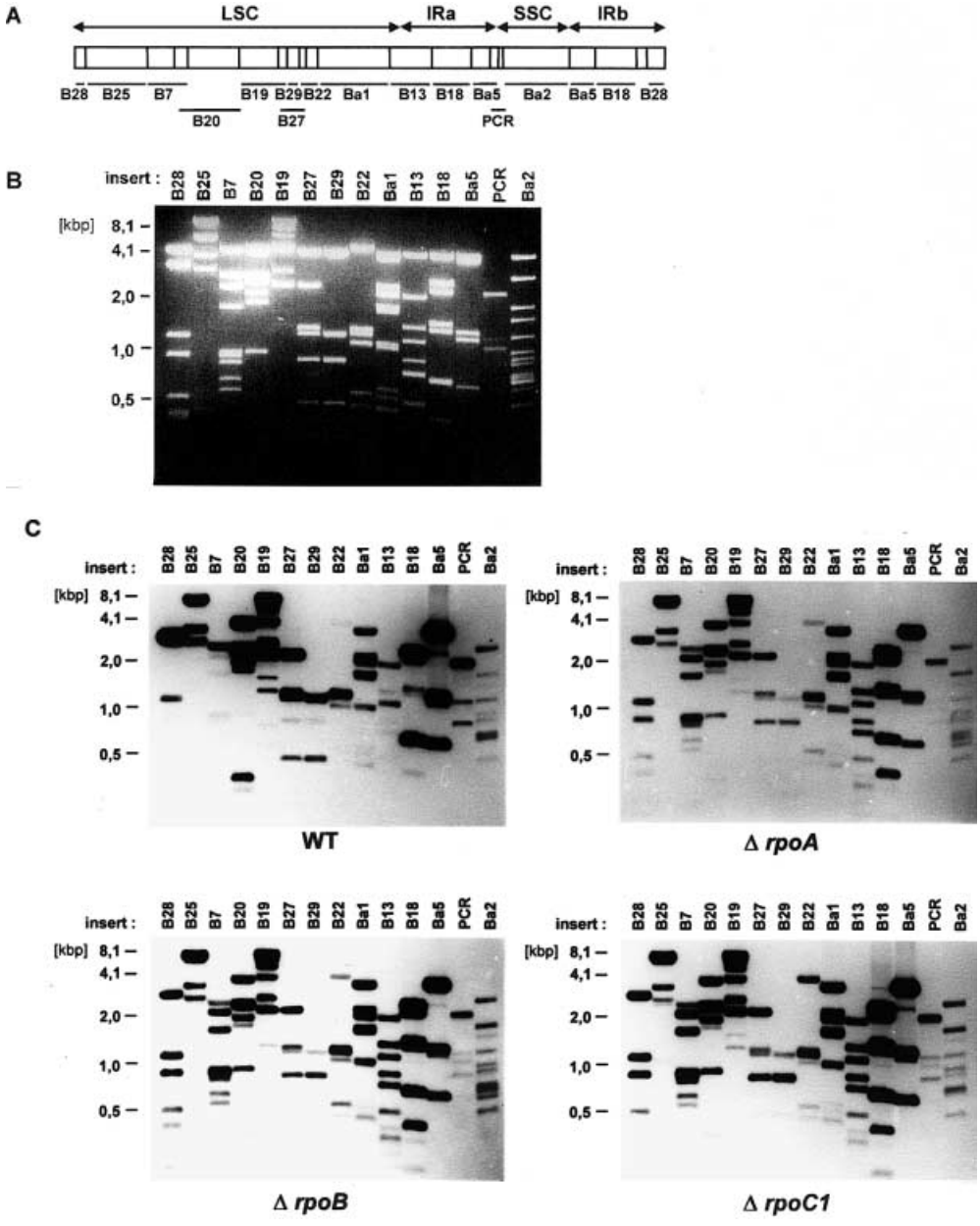

Fig. 3A–C Analysis of run-on transcripts by Southern hybridisation with fragments representing the complete tobacco plastome. Thirteen recombinant plasmids carrying *Bam*HI fragments of the tobacco plastid chromosome (Sugiura et al. 1986) were digested with *Bam*HI and *Eco*RI, and fractionated in a 1.2% agarose gel. **A** 3.4-kb segment of the plastid DNA not represented by these fragments was amplified by PCR (see Materials and methods) and treated similarly. A Location of the *Bam*HI fragments used for *Bam*HI/*Eco*RI digestion relative to the large and small single-copy regions (LSC, SSC) and the inverted repeats (IRa, IRb). **B** The DNA fragments in the gel were stained with ethidium bromide, and then transferred onto a nylon filter by capillary blotting. **C** The same filter was hybridised successively to run-on transcripts obtained from wild-type chloroplasts, *rpoA*-, *rpoB*- and *rpoC1*-deficient plastids. Positions and sizes (in kb) of the molecular-weight standards are indicated on the *left*

