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Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants

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The plastid genome in higher plants encodes subunits of an *Escherichia coli*-like RNA polymerase which initiates transcription of plastid genes from sequences resembling *E.coli* σ^{70}-type promoters. By deleting the gene for the essential β subunit of the tobacco *E.coli*-like RNA polymerase, we have established the existence of a second plastid transcription system which does not utilize *E.coli*-like promoters. In contrast to the *E.coli*-like RNA polymerase, the novel transcription machinery preferentially transcribes genetic system genes rather than photosynthetic genes. Although the mutant plants are photosynthetically defective, transcription by this polymerase is sufficient for plastid maintenance and plant development.

Keywords: biolistic transformation/plastid promoter/plastid RNA polymerase/*rpoB* gene/tobacco

Introduction

Chloroplast genes are transcribed by an RNA polymerase containing plastid-encoded subunits homologous to the α, β and β′ subunits of *Escherichia coli* RNA polymerase. The promoters utilized by this enzyme are similar to *E.coli* σ^{70} promoters, consisting of –35 and –10 consensus elements (reviewed in Igloi and Kössel, 1992; Gruissem and Tonkyn, 1993; Link, 1994). Promoter selection by the plastid-encoded RNA polymerase is dependent on nuclear-encoded σ-like factors (reviewed by Link, 1994). In addition, transcription activity from some promoters is modulated by nuclear-encoded transcription factors interacting with elements upstream of the core promoter (Sun *et al.*, 1989; Iratni *et al.*, 1994; Allison and Maliga, 1995; Kim and Mullet, 1995). These factors mediate nuclear control of plastid gene expression in response to developmental and environmental cues.

Several reports have suggested the existence of an additional plastid-localized, nuclear-encoded RNA polymerase. First, in the plastids of the non-photosynthetic parasitic plant *Epifagus*, transcription occurs despite the lack of all functional *E.coli*-like RNA polymerase genes in the plastid genome (Morden *et al.*, 1991). It remains to be determined, however, whether the genes for the *E.coli*-like RNA polymerase have been transferred to the nucleus, or whether the remaining plastid transcription is accomplished by a nuclear-encoded, non-*E.coli*-like polymerase. Second, both heat-bleached rye and the barley mutant *albostrians* exhibit a severe deficiency in plastid ribosomes. Nonetheless, transcription of a subset of plastid genes is detected, although translation of plastid-encoded RNA polymerase subunits is absent (Hess *et al.*, 1993, 1994). Third, at least a few plastid genes have no recognizable σ^{70}-like promoter elements, suggesting that these genes may be transcribed by a different RNA polymerase (Gruissem *et al.*, 1986; Neuhaus *et al.*, 1989; Klein *et al.*, 1992, 1994; Vera and Sugiura, 1995). However, transcription from these non-consensus promoters may be attributed to different σ-like factors modifying the selectivity of the *E.coli*-like polymerase core enzyme. Fourth, biochemical analyses have detected multiple separable RNA polymerase activities in isolated chloroplasts, ranging from large multisubunit complexes to single subunit activities (Little and Hallick, 1988; Lerbs-Mache, 1993; Pfannschmidt and Link, 1994; reviewed in Igloi and Kössel, 1992; Gruissem and Tonkyn, 1993). As yet, there is no consensus regarding the subunit composition, origin and promoter specificities of these activities.

Although the above evidence suggests the presence of more than one type of plastid transcription machinery, uncertainty remains as to the existence and characteristics of a second RNA polymerase which is distinct from the *E.coli*-like enzyme. To address this question directly, we deleted the gene for one of the essential subunits of the *E.coli*-like enzyme from the tobacco plastid genome and asked whether transcription was maintained in the mutant plastids. Our data indicate that, in the absence of the plastid-encoded *E.coli*-like RNA polymerase, expression of photogenes is reduced dramatically. In contrast, transcript levels for the plastid genes encoding the gene expression apparatus are similar to levels in wild-type plants. Therefore, the non-*E.coli*-like RNA polymerase selectively transcribes a subset of plastid genes which is sufficient to maintain the plastid compartment. This second transcription apparatus, designated as the genetic system (GS) RNA polymerase, does not initiate from typical *E.coli* σ^{70} promoters but recognizes a novel promoter sequence.

Results

Deletion of rpoB from the plastid genome results in a pigment-deficient phenotype

The *E.coli*-like RNA polymerase of higher plant plastids contains plastid-encoded α, β, β′ and β′′ subunits. The α subunit gene, *rpoA*, is located at the distal end of a large polycistronic operon containing genes encoding ribosomal protein subunits, whereas the β, β′ and β′′ subunit genes (*rpoB*, *rpoC1* and *rpoC2*, respectively) are assembled in a separate operon (Shinozaki *et al.*, 1986). To avoid disrupting plastid genes for other functions, we targeted the *rpoB* gene for deletion, since it is the first reading frame of an operon encoding exclusively subunits of the

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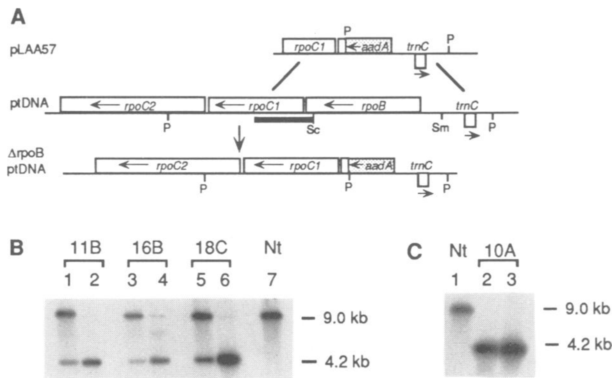

