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RNA Polymerase Subunits Encoded by the Plastid *rpo* Genes Are Not Shared with the Nucleus-Encoded Plastid Enzyme[1]

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Plastid genes in photosynthetic higher plants are transcribed by at least two RNA polymerases. The plastid *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* genes encode subunits of the plastid-encoded plastid RNA polymerase (PEP), an *Escherichia coli*-like core enzyme. The second enzyme is referred to as the nucleus-encoded plastid RNA polymerase (NEP), since its subunits are assumed to be encoded in the nucleus. Promoters for NEP have been previously characterized in tobacco plants lacking PEP due to targeted deletion of *rpoB* (encoding the β-subunit) from the plastid genome. To determine if NEP and PEP share any essential subunits, the *rpoA*, *rpoC1*, and *rpoC2* genes encoding the PEP α-, β′-, and β″-subunits were removed by targeted gene deletion from the plastid genome. We report here that deletion of each of these genes yielded photosynthetically defective plants that lack PEP activity while maintaining transcription specificity from NEP promoters. Therefore, *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* encode PEP subunits that are not essential components of the NEP transcription machinery. Furthermore, our data indicate that no functional copy of *rpoA*, *rpoB*, *rpoC1*, or *rpoC2* that could complement the deleted plastid *rpo* genes exists outside the plastids.

At least two distinct RNA polymerases are involved in the transcription of plastid genes in photosynthetic higher plants. One of these contains homologs of the *Escherichia coli* enzyme, including the α-, β-, β′-, and β″-subunits encoded in the plastid *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* genes, and is referred to as PEP. The promoters for PEP are reminiscent of the *E. coli* σ^{70}-type promoters, and have two conserved hexameric blocks of sequences (TTGACA or "-35" element; TATAAT or "−10" element) 17 to 19 nucleotides apart. Transcription from PEP promoters initiates 5 to 7 nucleotides downstream of the "−10" promoter element (Igloi and Kössel, 1992; Gruissem and Tonkyn, 1993; Link, 1996). Promoter specificity to PEP is conferred by nuclear-encoded σ-like factors (Isono et al., 1997; Tanaka et al., 1997).

Several reports indicate the existence of a second, NEP activity (Morden et al., 1991; Hess et al., 1993; Allison et al., 1996). A candidate for NEP is an approximately 110-kD protein that has properties similar to the mitochondrial and phage T3/T7 RNA polymerases that may be part of a larger complex (Lerbs-Mache, 1993; Hedtke et al., 1997). NEP promoters share a loose, 10-nucleotide consensus, ATA-GAATA/GAA, overlapping the transcription-initiation site, which is reminiscent of promoters recognized by the mitochondrial and phage T3/T7 RNA polymerases (Hajdukiewicz et al., 1997; Hübschmann and Börner, 1998; for review, see Maliga, 1998).

Plastid RNA polymerase activities with distinct sensitivities to inhibitors are present in higher plants in multisubunit complexes (Pfannschmidt and Link, 1994). Sharing of essential subunits of RNA polymerases has been reported in yeast (Sentenac et al., 1992). Therefore, plastid NEP and PEP could be part of the same complex. To test if NEP and PEP share any essential subunits, the *rpo* genes encoding PEP subunits were removed by targeted gene deletion from the plastid genome. Study of promoter activity in plastids lacking the *rpoB* gene has shown that the PEP β-subunit is essential for PEP transcription activity, but it is not required for transcription by NEP (Allison et al., 1996; Hajdukiewicz et al., 1997).

This study addresses the contribution of the *rpoA*, *rpoC1*, and *rpoC2* genes to transcription from PEP and NEP promoters. We report here that deletion of each of these genes yields photosynthetically defective plants that lack PEP activity while maintaining transcription from NEP promoters. Therefore, *rpoA*, *rpoB*, *rpoC1*, and *rpoC2* encode essential PEP subunits that are not components of the NEP transcription machinery. Furthermore, no functional copy of the *rpo* genes that could complement the deleted plastid *rpo* genes exists outside the plastid.