The autoradiographs demonstrate that, despite the absence of PEP, essentially all fragments of the plastid chromosome hybridise with run-on transcripts from the different mutant plastids, indicating that most, if not all, operons are transcribed in the *rpo* mutant plastids under the conditions investigated (Fig. 3C). The qualitative hybridisation patterns obtained with the three *rpo* mutants were basically identical to each other and similar to those of wild-type chloroplasts. While transcription levels in plastids from *rpo* mutants are rather uniform, transcription in wild-type chloroplasts is characterized by conspicuous differences in the relative transcription rates of different transcription units (Fig. 3C). No cross-hybridisation of any of the four probes was observed with the linearised vectors pBR322 or pUC18 (see Figs. 2 and 4C).

Analysis of run-on transcription of individual genes

Relative transcription rates of some plastid DNA regions that are expressed very weakly in wild-type tobacco are enhanced in all three mutants. Among these regions are subfragments of the B7 insert that contain the *rpoB*/*C* operon (Fig. 3C). Relative to wild-type chloroplasts, transcription rates of other operons in mutant plastids are unchanged or lower, leading to the relative uniformity of the mutant transcription patterns. An example for the transcription of all three gene classes in the mutants lacking PEP is provided by the three adjacent genes *atpB*, *rbcL* and *accD* on insert B27 (Fig. 4). Relative transcription rates of the *rbcL* gene, represented by subfragments 6 and 7, and the *accD* gene (subfragment 8) are both clearly decreased in the mutants. Although they behave similarly under these conditions, the two genes have previously been assigned to different gene classes based on their promoter structure: *rbcL* to the class I "PEP" genes and *accD* to class III "NEP" genes (Hajdukiewicz et al. 1997). In contrast, the *atpB* gene, which is represented by the 896-bp subfragment 2 (Fig. 4A, B) and belongs to class II genes with functional promoters for both polymerases (Hajdukiewicz et al. 1997; Kapoor et al. 1997), is transcribed at much higher rates in the mutants than in wild-type tobacco plants (Fig. 4C).

Analysis of strand-specific transcription

Since the double-stranded DNA fragments that were used for the hybridisation experiments shown in Figs. 2, 3 and 4 cannot discriminate between sense and antisense transcription, the strand-specificity of run-on transcription was checked in wild-type chloroplasts and plastids of the Δ*rpoB* mutant. The hybridisation pattern of run-on transcripts obtained with single-stranded, gene-specific RNA probes that were immobilized on a filter shows that the level of sense transcripts studied seems to be decreased in the mutant plastids compared to the wild-type chloroplasts, while the amount of antisense transcripts remains relatively unchanged (Fig. 5A). The average ratio for all 15 sense and antisense transcripts studied is 26:1 in wild-type chloroplasts. This ratio decreases to an average value of 7:1 in Δ*rpoB* plastids (Fig. 5B). The ratios, however, vary widely between individual genes. For instance, the levels of protein-coding sense transcripts of some of the typical PEP-dependent class I genes, like *psbA* or *psbD*, decrease drastically in the PEP mutants (Fig. 5A), resulting in a decreased ratio of sense to antisense transcripts in the mutants (for *psbD*: wild-type: 67:1, Δ*rpoB*: 0.4:1) (Fig. 5B). The opposite is found for some of the NEP-dependent class III transcripts, like *rpoB* (wild-type: 1.7:1, Δ*rpoB*: 8:1) (Fig. 5A, B). However, apart from the finding that transcripts of PEP-dependent genes are not absent in the mutants, there are examples for both gene classes that do not conform to expectation. For instance, the sense:antisense ratios of the PEP-dependent *psaA* transcripts are relatively unaffected by the *rpoB* mutation