Fig. 1. Deletion of *rpoB* from the tobacco plastid genome by targeted gene replacement. (**A**) Homologous recombination (diagonal lines) via plastid DNA sequences flanking *aadA* in plasmid pLAA57 results in replacement of *rpoB* (*SacI–SmaI* fragment) in the wild-type plastid genome (ptDNA) with *aadA* sequences, yielding the Δ*rpoB* plastid genome (Δ*rpoB* ptDNA). Abbreviations: *rpoB*, *rpoC1*, *rpoC2* are plastid genes encoding the β, β′ and β″ subunits of the *E.coli*-like RNA polymerase (Igloi and Kössel, 1992; Gruissem and Tonkyn, 1993); *trnC* is a plastid tRNA gene (Shinozaki *et al.*, 1986); *aadA* is a chimeric spectinomycin resistance gene (Svab and Maliga, 1993). Restriction enzyme recognition sites: P, *PstI*; Sm, *SmaI*; Sc, *SacI*. The thick black line under *rpoC1* indicates DNA used as the probe in DNA gel-blots shown in (B). (**B**) Pigment deficiency is associated with the deletion of *rpoB*. Total cellular DNA was isolated from green (lanes 1, 3 and 5) and white (lanes 2, 4 and 6) leaf tissue from three independently transformed lines (line Nt-pLAA57-11B, lanes 1 and 2; line Nt-pLAA57-16B, lanes 3 and 4; line Nt-pLAA57-18C, lanes 5 and 6) and from wild-type green leaf tissue (Nt, lane 7). The DNA was digested with *PstI*, and the gel-blot was hybridized with a DNA fragment (nucleotide positions 22 883–24 486 of the ptDNA, numbering according to Shinozaki *et al.*, 1986), containing part of *rpoC1* (thick black line in A). The probe hybridizes to a 9.0 kb fragment from the wild-type genome and a 4.2 kb fragment from the Δ*rpoB* ptDNA. (**C**) No wild-type ptDNA copies were detected by DNA gel-blot analysis in white shoots of line Nt-pLAA57-10A (lane 2) and white seed progeny of a grafted chimeric plant from the same line (lane 3). DNA from wild-type green leaf tissue was loaded in lane 1. Note the absence of the wild-type ptDNA 9.0 kb fragment in Δ*rpoB* plants. The blot was prepared as for (B).

E.coli-like plastid polymerase. The deletion was accomplished by replacing most of the *rpoB* coding region (3015 out of 3212 bp) and 691 bp of upstream non-coding sequence, with a chimeric spectinomycin resistance (*aadA*) gene (Svab and Maliga, 1993) in a cloned plastid DNA (ptDNA) fragment. The resulting plasmid was introduced by particle bombardment into tobacco chloroplasts, where the *aadA* gene integrated into the plastid genome via flanking plastid DNA sequences as diagrammed in Figure 1A. Since the plastid genetic system is highly polyploid, with every leaf cell containing up to 10 000 identical copies of the ptDNA, selective amplification of transformed genome copies was carried out by growing the bombarded tissue on spectinomycin-containing medium (Maliga, 1993).

From the initial round of selection, we obtained several spectinomycin-resistant plants exhibiting sectors of white leaf tissue (Figure 2A). DNA gel-blot analysis of white and green sectors from three independently transformed lines indicated that the pigment deficiency was correlated with deletion of *rpoB* (Figure 1B). Most DNA samples from the pigment-deficient tissue, for example lane 4 in Figure 1B, contained a mix of wild-type and transformed genome copies. To obtain plants containing only transformed plastid genomes, shoots were regenerated from the white sectors on drug-free medium. This procedure yielded white plants (Figure 2A) in which no wild-type ptDNA could be detected by DNA gel-blot analysis (Figure 1C). To confirm the lack of wild-type plastid genomes, we took leaf pieces from the white plants and transferred them to regeneration medium. Since cells containing wild-type plastid genomes proliferate faster than cells containing Δ*rpoB* genomes, plastids containing wild-type genomes would have sorted out rapidly in the absence of drug selection to generate green tissue sectors. Repeated regeneration from leaves of these white plants on non-selective (spectinomycin-free) medium yielded only white shoots. Recovery of exclusively white shoots from white transgenic leaves on spectinomycin-free medium indicated the complete absence of wild-type plastid genome copies in all leaf layers and cell types.

It is difficult to obtain seed from tobacco plants grown in sterile culture. Fortuitously, during plant regeneration from primary transformants, we obtained a periclinal chimera (Poethig, 1989) homoplasmic for the plastid mutation in the L2 leaf layer (Figure 2A). This line was grafted on wild-type tobacco and was raised to maturity in the greenhouse (Figure 2B). Seed from self-pollinated flowers gave rise to uniformly white seedlings, in which no wild-type plastid genomes could be detected by DNA gel-blot analysis (Figure 1C).

Plastids in ΔrpoB plants lack stacked thylakoid membranes

The pigment-deficient Δ*rpoB* plants were unable to grow photoautotrophically. However, if maintained on sucrose-containing medium to compensate for the lack of photosynthesis, they grew normally but at a reduced rate compared with wild-type plants, and exhibited no noticeable changes in organ morphology. Moreover, Δ*rpoB* seedlings germinated at a high efficiency on sucrose medium in sterile culture and developed into plants. These observations indicate that the *E.coli*-like plastid RNA polymerase is not required for maintenance of the non-photosynthetic plastid functions necessary for plant growth and differentiation.