MATERIALS AND METHODS

Plasmid Construction

Plasmid pGS95 carries the tobacco (*Nicotiana tabacum*) ptDNA *Hin*cII fragment (sites are at nucleotides 78990–82117 in the ptDNA) (Shinozaki et al., 1986), cloned into the *Eco*RV site of a pBSKS+ (Stratagene) plasmid derivative with the *Sca*I site removed. The *Bgl*II/*Sca*I fragment (sites are at positions 80549 and 81466) containing the *rpoA*-coding region was replaced by a chimeric spectinomycin-resistance gene (*aadA*) from plasmid pOVZ34 as a *Bam*HI/*Sma*I fragment (note that the *Ba*mHI and *Bgl*II ends are compatible). Plasmid pOVZ34 is a pUC119 plasmid deriv-

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Abbreviations: NEP, nuclear-encoded plastid RNA polymerase; PEP, plastid-encoded plastid RNA polymerase; ptDNA, plastid DNA.

ative and carries the *aadA* gene in a *psbA* cassette as in plastid vector pOVZ15 (Zoubenko et al., 1994).

Plasmid pGS97 carries the *Pst*I/*Psp*1406I ptDNA fragment (sites are at nucleotides 20283 and 25662) cloned into *Pst*I/*Acc*I-digested pBSIIKS$^+$ plasmid (Stratagene). Note that *Acc*I and *Psp*1406I ends are compatible. In the cloned ptDNA fragment most of the *rpoC1*-coding region is contained between *Acc*I sites at positions 21797 and 23840. The *rpoC1*-coding region between the indicated *Acc*I sites was replaced with a chimeric *aadA* gene as a *Bsp*HI/*Acc*65I fragment (ends were rendered blunt with T4 DNA polymerase) from plasmid pOVZ11, a pUC118 plasmid derivative. The pOVZ11 plasmid carries the P*rrn*::*aadA*::T*psbA* chimeric gene in plastid vector pPRV112 (Zoubenko et al., 1994).

Plasmid pGS99 carries the tobacco ptDNA *Sac*I fragment (sites are at nucleotides 15662 and 22658) cloned into *Sac*I-digested pBSIIKS$^+$ plasmid (Stratagene). The *rpoC2*-coding region was excised as a *St*uI/*Bsr*GI fragment (sites are at nucleotides 17397 and 21048) and replaced with the chimeric *aadA* gene (*Bsp*HI/*Acc*65I fragment) from plasmid pOVZ11, as described for pGS97.

Plastid Transformation

Tungsten particles were coated with pGS95, pGS97, or pGS99 plasmid DNA and introduced into plastids of tobacco leaves with a particle-delivery system (PDS1000He, Bio-Rad) (Svab and Maliga, 1993). Transgenic shoots were regenerated on spectinomycin-containing (500 μg/mL) RMOP medium containing Murashige and Skoog salts (Murashige and Skoog, 1962), 3% Suc, 1.0 mg/L 6-benzylaminopurine, and 0.1 mg/L naphthaleneacetic acid (Svab et al., 1990). White sectors lacking *rpo* genes were identified in variegated leaves during propagation on antibiotic-free plant maintenance (RM) medium (Murashige and Skoog salts and 3% Suc; Murashige and Skoog, 1962). Uniformly transformed white shoots were regenerated from white sectors in spectinomycin-free RMOP medium. The Δ*rpoB* plants were described previously (Allison et al., 1996).

DNA and RNA Gel Blots

Total leaf DNA (Mettler, 1987) was digested with restriction endonucleases and electrophoresed in 0.7% agarose gels (3 μg per lane). For the RNA gel blots, total leaf RNA was extracted using the TRIzol reagent (GIBCO-BRL) and electrophoresed in 1% agarose/formaldehyde gels (5 μg of RNA per lane). RNA and DNA gels were transferred to N-Hybond membranes (Amersham) with the PosiBlot Transfer apparatus (Stratagene). Nucleic acid hybridization was carried out for 3 or more h at 65°C in Rapid Hybridization buffer (Amersham) with [^{32}P]dCTP-labeled double-stranded DNA probes synthesized by random priming (Boehringer-Mannheim). The following gene probes were used: *16SrDNA*, *Eco*RI/*Eco*RV fragment, sites are at nucleotides 138447 and 140855 in the tobacco ptDNA; *atpB*, PCR amplified with primers GCAGGAGCAGGGTCGGTCAAATC and GAGAGGAATGGAAGTGATTGACA (fragment ends are at nucleotides 55751–56512 of the tobacco ptDNA); *clpP*, fragment PCR amplified with primers GAGGGAATGCTAGACG and GACTTTATCGAGAAAG (ends are at nucleotides 73340–73621 of the tobacco ptDNA); *rbcL*, *Bam*HI fragment (restriction sites are at nucleotides 58047–59285 of the tobacco ptDNA); *accD*, fragment PCR amplified with primers GGATTTAGGGGCGAA and GTGATTTTCTCTCCG (ends are at nucleotides 60211–60875 of the tobacco ptDNA); cytoplasmic 25S rRNA gene, fragment PCR amplified with primers TCACCTGCCGAATCAACTAGC and GACTTCCCTTGCCTACATTG.