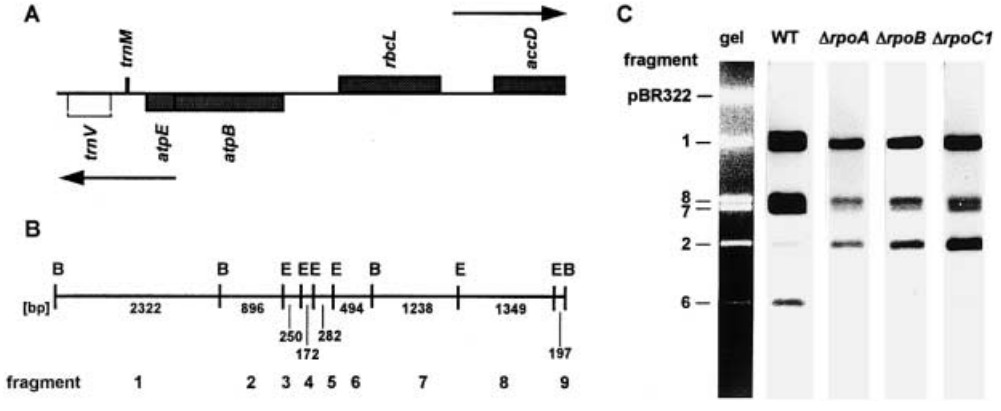

Fig. 4A–C Restriction map and Southern analysis of the tobacco plastome fragment pTB27. **A** The locations of the genes *atpB*, *atpE*, *rbcL* and *accD* on the tobacco plastid DNA fragment B27 (Sugiura et al. 1986) are shown relative to *Bam*HI [B] and *Eco*RI [E] sites in B. The *arrows* indicate the direction of transcription. **B** The sizes of the restriction fragments as calculated from sequence data (Wakasugi et al. 1998) are given *below the line*. The fragments were numbered (1–9) in order from left to right. **C** *Bam*HI-*Eco*RI subfragments of pTB27, size-fractionated in an agarose gel, were visualized by ethidium bromide staining (*gel*) and hybridised to run-on transcripts from the plastid types WT, Δ*rpoA*, Δ*rpoB*, and Δ*rpoC1* after transfer onto a nylon membrane. The corresponding fragment numbers are shown on the *left*

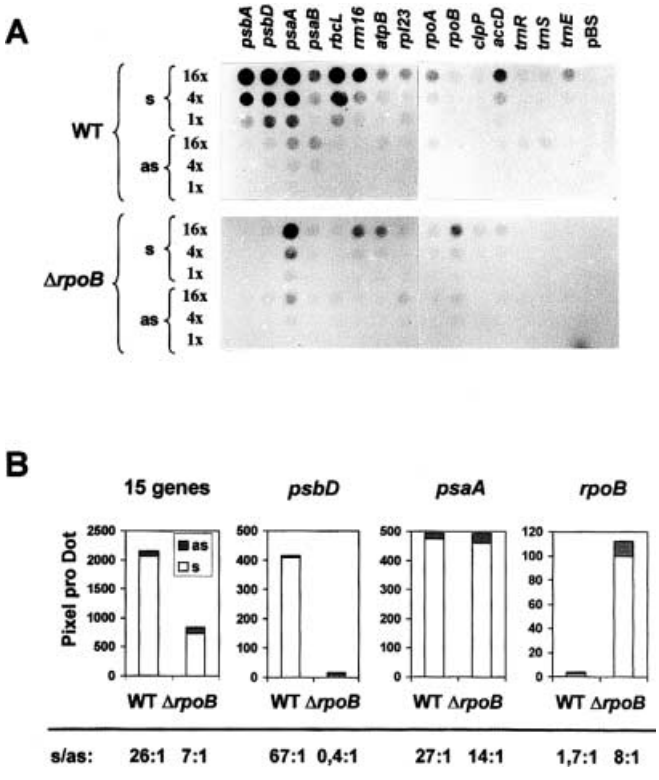

Fig. 5A, B Strand-specific hybridisation of run-on transcripts from *rpoB*-deficient and wild-type plastids of tobacco. **A** α-^{32}P-labeled run-on transcripts from wild-type and *rpoB*-deficient tobacco plastids were hybridised to identical dot blots containing homologous strand-specific RNAs that were generated by T7 or T3 polymerase-driven in vitro transcription of 15 cloned tobacco plastid gene fragments. Labeled run-on transcripts of coding strands (sense strand) are indicated by s; non-coding run-on transcripts (antisense strand) by as. Note that the differences between the hybridisation intensities of *psaA* and *psaB* are merely due to the fact that the sizes of the gene-specific probes differ by a factor of three in favour of *psaA* and, therefore, the signals do not indicate differential transcription of cistrons within the *psaA*/*psaB*/*rps14* operon. **B** The autoradiographs were examined by videodensitometric scanning and the mean optical densities of the hybridisation signals for sense and antisense run-on transcripts from *rpoB*-deficient and wild-type plastids were compared with each other. The diagram shows a comparison of the sums of optical densities of all sense and antisense signals for the 15 genes examined as well as optical densities of *psbD*, *psaA* and *rpoB* sense and antisense transcripts. s, sense transcripts, represented by *open boxes*; as, antisense transcripts, represented by *filled boxes*