An examination of plastid ultrastructure in leaf mesophyll cells of the Δ*rpoB* plants revealed that the mutant plastids were smaller and rounder than wild-type chloroplasts, averaging 2–5 μm in length as compared with 5–9 μm for wild-type chloroplasts. The Δ*rpoB* plastids are thus larger than undifferentiated proplastids whose average size is 1 μm (Thomas and Rose, 1983). In addition, Δ*rpoB* plastids typically contained multiple vesicles of irregular size and shape, and lacked the arrays of stacked thylakoid membranes which are characteristic of photosynthetically active chloroplasts (Figure 3).

Transcription of plastid genes is maintained in ΔrpoB plastids

In the absence of the β subunit, no transcription was expected from plastid σ^{70}-type promoters. To determine whether any transcription activity was maintained in the Δ*rpoB* plastids, accumulation of RNAs was examined by RNA gel-blot analysis. Transcripts were surveyed for two different classes of plastid genes (Shinozaki *et al.*, 1986). The first group included genes encoding subunits of the

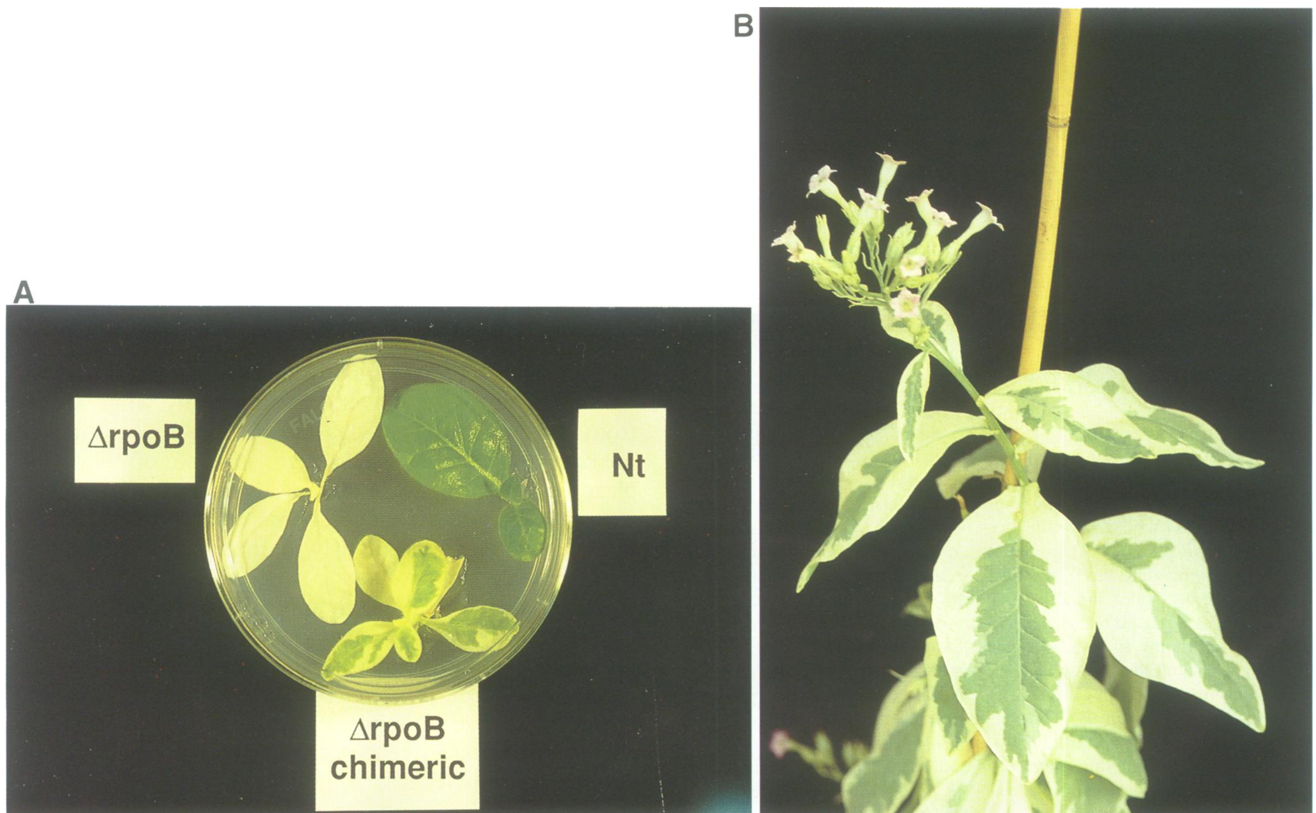

Fig. 2. Deletion of *rpoB* results in a pigment-deficient phenotype. (**A**) Green wild-type (right), pigment-deficient Δ*rpoB* (left) and chimeric (centre) plants are shown. (**B**) A flowering chimeric plant in the greenhouse. Note the white leaf margins indicating Δ*rpoB* plastids in the second leaf layer which forms the germline cells (Poethig, 1989).

photosynthetic apparatus: the *psbD/C* operon, encoding subunits D2 and CP43 of photosystem II; *rbcL*, encoding the large subunit of ribulose-1,5-bisphosphate carboxylase; and *psbA*, encoding the D1 subunit of the photosystem II reaction centre. The second group contained genes for components of the gene expression apparatus: *rpl16* encoding a ribosomal protein, and *16SrDNA* encoding the plastid 16S rRNA. All plastid RNA quantitations were normalized to cytoplasmic 25S rRNA levels.