Primer-Extension Reactions

Reactions were carried out with 10 μg of total leaf RNA (Allison and Maliga, 1995) using the following primers: *16SrDNA*, TTCATAGTTGCATTACTTATAGCTTC (5′ nucleotide complementary to 102757); clpP, GGGACTTTTGGAACACCAATAGGCAT (5′ at nucleotide 74479); *rbcL*, ACTTGCTTTAGTCTCTGTTTGTGGTGACAT (5′ nucleotide complementary to 57616); *accD*, ccgagcTCTTATTTCCTATCAGACTAAGC (5′ nucleotide complementary to 59758); and atpB, CCCCAGAACCAGAAGTAGTAGGATTGA (5′ nucleotide at 56736).

The primers listed above were previously used to map transcription-initiation sites of these genes (Allison et al., 1996; Hajdukiewicz et al., 1997). Lowercase nucleotides are nonplastidic sequences. The position of RNA 5′ ends was determined using these same primers and homologous DNA templates as the reference. Sequence ladders were generated with the Sequenase II kit (Amersham).

RESULTS

Targeted Deletion of *rpoA*, *rpoC1*, and *rpoC2* from the Plastid Genome Yields Pigment-Deficient Plants

To construct vectors for targeted deletion of the *rpo* genes, ptDNA fragments were cloned in Bluescript plasmids. Subsequently, the *rpo*-coding region in the cloned ptDNA fragment was replaced by a selectable spectinomycin-resistance gene (*aadA*) (Fig. 1, A–C). The size of the deletion in the targeting plasmids was: *rpoA*, 90% of the coding region; *rpoC1*, 63% of the coding region (80% of the 3′ exon); and *rpoC2*, 73% of the coding region. The transforming DNA was introduced into tobacco chloroplasts in leaf cells by the biolistic process. Targeted deletion of the *rpo* genes was achieved by replacement of the coding region with *aadA* as the result of two homologous recombination events via the flanking ptDNA sequences (Fig. 1, A–C). Culture of the bombarded leaf segments on spectinomycin-containing RMOP medium facilitated shoot regeneration with transformed plastid genomes. Since the chimeric *aadA* gene is transcribed by PEP, cells carrying only knockout plastid genomes are sensitive to spectinomycin. Therefore, the developing green shoots and calli contained plastids with a mixed population of knockout plastid genomes expressing *aadA,* and wild-type plastid genomes expressing the targeted *rpo* subunit gene (Fig. 2A). To facilitate formation of homoplasmic sectors, the