(wild-type: 27:1, Δ*rpoB*: 14:1) (Fig. 5B). Conversely, the "NEP gene" *accD* seems to be expressed more strongly in wild-type plastids (Fig. 5A); this is consistent with data obtained with double-stranded hybridisation probes (Fig. 4).

The patterns of mRNA accumulation do not reflect rates of transcription in the mutant plastids

In order to compare the differences in transcriptional activity of the various genes with the steady-state levels of the corresponding transcripts, Northern hybridisations were performed with probes specific for genes representing each of the three different gene classes (Hajdukiewicz et al. 1997). Figure 6 illustrates the findings. Both qualitative and quantitative differences were noted. The level of the smallest class III *accD* transcript detected (~2800 nt) is moderately enhanced in all three *rpo* mutants. A second, substantially larger RNA of ~7150 nt is detectable only in the mutants. Northern hybridisation with the *atpB* (class II)-specific probe reveals no pronounced changes in transcript levels but drastic differences in transcript sizes. In contrast to wild-type tobacco, which express two major transcripts of 2600 and 2500 nt, the mutants accumulate only a single transcript of ~2550 nt. Again, only the

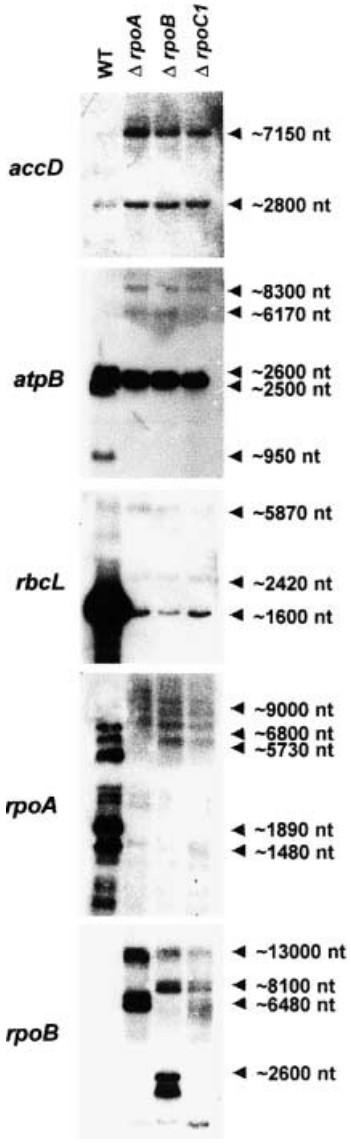

Fig. 6 Northern analysis of particular plastid transcripts in *rpo*-deficient and wild-type plastids of tobacco. Levels of transcription of the *accD*, *atpB*, *rbcL*, *rpoA* and *rpoB* genes were analysed by hybridisation of total RNA from wild-type and *rpo*-deficient plants with homologous gene-specific DNA probes. Transcript sizes are given on the *right*. Note that the different sizes of *rpoB* transcripts in the three mutants are the result of the insertion of an *aadA* cassette

larger precursor transcripts could be detected in the mutant RNA. Very low levels of the class I *rbcL* transcript were detected in the leaves of all three *rpo* mutants. Northern hybridisations with two *rpo* gene-specific probes (*rpoA* and *rpoB*) reveal opposite regulation of these two genes at the post-transcriptional level. While *rpoB* transcripts accumulate to significant levels in the mutants, the reverse was noted for mature *rpoA* transcripts. Predominantly high-molecular-weight *rpoA* transcripts (~5700–9000 nt) accumulated in mutant leaf material.

