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 genespecific 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.

Key words Plastid gene expression · Plastid transformation · RNA polymerases · rpo gene disruption · Run-on transcription

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 x and \( \beta \) subunits of the Escherichia coli RNA polymerase, while the two plastid genes \( rpoC1 \) and \( rpoC2 \) correspond to the N- and C-termini of the \( E. \ coli \) \( \beta' \) 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-operand, 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 albostrains mutant of barley (Hübshmann 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 orf genes 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 Havana) 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 MnCl2, 0.8 mM MgCl2, 4 mM EDTA, 1 mM KH2PO4, 4 mM DTT, 0.2% (w/v) BSA, 0.1% (w/v) PVPP-10, pH 8.0]. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, Calif.), and centrifuged at 3000 × 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 × 106 plastids per mL.

**Run-on transcription assays**

Run-on transcription assays with 2 × 107 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/isoamyl alcohol (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 inhibitorigmatetoxin (Epicentre Technologies, Madison, Wis.), at a final concentration of 20 μM, was performed as described by Krause et al. (1998) with 2 × 106 plastids in a 10-μL volume. Incorporation of [32P]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 BamHI fragments Ba1, Ba2, Ba5, B7, B13, B18, B19, B20, B22, B25, B27, B28 and B29 of tobacco plastid DNA (Sugira et al. 1986) were digested with BamHI and EcoRI. 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 165706–165708 of the tobacco plastome, respectively (Wakasugi et al. 1998). The 3.4-kb PCR product was purified and directly digested with BamHI and EcoRI. 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 32P-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.