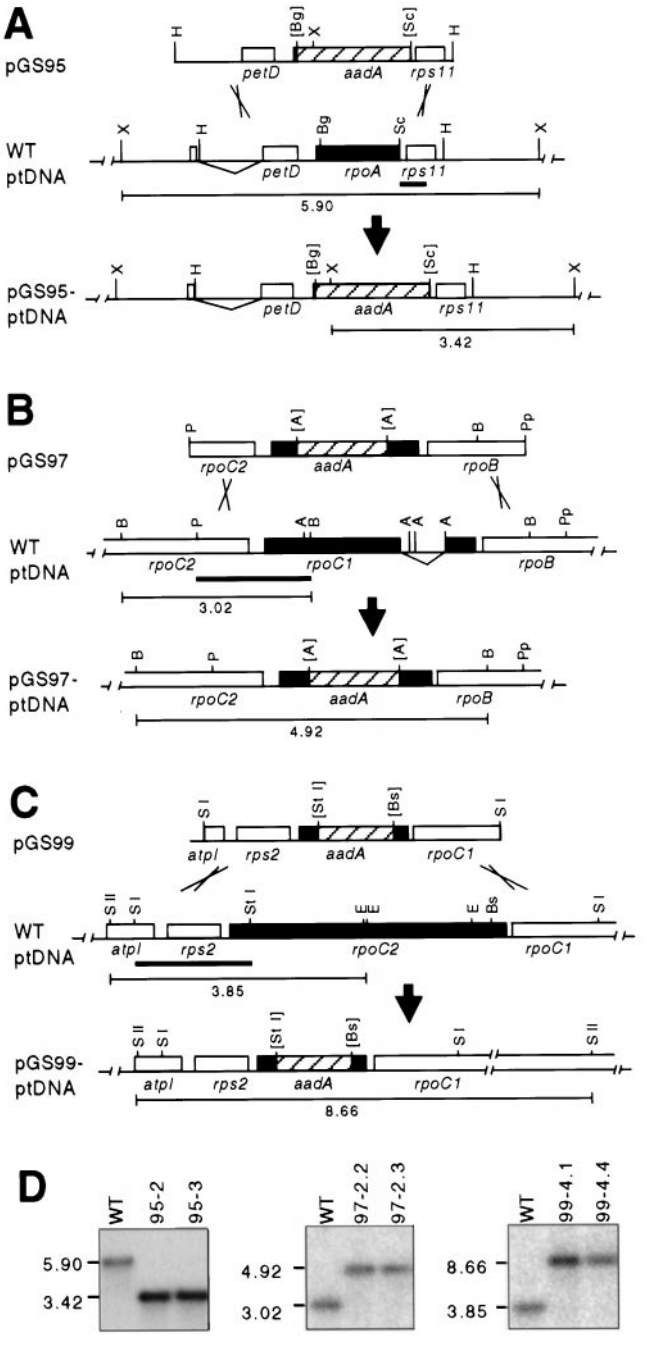

Figure 1. Targeted deletion of *rpo* genes from the plastid genome. A, Deletion of the *rpoA* gene. Homologous recombination events (hatched lines) between ptDNA sequences in vector pGS95 and the tobacco plastid genome yields a genome lacking *rpoA*. Probes for Southern blots in D are marked with thick horizontal lines. Map position of the probed restriction fragments with size in kilobases is shown below the maps. *aadA*, Chimeric spectinomycin resistance gene (Svab and Maliga, 1993); *rpoA*, *rpoB*, *rpoC1*, and *rpoC2*, the plastid genes encoding the α-, β-, β′-, and β″-subunits of PEP, respectively; *atpI*, *petD*, *rps2*, and *rps11*, plastid genes (Shinozaki et al., 1986). Restriction endonuclease cleavage sites: H, *Hin*cII; X, *Xba*I; Bg, *Bgl*II; Sc, *Sca*I; P, *Pst*I; B, *Bam*HI; Pp, *Psp*1406I; A, *Acc*I; SI, *Sac*I; SII, *Sac*II; StI, *Stu*I; E, *Eco*RV; Bs, *Bsr*GI. Brackets indicate restriction sites eliminated during cloning. B, Deletion of the *rpoC1* gene. Homologous recombination events (crossed lines) between ptDNA sequences in vector pGS97 and the tobacco plastid genome yields a genome lacking *rpoC1*. C, Deletion of the *rpoC2* gene. Homologous recombination events (crossed lines) between ptDNA sequences in vector pGS99 and the tobacco plastid genome yields a genome lacking *rpoC2*. D, Southern probing demonstrates a uniform population of transformed plastid genomes. Total cellular DNA was isolated from the leaves of plants transformed with plasmids pGS95 (targeting *rpoA*), pGS97 (targeting *rpoC1*), and pGS99 (targeting *rpoC2*), and from wild-type green leaves (WT). Data are shown for two independently transformed lines (pGS95-2, pGS95-3), or two plants derived from the same transformation event (pGS97-2.2, pGS97-2.3 and pGS99-4.1, pGS99-4.4).

shoots regenerating on the leaf segments were excised and transferred onto antibiotic-free plant maintenance medium. Plastid genome sorting in developing shoots facilitated formation of chimeric leaves, with white sectors containing a uniform population of knockout plastid genomes and green sectors with wild-type ptDNA (Fig. 2B). A second cycle of shoot regeneration from the white sectors yielded white plants (Fig. 2C). These plants carry a uniform population of transformed ptDNA lacking *rpoA* (Fig. 1, A and D), *rpoC1* (Fig. 1, B and D), or *rpoC2* (Fig. 1, C and D).