Discussion

The work outlined here on transcription and transcript analysis of chromosomes from wild-type plastids and plastids defective in the plastid-encoded DNA-dependent RNA polymerase (PEP) uncovers new insights regarding the components of the composite transcriptional apparatus in chloroplasts, and their interactions. It demonstrates that the changes caused by the loss of the bacterial-type plastid RNA polymerase are significantly more complex than previously assumed. Earlier work on PEP-deficient tobacco concentrated on the comparison of steady-state transcript levels and of transcription initiation sites in wild-type and *rpo*-deficient plastids (Allison et al. 1996; Hajdukiewicz et al. 1997; Serino and Maliga 1998). The present study included an analysis of the transcription process itself, in wild-type and *rpo*-deficient plastids, by employing an in vitro plastid run-on transcription assay (Deng et al. 1987; Mullet and Klein 1987; Krupinska 1992). Organellar run-on transcription assays have been successfully used with chloroplasts expected to utilize the phage-type RNA polymerase (Hess et al. 1993; Falk et al. 1993) and with mitochondria (Iqbal and Hudson 1996). Here, the data obtained from the analysis of transcription in plastids from *rpo*-deficient mutants were compared with those derived from Northern analyses.

In order to estimate to what extent the unexpected hybridisation patterns are due to inefficient transcription termination that would result in enhanced production of long sense and non-coding antisense transcripts, and to detect possible artifacts inherent in the in-vitro assays, strand-specific transcription of some representative genes was investigated (Fig. 5). This approach demonstrated that indeed in a few cases the amounts of antisense transcripts, which might result from non-specific transcription, are increased in the mutants (e.g. *psbD*, Fig. 5), but generally the ratio of sense to antisense transcription is still much in favour of the sense mRNAs for most of the genes assayed, and is comparable to that seen in the wild-type situation. Thus, although the possibility of inefficient transcription termination has to be taken into consideration, there are probably other reasons, such as spurious promoter recognition by the NEP polymerase, that may account for the observed discrepancies. The consistent nature of the results obtained with the three different *rpo* mutants is compatible with this inference.

Clear differential effects on the overall transcriptional activity of wild-type and *rpo*-deficient plastids were observed with tagetitoxin, which is a powerful inhibitor of the plastid bacterial-type RNA polymerase (Berends Sexton et al. 1990; Mathews and Durbin 1990) but does not seem to inhibit significantly the nucleus-encoded plastid enzyme(s) (Liere and Maliga 1999). While transcriptional activity in chloroplasts was efficiently inhibited by this antimetabolite (90% reduction at 20 μM final concentration), transcription in all three mutant plastids was only slightly affected (10–20%; Fig. 1), confirming previous data of Liere and Maliga (1999). Since mitochondrial contamination of the plastid preparations used was negligible (data not shown), the relative insensitivity of the mutant plastid transcriptional apparatus is not attributable to contaminating mitochondria, whose phage-type RNA polymerase is presumably insensitive to tagetitoxin as well. Despite the strong quantitative effect of tagetitoxin on the overall transcription rate in wild-type chloroplasts, no significant effect of this inhibitor was noted with respect to the relative transcription rates of 10 different plastid genes examined (Fig. 2). The characteristics of NEP transcription as reflected in the run-on transcription pattern of *rpo*-deficient plastids are not found in tagetitoxin-treated wild-type chloroplasts (Fig. 2), which implies that most of the remaining 10% of transcriptional activity in tagetitoxin-treated wild-type chloroplasts is probably not attributable to NEP. This activity may either simply reflect incomplete inhibition of the PEP enzyme or might even indicate the existence of a third plastid RNA polymerase type. Tagetitoxin appears to cause prolonged pausing of the prokaryotic transcription complex at discrete sites along the DNA template (Mathews and Durbin 1994). It is therefore conceivable that structural changes in the template DNA – in addition to changes in the composition of the transcriptional apparatus – could affect the inhibition of plastid transcription by tagetitoxin.

Four points of general interest emerge from the study presented here: (1) the finding that all operons appear to be transcribed by plastid NEP, albeit at a relatively low level; (2) the observation of quantitative differences in the relative transcription rates of individual plastid genes in wild-type and mutant material; (3) a discordance between transcriptional activity and steady-state levels of the transcripts of individual genes; and (4) the occurrence of comparatively high levels of large transcripts in the PEP-deficient plastids.

One of the most striking findings of the study is that there is no obvious qualitative difference between the overall levels of transcription attributable to PEP and NEP. Hybridisation of run-on transcripts to filters bearing DNA fragments representing the entire tobacco plastid chromosome revealed that the NEP enzyme appears to be capable of transcribing all parts of the plastid DNA (Fig. 3). This finding cannot be reconciled with

the suggestion that genes for components of the transcriptional and translational machinery are preferentially transcribed in PEP-deficient plastids, relative to those for elements of the photosynthetic apparatus (Allison et al. 1996), nor is it consistent with the hypothesis that each of the different plastid RNA polymerases transcribes a distinct set of genes (Hajdukiewicz et al. 1997).

