The EMBO Journal Vol.16 No.13 pp.4041–4048, 1997

The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids

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The plastid genome in photosynthetic higher plants encodes subunits of an *Escherichia coli*-like RNA polymerase (PEP) which initiates transcription from *E.coli* σ^{70}-type promoters. We have previously established the existence of a second nuclear-encoded plastid RNA polymerase (NEP) in photosynthetic higher plants. We report here that many plastid genes and operons have at least one promoter each for PEP and NEP (Class II transcription unit). However, a subset of plastid genes, including photosystem I and II genes, are transcribed from PEP promoters only (Class I genes), while in some instances (e.g. *accD*) genes are transcribed exclusively by NEP (Class III genes). Sequence alignment identified a 10 nucleotide NEP promoter consensus around the transcription initiation site. Distinct NEP and PEP promoters reported here provide a general mechanism for group-specific gene expression through recognition by the two RNA polymerases.

Keywords: *Nicotiana tabacum*/nuclear-encoded plastid RNA polymerase (NEP)/plastid-encoded RNA polymerase (PEP)/plastid gene expression/*rpoB* deficient mutant

Introduction

The plastid genome of photosynthetic higher plants encodes proteins which are homologous to the *Escherichia coli* DNA-dependent RNA polymerase α, β and β′ subunits. The subunit structure of the plastid enzyme is similar to that of the *E.coli* enzyme, except that the plastid β′ and β″ subunits are equivalent to the N- and C-termini of the bacterial β′ subunit, respectively. The plastid genes were named *rpoA*, *rpoB*, *rpoC1* and *rpoC2* to indicate homologies with the *E.coli* genes. Promoter selection by this plastid-encoded plastid RNA polymerase (PEP) is dependent on nuclear-encoded σ-like factors. The promoters utilized by PEP 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, 1996). Transcription activity from some PEP 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).

Several reports have suggested the existence of an additional plastid-localized, nuclear-encoded RNA polymerase (reviewed in Igloi and Kössel, 1992; Gruissem and Tonkyn, 1993; Mullet, 1993; Link, 1994, 1996). By deleting the *rpoB* gene encoding the essential β subunit of the tobacco PEP, we established the existence of a second nuclear-encoded plastid RNA polymerase (NEP) in photosynthetic higher plants (Allison *et al.*, 1996). Deletion of *rpoB* yielded photosynthetically defective, pigment-deficient plants. An examination of Δ*rpoB* plants revealed proplastid-like structures containing low levels of mRNAs for the photosynthetic genes *rbcL*, *psbA* and *psbD* due to the lack of PEP promoter activity. In wild-type tobacco leaves, the ribosomal RNA operon (*rrn*) is transcribed by PEP. Interestingly, in the Δ*rpoB* plants the *rrn* mRNA accumulated close to wild-type levels due to transcription by NEP acting at a downstream non-σ^{70}-type promoter. The rRNA operon is the first plastid transcription unit for which a promoter for both PEP and NEP was identified.

We report here that the rRNA operon is not unique, but represents a class of plastid transcription units which have at least one promoter each for PEP and NEP. These genes or operons have a potential for expression by either of the two plastid RNA polymerases. Furthermore, some genes are transcribed by only one of the two RNA polymerases. We propose that transcription by NEP and PEP, through recognition of distinct promoters, is a general mechanism of group-specific gene regulation during chloroplast development. A tentative NEP promoter consensus is derived by the alignment of the transcription initiation sites.

Results

Identification of genes with promoters for the NEP polymerase

To facilitate mapping of additional NEP promoters, we examined mRNA steady-state concentrations in Δ*rpoB* plants for several plastid genes (Figure 1). The plastid genes were divided into three groups based on mRNA steady-state concentrations in fully-extended leaves of wild-type and Δ*rpoB* plants. Group I includes genes for which the mRNAs accumulate to high levels in wild-type leaves, and to very low levels in the leaves of Δ*rpoB* plants (Figure 1A). Genes in this class are *psaA* (photosystem I gene); *psbB* and *psbE* (photosystem II genes); *petB*, a cytochrome b6/f complex gene (for references see Shinozaki *et al.*, 1986b); *ndhA*, a respiratory chain NADH dehydrogenase homologue (Matsubayashi *et al.*, 1987); and the *rps14* ribosomal protein gene (Meng *et al.*, 1988). Group II includes plastid genes encoding mRNAs that accumulate to significant levels in both wild-type and Δ*rpoB* leaves (Figure 1B). Group II includes *atpB* (ATP