Plastid Transcript Accumulation Pattern Is Similar in All Plastid *rpo*-Deleted Mutants

Deletion of genes for essential PEP subunits prevents assembly of functional PEP enzyme. In the absence of PEP activity, mRNAs will accumulate at significant levels only from NEP promoters. The mRNAs initiating from PEP promoters will be absent. However, transcripts for genes with PEP promoters only may be present at low levels due to read-through transcription from upstream NEP promoters and processing (Allison et al., 1996; Hajdukiewicz et al., 1997). We expect that if NEP and PEP share a subunit, deletion of the relevant *rpo* gene should prevent mRNA accumulation from both PEP and NEP promoters. Therefore, steady-state mRNA level in the Δ*rpo* plants was determined for a number of plastid genes (Fig. 3). The *rbcL* gene in tobacco plastids is transcribed from a PEP promoter. Accumulation of *rbcL* mRNA in each of the *rpo* deletion derivatives at significantly reduced (25-fold lower) levels is consistent with the lack of transcription from the PEP promoter, and with the accumulation of processed read-through transcripts (Allison et al., 1996). The *atpB*, *16SrDNA*, and *clpP* genes have both PEP and NEP promoters (Allison et al., 1996; Hajdukiewicz et al., 1997). High steady-state levels of mRNAs for each of these genes in the Δ*rpo* plants is consistent with sustained NEP promoter activity. The *accD* gene in tobacco is transcribed from a NEP promoter from which mRNA accumulation in wild-type chloroplasts is low, while it is highly elevated in Δ*rpoB* plants (Hajdukiewicz et al., 1997). Accumulation of *accD* mRNA in the *rpoA*, *rpoC1*, and *rpoC2* deletion derivatives is elevated as in the Δ*rpoB* plants (Fig. 3). Sustained NEP activity in the *rpo*-deleted plants indicates the lack of contribution of the PEP α-, β′-, and β″-subunit to NEP function.

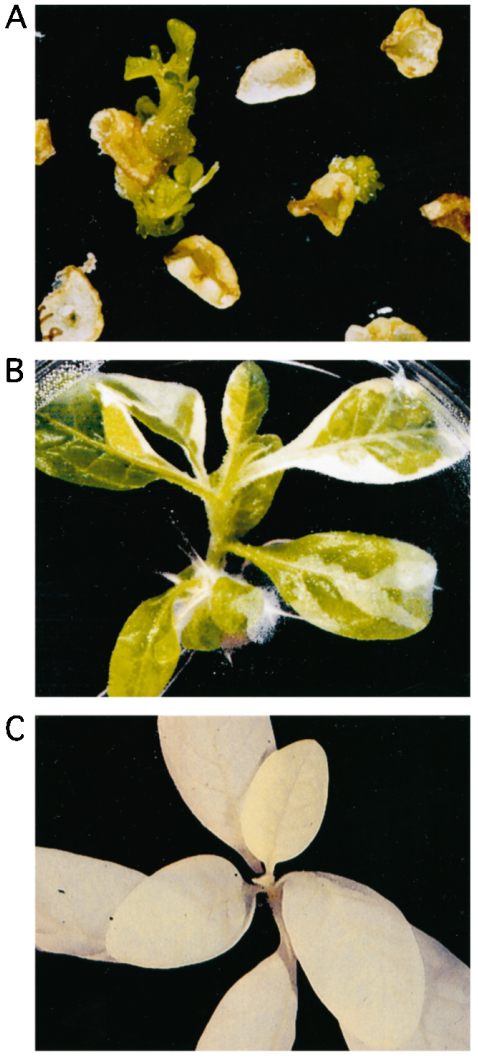

Figure 2. Isolation of homoplasmic Δ*rpoA* plants. A, Callus and shoots carrying a mixed population of wild-type and Δ*rpoA* plastid genomes are green. B, Chimeric leaves with white (transgenic) and green (wild-type) sectors. C, White, homoplasmic Δ*rpoA* plant with transgenomes only.