Surprisingly, accumulation of mRNAs was detected for all the genes examined. However, the effect of the *rpoB* deletion on transcript accumulation was dramatically different for the two classes of genes. The steady-state mRNA levels of the photosynthetic genes *psbD/C*, *rbcL* and *psbA*, were reduced 40- to 100-fold compared with wild-type levels (Figure 4A; signals were visible in all Δ*rpoB* lanes upon longer exposure, data not shown). In contrast, transcript levels for genetic system genes were much less affected. We measured a 3-fold reduction for 16S rRNA, and an actual increase for the multiple transcripts arising from the polycistronic operon containing the *rpl16* gene (Figure 4B). These data indicate that, while expression of genes encoding the photosynthetic apparatus is defective in the Δ*rpoB* plants, the RNAs for genes involved in housekeeping functions accumulate to approximately wild-type, or higher, levels.

Many plastid genes with common functions are co-localized in operons. However, a few cases of mixed operons exist in which genetic system genes are grouped with photosynthetic genes. We examined transcript levels for *atpI*, encoding a subunit of the ATP synthase complex, which is part of the mixed-class *atpA* operon encoding four photosystem genes as well as a ribosomal protein (*rps2*) gene. Hybridization with an *atpI* probe revealed approximately equal levels of transcripts from this operon in the Δ*rpoB* plants compared with wild-type (Figure 4B). Therefore, when a photosynthetic gene is part of a mixed operon its expression is also elevated, possibly to accommodate the requirement for high transcript levels of the co-localized housekeeping genes.

Deletion of rpoB does not alter plastid genome copy number in leaf cells

The plastid RNA quantitations described above were normalized to cytoplasmic 25S rRNA levels. However, should the plastid genome copy number per cell be significantly lower in the mutant plants, the reduced levels of RNAs measured for several plastid genes could be due to a reduced number of DNA template molecules. To test whether changes in plastid genome copy number contributed to the estimated differences in gene expression, total cellular DNA and RNA were prepared from equal amounts of leaf tissue from wild-type and Δ*rpoB* plants. To compare the number of plastid genome copies per equivalent leaf mass, DNA gel-blots were carried out with total cellular DNA extracted from equal amounts of leaf tissue, and hybridized with a *16SrDNA* probe. The amount of total DNA loaded was similar in the wild-type and Δ*rpoB* samples, based on ethidium bromide staining (not shown). Quantitation of the hybridization signal demonstrated a similar number of plastid genome copies in wild-type and Δ*rpoB* samples (Figure 5). We conclude that

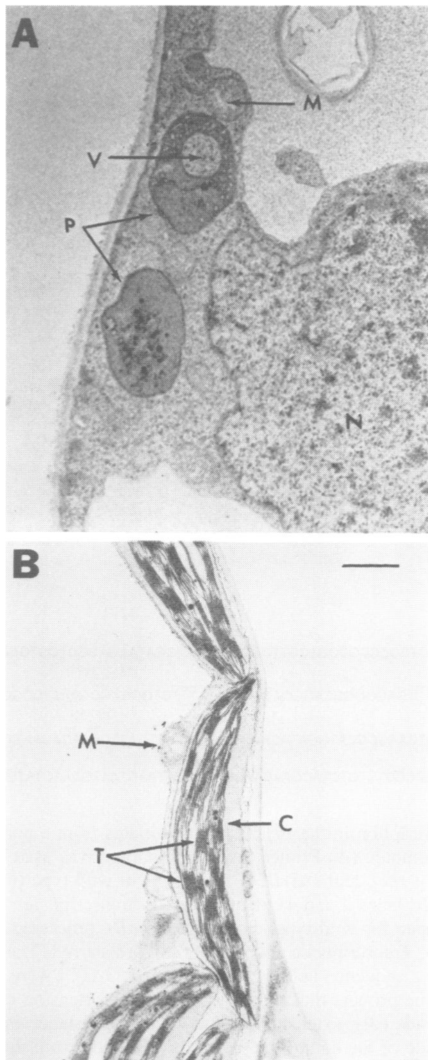

Fig. 3. (A) Plastids (P) in leaf mesophyll cells of Δ*rpoB* plants lack organized photosynthetic membranes. Abbreviations: N, nucleus; V, vesicles, M, mitochondrion. (B) For comparison, an electron micrograph of a wild-type leaf chloroplast (C) with thylakoid membranes (T) is shown. Magnification in both (A) and (B) is 7800×. The bar corresponds to 1 μm.

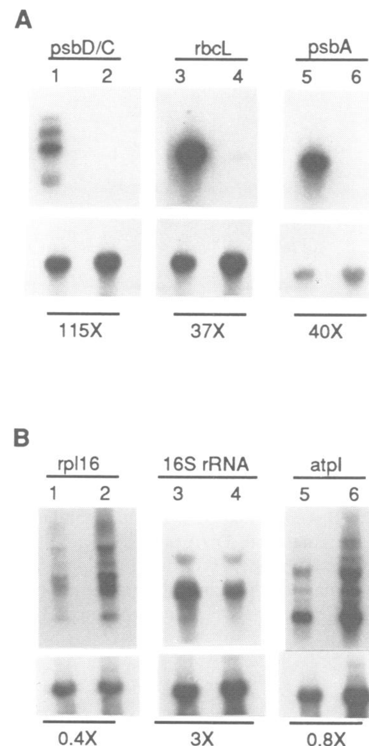

Fig. 4. Accumulation of plastid mRNAs for (A) photosynthetic genes and (B) genetic system genes in the Δ*rpoB* plants. Gel-blots were prepared with total cellular RNA (A, 3 μg per lane; B, 5 μg per lane) from wild-type (lanes 1, 3 and 5) and Δ*rpoB* (lanes 2, 4 and 6) leaf tissue, and hybridized to the indicated plastid gene sequences. Upper panels in (A) and (B): blots shown above were reprobed with *25S rDNA* sequences. Lower panels in (A) and (B): hybridization signals were quantified with a Molecular Dynamics PhosphorImager and normalized to the 25S rRNA signal. The fold excess of wild-type over Δ*rpoB* signal intensities for each probe is shown below the lanes.

deletion of the *rpoB* gene did not change substantially the total plastid genome number in the leaf tissue. The amount of 16S rRNA in equal amounts of wild-type and Δ*rpoB* leaf tissue was reduced by 2.5-fold in the Δ*rpoB* plants. This value is similar to the 3-fold reduction estimated when normalizing for the cytoplasmic 25S rRNA signal (Figure 4B).