Quantitative differences in relative (as opposed to absolute) transcription rates of individual plastid genes in wild-type and mutant plant material were observed by hybridisation of run-on transcripts with specific probes (Figs. 4 and 5) but are apparently not related to the nature of the RNA polymerase or the promoters. Hybridisation with homologous, strand-specific probes clearly shows that specific transcription of distinct genes is enhanced in the presence of the "cognate" polymerase, but that this does not hold for all genes (Fig. 5). For example, transcription rates of operons assumed to possess NEP promoters can increase (*rpoB*/*C* operon), decrease (*accD*) or apparently remain unchanged (Fig. 5) in the mutants. Remarkably, the rates of expression of the *atpB* operon, which encodes the β and ε subunits of the ATP synthase and appears to function with a mixed promoter (Hajdukiewicz et al. 1997; Kapoor et al. 1997), increases in the *rpo*-deficient background (Figs. 4 and 5). The genes *psaA* and *psaB* are part of the heterogenic *psaA*/*psaB*/*rps14* operon that has been assigned to class I plastid units, and are thought to be transcribed exclusively from PEP-type promoters (Hajdukiewicz et al. 1997). However, this operon exhibits strong transcriptional activity attributable to NEP in run-on assays with *rpo*-deficient material (Fig. 5). It is conceivable that an as yet unidentified NEP promoter resides within this operon. The relatively high expression level of the *rpoB*/*C* operon displayed by the Δ*rpo* plastids is consistent with reports for the plastid ribosome-deficient barley mutant *albostrians*, which also appears to lack the PEP enzyme (Hess et al. 1993).

Transcription rates and transcript stability, as judged from steady-state RNA concentrations, frequently do not coincide in the PEP-deficient mutants. For instance, although *accD* transcription rates decrease (Fig. 4), the corresponding transcript levels increase when PEP is lacking (Fig. 6). Similarly, although the S10/SPC/alpha operon was strongly transcribed in the mutant plastids (Fig. 2; Fig. 3B, fragments Ba1 and B13), the monocistronic *rpoA* transcripts ($\sim$1500 nt) seen in wild-type chloroplasts were scarcely detectable in the mutant leaf material, while large precursor transcripts ($>$5700 nt) accumulated substantially (Fig. 6). Although polycistronic transcripts can be translated into functional proteins – at least in some cases (Barkan 1988) – the lack of mature monocistronic mRNAs could influence the efficiency of protein synthesis, and hence affect protein levels. These results, and the finding that steady-state RNA concentrations and the rates of mRNA translation are often discordant as well (Herrmann et al. 1992), limit the inferences that can be drawn from gene expression data. Apart from the observation that in etiolated material more than 50% of the thylakoid proteins may be translated from very minute amounts of RNA (Herrmann et al. 1992), which are therefore not negligible in the overall expression process, differential expression of plastid-encoded proteins is known from etiolated material, and has recently been found for the ribosomal protein Rpl2 vis-a-vis thylakoid proteins in an *rpo*-deficient background (DeSantis-Maciossek et al. 1999).

Our data also bear on the possible redox control of transcription that has been inferred from work on plant material illuminated with photosystem-specific light regimes (Pfannschmidt et al. 1999). Remarkably, increases in *psaA*/*B* transcription rates and transcript levels can also be seen in the PEP-deficient material lacking redox control (DeSantis-Maciossek et al. 1999), and, in this case, appear not to be linked to corresponding changes in protein synthesis. No PsaA and PsaB has been detected in the *rpo* mutants (DeSantis-Maciossek et al. 1999).

In most cases high-molecular-weight transcripts accumulated to a remarkable extent in the mutants, indicating possible impairment of post-transcriptional processing in the defective plastids (Fig. 6). However, their precise nature is difficult to interpret at present, since information on transcription units in plastids and especially on transcription termination by either NEP or PEP is rather limited.

Collectively, our data and those outlined by DeSantis-Maciossek et al. (1999) demonstrate that the functional and phylogenetic integration of the different RNA polymerases that operate in plastids into the ordered function of the compartmentalised eukaryotic plant genome is a substantially more complex process than previously assumed. The finding that transcription rates, steady-state transcript levels and translational efficiency frequently do not coincide precludes interpretations based on the analysis of only one level of plastid gene expression.

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