Promoter Utilization Is Identical in All *rpo* Deletion Lines

Many of the plastid genes have multiple promoters. Therefore, it was important to show which of the promoters are active in the Δ*rpo* plants. Transcription activity from previously characterized NEP and PEP promoters was determined by mapping transcript 5′ ends with primer-extension analysis.

The photosynthetic *rbcL* gene is transcribed from a single PEP promoter (P*rbcL*-182 in wild-type tobacco chloroplasts [Shinozaki and Sugiura, 1982]). This promoter is not active in the *rpo*-deletion derivatives (Fig. 4). The 5′ end at position −59 (indicated by ⋊ in Fig. 4) derives from longer transcripts by processing in both wild-type and Δ*rpo* mutants (Mullet et al., 1985; Allison et al., 1996). The *atpB* gene in wild-type tobacco is transcribed from four promoters (Fig. 4; Hajdukiewicz et al., 1997). The P*atpB*-255, P*atpB*-488/-502, and P*atpB*-611 PEP promoters are active in the wild-type, but not in the Δ*rpo* plants (Fig. 4). In contrast, the P*atpB*-289 NEP promoter is active in the leaves of wild-type and Δ*rpo* plants. The tobacco rRNA operon (*rrn*) has one promoter for each of the two RNA polymerases (Vera and Sugiura, 1995; Allison et al., 1996). The PEP promoter initiates transcription 113 and 114 bp upstream of the mature 16SrRNA in wild-type leaves, whereas it is inactive in the *rpo*-deleted plants. In contrast, the P*rrn*-62 NEP promoter is inactive in wild-type leaves, but active in the *rpo* deletion derivatives (Fig. 4). The *clpP* gene is transcribed from four promoters (Hajdukiewicz et al., 1997; fig. 5B). Although the PEP transcript (P*clpP*-95) is absent in all *rpo*-deleted lines, the activity of the three NEP promoters (P*clpP*-53, P*clpP*-173, P*clpP*-511) is evident in the Δ*rpo* mutants (Fig. 4; Hajdukiewicz et al., 1997). Finally, *accD* has one NEP promoter. This promoter, located 129 bp upstream of the *accD*-coding region (Hajdukiewicz et al., 1997), is active in the lines deficient in the PEP α-, β-, β′-, and β″-*E. coli*-like subunits (Fig. 4). In summary, the PEP promoters (six tested) were inactive, whereas transcription initiated at the same position from each of the six NEP promoters tested in the *rpoA*-, *rpoB*-, *rpoC1*-, and *rpoC2*-deletion derivatives.