Transcription from σ^{70}-type promoters is not detected in ΔrpoB plants

The accumulation of plastid RNAs confirmed that there is RNA polymerase activity in plastids lacking the β subunit of the *E.coli*-like enzyme. To establish whether or not the plastid transcripts detected in Δ*rpoB* plants were products of transcription from a σ^{70}-type promoter, we mapped the 5′ transcript ends for four genes, *rbcL* (Shinozaki and Sugiura, 1982), *16SrDNA* (Vera and Sugiura, 1995), *psbA* (Sugita and Sugiura, 1984) and *psbD* (Yao *et al.*, 1989), for which the transcription initiation sites have been established previously. None of the 5′ ends mapped to σ^{70}-type promoter initiation sites (data are shown for *rbcL* and *16SrDNA* in Figure 6A). Therefore, we conclude that the residual RNA polymerase activity in the Δ*rpoB* plastids was not due to an *E.coli*-like enzyme, but represents a second unique plastid transcription system. We refer to this distinct RNA polymerase enzyme as the genetic system (GS) RNA polymerase, to distinguish it from the *E.coli*-like enzyme. Since the tobacco plastid genome has been fully sequenced, and since the few unidentified reading frames bear no sequence similarity to known RNA polymerase subunits (Shinozaki *et al.*, 1986), transcription by the GS RNA polymerase relies on nuclear gene products.

In the absence of transcription from σ^{70}-type promoters in the Δ*rpoB* plants, the question remained: what promoters were the source of the plastid RNAs. Interestingly, the major transcript 5′ end for the photosynthetic gene *rbcL* mapped to position –59 with respect to the translation initiation codon (Figure 6A, triangle). This 5′ end has been shown to result from processing rather than transcription initiation (Hanley-Bowdoin *et al.*, 1985; Mullet *et al.*, 1985; Reinbothe *et al.*, 1993). The same result was true for *psbD* transcripts: the major detectable 5′ end in the Δ*rpoB* RNA mapped to position –132 (data not shown), also known to be a processed end (Yao *et al.*, 1989). For both *rbcL* and *psbD*, additional minor transcript ends

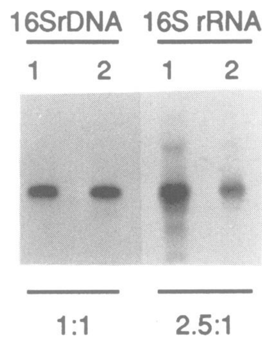

Fig. 5. Normalization of 16S rRNA transcript levels to total plastid genome levels. Lanes 1 and 2 contain samples from wild-type and Δ*rpoB* plants respectively. DNA gel-blots (left panel) were loaded with *Eco*RI-digested total cellular DNA extracted from 60 mg of leaf tissue. The amount of total DNA extracted from wild-type and Δ*rpoB* plants was equal, as judged by ethidium bromide staining (not shown). RNA gel-blots (right panel) were prepared with total cellular RNA extracted from 60 mg of leaf tissue. Both DNA and RNA gel-blots were hybridized to a *16SrDNA* probe. Hybridization signals were quantified with a Molecular Dynamics PhosphorImager. The ratio of wild-type to Δ*rpoB* signal intensities for the DNA and RNA signals is shown below the lanes.

mapped upstream of the processed termini. Therefore, the low levels of transcript accumulation for these photosynthetic genes are probably the result of activity from unidentified upstream promoters and subsequent processing of the readthrough RNAs to yield correctly sized transcripts.

The 16SrDNA gene is transcribed from a novel promoter in ΔrpoB plants

In contrast to the photogenes *rbcL* and *psbD*, we mapped a primary transcript in the immediate upstream region of the genetic system gene, *16SrDNA*. This 5′ end, prominent in the Δ*rpoB* plants, was located 62 nucleotides upstream of the mature 16S rRNA 5′ terminus (Figure 6A, filled circle). We determined that the 5′ end is a primary transcript by *in vitro* capping (Figure 6B). A similar primary transcript was reported recently in proplastids of heterotrophically cultured tobacco cells, and was designated P2 (Vera and Sugiura, 1995). This transcript is also present at very low levels in wild-type leaf cells (Vera and Sugiura, 1995; Figure 6A longer exposure, not shown). The sequence surrounding the initiation site is highly conserved among all plant species examined, and bears no resemblence to the σ^{70} consensus sequence (Vera and Sugiura, 1995; Figure 6C). Based on its prominent usage in the Δ*rpoB* plants, we conclude that this unique promoter is utilized by the GS transcription apparatus. We anticipate that additional promoters for the GS RNA polymerase are present upstream of genes which are highly expressed in the Δ*rpoB* plants.