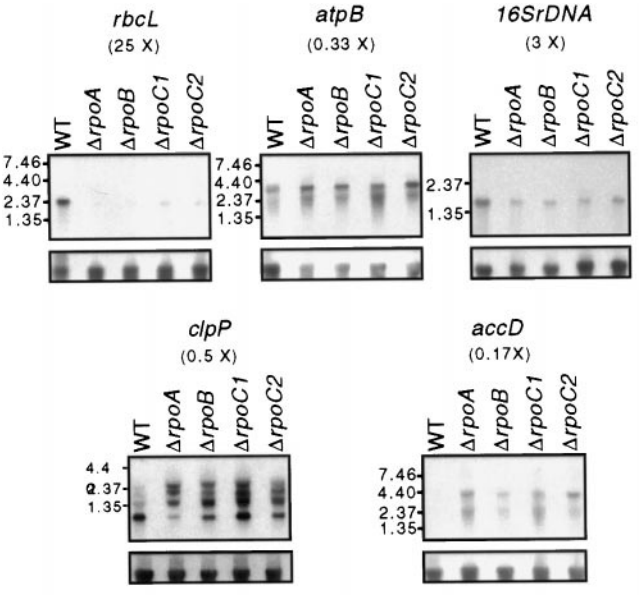

Figure 3. Accumulation of plastid mRNAs in wild-type and plastid *rpo* gene deletion derivatives. Data are shown for genes carrying only PEP promoters (*rbcL*), only NEP promoters (*accD*), or PEP and NEP promoters (*clpP*, *16S rDNA*, *atpB*) in wild-type, Δ*rpoA*, Δ*rpoB*, Δ*rpoC1*, and Δ*rpoC2* leaves. The excess of wild-type over Δ*rpo* intensities (average of the four Δ*rpo* lines) for each probe is given in parentheses. Gel blots were prepared with total leaf RNA (5 μg per lane) from wild-type plants, and in plants transformed with plasmids pGS95 (Δ*rpoA*), pGS97 (Δ*rpoC1*), and pGS99 (Δ*rpoC2*). Upper panels show blots probed for plastid genes. Lower panels show loading controls, obtained by probing the same filters for the cytoplasmic 25S rRNA. The blots were scanned with a phosphor imager (Molecular Dynamics, Sunnyvale, CA). Hybridization signals were quantified with Imagequant software (Molecular Dynamics) and normalized to the 25S rRNA signal.

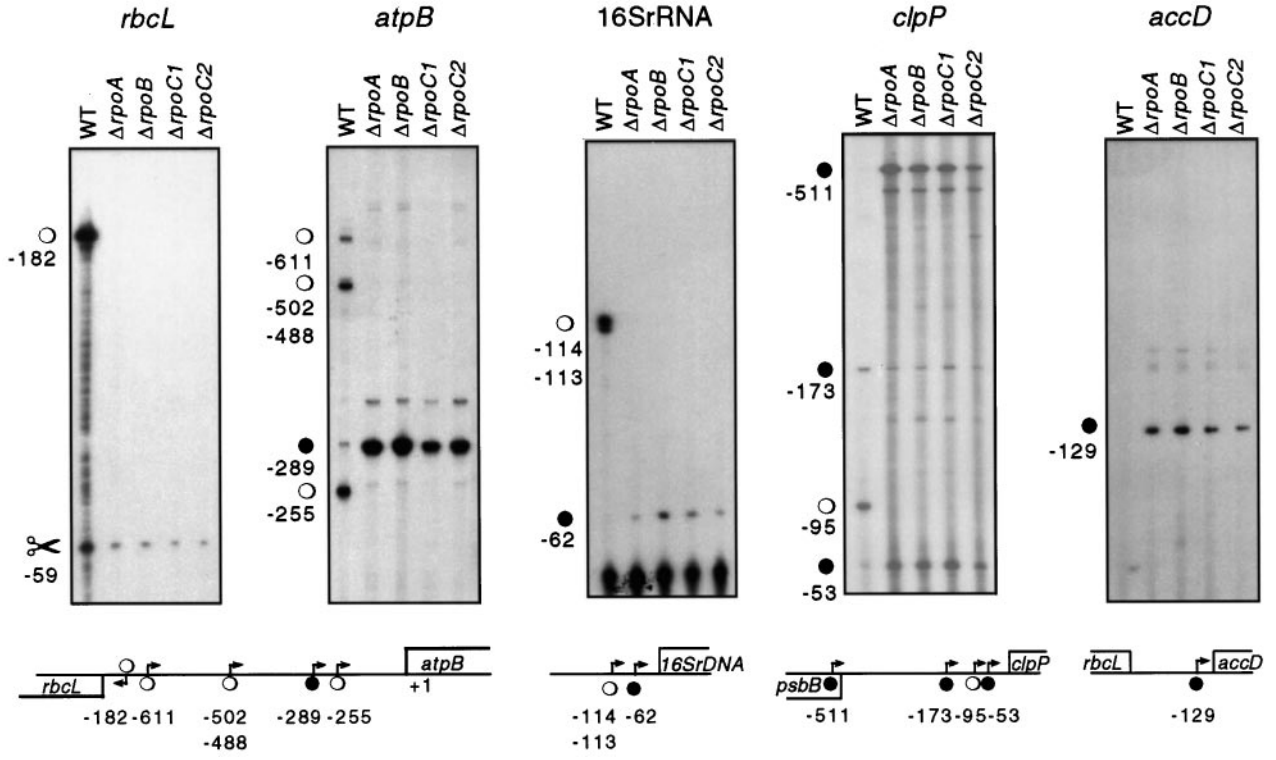

Figure 4. Mapping of transcription-initiation sites in plastids of wild-type and *rpo*-deletion derivatives. Primer-extension data are shown for the *rbcL*, *atpB*, *16SrDNA*, *clpP*, and *accD* genes. Mapped NEP (●) and PEP (○) promoters are identified by the distance between the transcription-initiation site and the translation-initiation codon (ATG) in nucleotides (Allison et al., 1996; Hajdukiewicz et al., 1997). Processing sites are also marked (✂). Schematic maps with transcription-initiation sites for NEP and PEP promoters are shown at the bottom of the figure.