Discussion

This work presents the first successful targeted deletion of a plastid RNA polymerase subunit gene. A previous attempt to disrupt the *rpoB1*, *rpoB2* and *rpoC2a* genes in the unicellular green alga, *Chlamydomonas rheinhardtii*, resulted in heteroplasmic cells which contained a mix of transformed and wild-type genome copies on selective

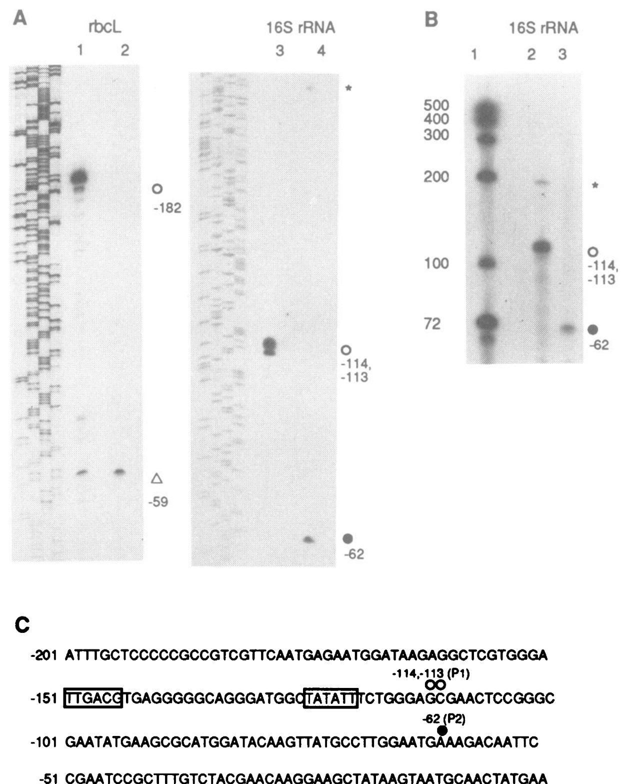

Fig. 6. Transcription in the Δ*rpoB* plants initiates from a non-canonical promoter. (**A**) Primer extension analysis was used to map the 5′ ends of *rbcL* and *16SrDNA* transcripts in wild-type (lanes 1 and 3) and Δ*rpoB* (lanes 2 and 4) plants. Primary transcripts are marked by circles (open for wild-type, closed for Δ*rpoB*), processed transcripts by a triangle. Transcripts of unknown origin are starred. The accompanying sequence ladders (loading order GATC) were generated using the same primers that served in the primer extension reactions. Numbers beside each extension product mark the distance from the first nucleotide of the coding sequence for *rbcL* and from the first nucleotide of the mature 16S rRNA. (**B**) Mapping primary transcripts for 16S rRNA in wild-type (lane 2) and Δ*rpoB* (lane 3) plants. Total leaf RNA (20 μg) was capped *in vitro*, and capped 16S rRNA species were identified by RNase protection after hybridization with a complementary RNA probe. Capped protected products are marked as in (A). Lane 1 contains RNA standards of the sizes indicated. (**C**) DNA sequence of the *16SrDNA* upstream region with transcripts initiating from promoters for the plastid-encoded (σ^{70}-type, P1) and nuclear-encoded (P2) polymerases (designation of P1 and P2 is based on Vera and Sugiura, 1995). Consensus σ^{70} promoter elements (−35 and −10) are boxed. Initiation sites are marked by circles, as in (A) and (B). Numbering begins from the first nucleotide upstream of the *16SrDNA* coding region (−1 = nucleotide 102 757 in the tobacco plastid genome).

media (Rochaix, 1995). In contrast, in tobacco, a higher plant, deletion of the RNA polymerase subunit gene, *rpoB*, could be obtained readily in homoplasmic form. The conclusion that the Δ*rpoB* plants contained a uniformly transgenic plastid genome population relied on somatic segregation analysis, and was indicated by regeneration of exclusively white shoots from white leaf tissue on non-selective medium. We did not use quantitative Southern experiments or quantitative PCR to exclude the presence of wild-type plastid genome copies, since segments of the plastid genome are known to be incorporated in the nuclear genome (Ayliffe and Timmis, 1992), and may also be present in the mitochondrial genome (Nakazono and Hirai, 1993). In line with the absence of *rpoB* genes in the

Δ*rpoB* plastid genome, primer extension analysis did not detect transcripts initiated at *E.coli*-like promoter sites for any of the plastid genes examined. If any wild-type genome copies had remained in the Δ*rpoB* plants, we would have expected to detect initiation by the *E.coli*-like RNA polymerase at the σ^{70}-type promoters of the *psbA*, *rbcL* and *16SrDNA* genes, all of which are transcribed very strongly by this polymerase in wild-type plants (Baumgartner *et al.*, 1993).

While we could not detect transcription initiation from *E.coli*-like promoters in the Δ*rpoB* plants, high steady-state levels of transcripts for a subset of plastid genes indicated that transcription activity was maintained in the absence of the *E.coli*-like RNA polymerase. We mapped the initiation site of the GS RNA polymerase on the *16SrDNA* gene to a recently identified site, P2 (Vera and Sugiura, 1995), which does not have the –35/–10 consensus elements typical of the plastid σ^{70}-type promoters.

It is of interest to ask whether the P2 promoter, recognized by the Δ*rpoB* transcription machinery, resembles non-consensus promoters described for other plastid genes, or whether it represents a new class of plastid promoter. Three examples of non-consensus plastid gene promoters, other than P2, have been well documented. First, the strong light-regulated plastid *psbD* promoter was thought to represent a non-σ^{70}-type regulatory region (Christopher *et al.*, 1992; Kim and Mullet, 1995). Transcription from this promoter, however, is absent in the Δ*rpoB* plants (not shown), indicating that in wild-type plastids it is expressed by the *E.coli*-like enzyme. Indeed, *in vivo* deletion analysis of this promoter demonstrated that elements resembling σ^{70}-type –35/–10 boxes make up the core promoter of this regulatory region (Allison and Maliga, 1995). Second, a non-consensus promoter region, Pc, has been characterized for the *16SrDNA* gene in spinach (Baeza *et al.*, 1991; Iratni *et al.*, 1994). Transcripts initiated from this site are absent in both wild-type and Δ*rpoB* tobacco plants. Moreover, there is no substantial similarity between sequences surrounding the spinach initiation site and the P2 polymerase initiation site. Therefore, the P2 promoter should represent a different promoter type than spinach Pc. The enzymatic origin of the Pc 16S rRNA transcript in spinach is unclear. Third, non-σ^{70}-type promoters have been described for several plastid genes in *C.rheinhardtii* (Klein *et al.*, 1992, 1994). These promoters are typified by a highly conserved TATAATAT sequence upstream of the initiation site and by additional sequences downstream which are essential for full promoter activity. It is not known whether this promoter type is present in higher plant plastids. However, the *16SrDNA* P2 promoter certainly does not fall into this class since it is missing the A/T-rich element at the appropriate position in tobacco and all other plant species examined (Vera and Sugiura, 1995). We conclude that the P2 transcription element recognized in the Δ*rpoB* plants typifies a new class of plastid gene promoters. We should be able to define a consensus for this novel plastid promoter by determining the transcription initiation sites for other plastid genes in the Δ*rpoB* plants.

The absence of an essential *E.coli*-like core subunit in the Δ*rpoB* plants implies that the remaining GS RNA polymerase(s) is distinct from the *E.coli*-like RNA polymerase. The GS RNA polymerase activity may constitute the tobacco homologue of a 110 kDa single subunit RNA polymerase purified from spinach plastids (Lerbs-Mache, 1993), or may comprise a distinct multisubunit plastid RNA polymerase (Little and Hallick, 1988; Pfannschmidt and Link, 1994). An alternate possibility is that the GS RNA polymerase contains a nuclear-encoded β subunit homologue which was transferred to the nucleus (Baldauf and Palmer, 1990; Gantt *et al.*, 1991) and evolved to confer an altered promoter specificity (Glass *et al.*, 1986). Ultimately, the question of subunit composition of the RNA polymerase detected in Δ*rpoB* plants will be understood only through purification of the enzyme and cloning of the genes for its component subunits.

There is preferential accumulation of RNAs for the genetic system genes in the Δ*rpoB* plants. This indicates a role for the GS RNA polymerase in maintaining the expression of plastid housekeeping genes. Apparently, these expression levels are sufficient to support the growth and differentiation of non-photoautotrophic plants as long as the reduced carbon is supplied externally. In contrast, the *E.coli*-like RNA polymerase seems to provide the high levels of plastid gene transcripts necessary for development of photosynthetically active chloroplasts. The proposed role for the GS RNA polymerase implies a high demand for its function during the early phases of chloroplast development, before the *E.coli*-like RNA polymerase is active (Mullet, 1993). Such developmental regulation of a nuclear-encoded RNA polymerase is supported by the observation that the GS polymerase P2 promoter of the *16SrDNA* gene is more active in proplastids of cultured tobacco cells than in leaf chloroplasts (Vera and Sugiura, 1995).

We have found that elimination of the *E.coli*-like RNA polymerase results in high levels of P2 transcripts in leaf cells, in contrast to the almost undetectable levels of these transcripts in wild-type leaf chloroplasts. Thus, we speculate that the absence of the *E.coli*-like RNA polymerase initiated a feedback mechanism, resulting in increased activity of the GS RNA polymerase. The Δ*rpoB* plants are similar in this respect to the barley mutant *albostrians* (Hess *et al.*, 1993). The uncharacterized nuclear mutation of *albostrians* generates leaf sectors containing essentially ribosome-free plastids. The activated transcription of plastid genetic system genes in the mutant sectors was attributed to the block in plastid translation (Hess *et al.*, 1993, 1994). However, this same mechanism cannot be invoked in the Δ*rpoB* plants, since translation occurs in the mutant plastids, as evidenced by immunodetection of low levels of the plastid-encoded ribulose 1,5-bisphosphate carboxylase large subunit (not shown).

The Δ*rpoB* plants, which were critical for establishing the existence of the GS RNA polymerase, will now be useful for deriving a consensus for GS promoters, biochemically characterizing the GS polymerase and dissecting the nuclear-plastid communication pathway that activates GS RNA polymerase function.

Materials and methods

Plasmid construction

Plasmid pLAA57 is a pBSKS$^+$ (Stratagene) derivative which carries a *Sac*I–*Bam*HI fragment (nucleotides 22 658–29 820) of the ptDNA. An

internal *SacI–SmaI* DNA fragment within the ptDNA insert, between nucleotides 24 456 and 28 192, was replaced by a chimeric spectinomycin resistance (*aadA*) gene. The *aadA* gene, expressed from plastid *16SrDNA* promoter sequences, is identical to that described (Svab and Maliga, 1993), except that the *psbA* 3′ region is shorter and is contained in an *XbaI–DraI* fragment as described (Staub and Maliga, 1994).

Plastid transformation

For plastid transformation, tungsten particles were coated (Svab and Maliga, 1993) with pLAA57 DNA, and introduced into the leaves of *Nicotiana tabacum* plants using the DuPont PDS1000He Biolistic gun at 1100 p.s.i. Transgenic shoots were selected aseptically on RMOP medium (Svab *et al.*, 1990) containing 500 μg/ml spectinomycin dihydrochloride. Plant regeneration from white tissue sectors of spectinomycin-resistant plants was carried out on drug-free RMOP medium. Transgenic cuttings were rooted and maintained on RM medium consisting of agar-solidified MS salts (Murashige and Skoog, 1962) containing 3% sucrose.