DISCUSSION

The first successful targeted deletion of a plastid RNA polymerase subunit gene was that of *rpoB* from the tobacco plastid genome (Allison et al., 1996). We report here deletion of the genes for the remaining core subunits of the plastid-encoded RNA polymerase (*rpoA*, *rpoC1*, and *rpoC2*). We propose that deletion of the *rpo* genes from the plastid genome is possible because NEP, an alternative, nucleus-encoded enzyme transcribes all essential housekeeping and metabolic genes. Attempts at targeted deletion of the plastid *rpoB1*, *rpoB2*, or *rpoC2a* genes in the unicellular alga *Chlamydomonas reinhardtii* failed to yield homoplasmic cells lacking PEP (Rochaix, 1997). Therefore, *C. reinhardti* may not have NEP, or it may have NEP but transcription of at least some of the essential genes is dependent on PEP. NEP promoters are active in the liverwort *Marchantia polymorpha* and the conifer *Pinus contorta*, indicating duplication of the plastid-transcription machinery early during the evolution of the land plants (D. Silhavy and P. Maliga, unpublished data).

Deletion of the plastid *rpo* genes in this study led to the loss of transcription from σ^{70}-type PEP promoters and to a mutant phenotype. These findings indicate that subunits of the *E. coli*-like plastid RNA polymerase are the products of plastid genes and that no functional copy of the *rpo* genes exists outside the plastids, which could complement the deleted-plastid *rpo* genes. Our experiments extend the earlier work of Bogorad and coworkers, who provided evidence for the contribution of the plastid *rpo* genes to the plastid RNA polymerase activity by sequencing the N-terminal amino acids of a purified maize RNA polymerase (Hu and Bogorad, 1990; Hu et al., 1991). Contribution of plastid *rpo* genes to plastid RNA polymerase activity was also shown by the sensitivity of in vitro transcription to antibodies obtained in rabbits immunized with fusion peptides expressed from genes containing *rpo* gene segments (Little and Hallick, 1988).

We report here that deletion of the plastid-encoded PEP subunit genes does not affect NEP transcription specificity, so PEP subunits are not shared with NEP. The α-subunit gene could be deleted without interfering with the expression of other plastid genes since *rpoA* is the last open reading frame of the *rpl23* operon. Interpretation of data in plants lacking the β″-subunit gene was also unambiguous, since *rpoC2* is the last gene of the *rpoB* operon. *rpoC1* is the second gene of the *rpoB* operon, which, after deletion of *rpoC1*, consists of three genes: *rpoB*, *aadA* (replacing *rpoC1*), and *rpoC2*. Since polycistronic mRNAs are efficiently translated on the plastid ribosomes (Staub and Maliga, 1995), it is likely that deletion of *rpoC1* does not interfere with the expression of the downstream *rpoC2* gene. Furthermore, deletion of *rpoC1* could only affect the expression of *rpoC2* already shown to be nonessential for NEP activity. Transcription of the *rpoB* operon initiates 345 nucleotides up-

stream of *rpoB* (nucleotide position 27846 in the plastid genome; G. Serino and P. Maliga, unpublished data). In the previously described Δ*rpoB* plant (Allison et al., 1996), expression of the entire *rpoB* operon should be affected since the deletion included the operon promoter. Therefore, the Δ*rpoC2*, Δ*rpoC1*, and Δ*rpoB* plants form a series lacking the β″ (Δ*rpoC2*), β′ and possibly β″ (Δ*rpoC1*), and β, β′, and β″ (Δ*rpoB*) PEP subunits. Collectively, these data indicate that none of the PEP subunits is part of the NEP complex. Identification of NEP subunits will depend on purification of the NEP enzyme and development of an in vitro transcription assay. Important steps in this direction were the cloning of the gene for a 113-kD protein, the likely catalytic subunit of NEP (Hedtke et al., 1997), and identification of promoters recognized by the NEP-transcription machinery (Allison et al., 1996; Hajdukiewicz et al., 1997; Hübschmann and Börner, 1998).

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