Electron microscopy

Electron microscopy was done on fully expanded leaves from wild-type and Δ*rpoB* cuttings grown in sterile culture on RM medium with 3% sucrose. Tissue was fixed for 2 h in 2% glutaraldehyde, 0.2 M sucrose, 0.1 M phosphate buffer (pH 6.8) at room temperature, and washed three times in 0.2 M sucrose, 0.1 M phosphate buffer. Fixed tissues were post-fixed in buffered 1% osmium tetroxide with 0.2 M sucrose, dehydrated in a graded ethanol series, embedded in Spurr's epoxy resin (hard), sectioned and stained with uranyl acetate and lead citrate for transmission electron microscopy.

Gel-blots

Total leaf DNA was prepared as described (Mettler, 1987), digested with restriction endonuclease *PstI*, separated on 0.7% agarose gels, and transferred to Hybond N (Amersham) using the Posiblot Transfer apparatus (Stratagene). Hybridization to random primer-labelled fragment was carried out in Rapid Hybridization Buffer (Amersham) overnight at 65°C. Total leaf RNA was prepared using TRIzol (GIBCO BRL), following the manufacturer's protocol. The RNA was electrophoresed on 1% agarose–formaldehyde gels, then transferred to nylon membrane and probed as for the DNA blots.

Double-stranded DNA probes for *psbA*, *atpI* and *rpl16* were prepared by random-primed ^{32}P-labelling of PCR-generated DNA fragments. The sequence of the primers used for PCR, along with their positions within the tobacco ptDNA (Shinozaki *et al.*, 1986) are as follows: *psbA* 5′ primer = 5′-CGCTTCTGTAACTGG-3′ (complementary to nucleotides 1550–1536 of the ptDNA), 3′ primer = 5′-TGACTGTCAACTACAG-3′ (nucleotides 667–682); *atpI* 5′ primer = 5′-GTTCCATCAATACTC-3′ (complementary to nucleotides 15 985–15 971), 3′ primer = 5′-GCCGCGGCTAAAGTT-3′ (nucleotides 15 292–15 306); *rpl16* 5′ primer = 5′-TCCCACGTTCAAGGT-3′ (complementary to nucleotides 84 244–84 230), 3′ primer = 5′-TGAGTTCGTATAGGC-3′ (nucleotides 83 685–83 699). To generate probes for *rbcL*, *psbD/C* and 16S rRNA, the following restricted DNA fragments were ^{32}P-labelled: *rbcL*, a *Bam*HI fragment (nucleotides 58 047–59 285 in the ptDNA); *psbD/C*, a *Sac*II–*Hind*III fragment of the tobacco *psbD/C* operon (nucleotides 34 691–36 393); 16S rRNA, an *Eco*RI–*Eco*RV fragment (nucleotides 138 447–140 855 in the ptDNA).

The probe for tobacco 25S rRNA was from plasmid pKDR1 (Dempsey *et al.*, 1993) containing a 3.75 kb *Eco*RI fragment from a tobacco 25S/18S locus cloned in plasmid pBR325. When hybridizing gel-blots for 25S rRNA, ^{32}P-labelled double-stranded DNA probe was mixed with unlabelled plasmid pKDR1 corresponding to a 2-fold excess over the amount of RNA present on the filter.

Primer extension reactions

Primer extension reactions were carried out on 3 μg (wild-type) or 10 μg (Δ*rpoB*) of total leaf RNA as described (Allison and Maliga, 1995) using the following primers: 16S rRNA: 5′-TTCATAGTTGCATT-ACTTATAGCTTC-3′ (complementary to nucleotides 102 757–102 732); *rbcL*: 5′-ACTTGCTTTAGTCTCTGTTTGTGGTGACAT (complementary to nucleotides 57 616–57 587). Sequence ladders were generated with the same primers using the Sequenase II kit (USB).

Identification of primary transcripts by in vitro capping

Total leaf RNA (20 μg) from wild-type and Δ*rpoB* plants was capped in the presence of [α-^{32}P]GTP (Kennell and Pring, 1989). Labelled 16S rRNAs were detected by RNase protection (Vera and Sugiura, 1992) using the RPAII kit (Ambion). To prepare the protecting complementary RNA, the *16SrDNA* upstream region (nucleotides 102 526–102 761 of the ptDNA) was PCR amplified using the following primers: 5′ primer was 5′-CCTCTAGACCCTAAGCCCAATGTG-3′ corresponding to nucleotides 102 526–102 541 of the ptDNA (Shinozaki *et al.*, 1986, underlined) plus an *XbaI* site; 3′ primer was 5′-CCGGTACCGAGATT-CATAGTTGCATTAC-3′ complementary to nucleotides 102 761–102 742 of the ptDNA (underlined) plus a *KpnI* site. The amplified product was cloned as an *XbaI–KpnI* fragment into *XbaI*- and *KpnI*-restricted pBSKS^{+} vector (Stratagene). To generate unlabelled RNA complementary to the 5′ end of 16S rRNAs, the resulting plasmid was linearized with *XbaI* and transcribed in a Megascript (Ambion) reaction with T3 RNA polymerase. Markers (100, 200, 300, 400 and 500 nucleotides) were prepared with the RNA Century Markers Template Set (Ambion), following the manufacturer's protocol. The 72 nucleotide marker was the mature processed transcript from the plastid *trnV* gene, and was generated by RNase protection.

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