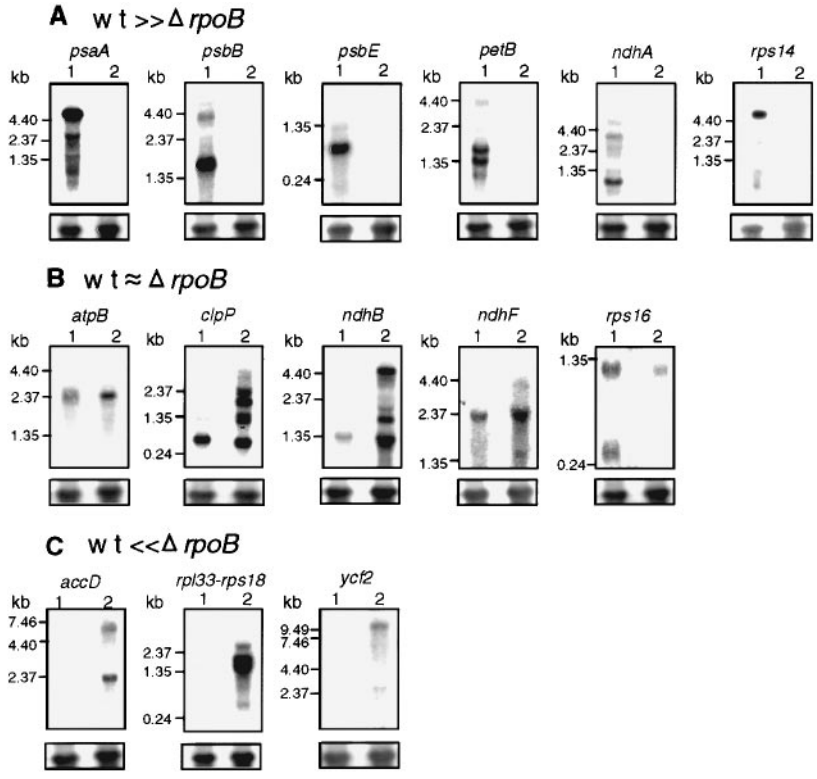

Fig. 1. RNA steady-state concentrations in wild-type and Δ*rpoB* tobacco leaves. Blots for the plastid genes (see text) are grouped as follows. (**A**) mRNA is significantly more abundant in the leaves of wild-type than in Δ*rpoB* plants (Group I). (**B**) Levels of mRNA are comparable in wild-type and Δ*rpoB* leaves (Group II), or (**C**) are elevated in Δ*rpoB* leaves (Group III). Gel blots were prepared with total cellular RNA (5 μg per lane) from wild-type (lanes 1) and Δ*rpoB* (lanes 2) leaf tissue, and hybridized to the indicated plastid gene sequences (upper panels). To control for loading, blots shown above were reprobed for cytoplasmic 25S ribosomal RNA (lower panels). References for transcripts in wild-type tobacco are: *psaA*, *rps14* (Meng *et al.*, 1988); *psbB*, *petB* (Tanaka *et al.*, 1987); *psbE* (Carillo *et al.*, 1986); *ndhA*, *ndhB*, *ndhF* (Matsubayashi *et al.*, 1987); *atpB* (Kapoor *et al.*, 1994); *rps16* (Shinozaki *et al.*, 1986a).

synthase gene; Orozco *et al.*, 1990); *clpP* encoding the proteolytic subunit of the Clp ATP-dependent protease (Gray *et al.*, 1990; Maurizi *et al.*, 1990); *ndhB* and *ndhF*, two respiratory chain NADH dehydrogenase homologues (Matsubayashi *et al.*, 1987); the *rps16* ribosomal protein gene (Shinozaki *et al.*, 1986a); and *ycf1*, a gene with unknown function (*ORF1901*; Wolfe *et al.*, 1992; Hallick and Bairoch, 1994; data not shown). Group III includes genes for which there is significantly more mRNA in the Δ*rpoB* leaves than in the leaves of wild-type plants (Figure 1C). Among these are: *accD*, encoding a subunit of the acetyl-CoA carboxylase (Sasaki *et al.*, 1993); ribosomal protein genes *rpl33* and *rps18* (Shinozaki *et al.*, 1986b); and *ycf2*, a putative ATPase with unknown function (*ORF2280*; Hallick and Bairoch, 1994; Wolfe, 1994).

Apparent are the more complex RNA patterns in Δ*rpoB* plants as compared with wild-type plants (Figure 1B and C). The reason for the more complex patterns may be activation of additional promoters upstream of the tested genes, differences in mRNA processing and stability, and differences in the transcription termination signals for the two polymerases. The origin of the complex RNA patterns in the Δ*rpoB* plants is outside the scope of the present study.

The atpB and atpI ATP synthase genes have both NEP and PEP promoters

The RNA gel blot analysis identified a number of genes and operons for which transcript levels are maintained or elevated in Δ*rpoB* leaves (Figure 1B). To identify NEP promoters, 5′ ends of transcripts were mapped by primer extension analysis. To distinguish between 5′ ends that represent transcripts from a NEP promoter from those generated by RNA processing, the 5′ ends were capped using guanylyltransferase.

Transcript 5′ ends for the tobacco *atpB* gene have been identified by Orozco *et al.* (1990) at nucleotide positions –255, –289, –488, –502 and –611 relative to the translation initiation codon (ATG; the nucleotide directly upstream of the A-occupying position –1). Primer extension analysis identified each of these 5′ ends in wild-type plants (Figure 2A). These RNA species are not resolved distinctly in Figure 1B (see also Kapoor *et al.*, 1994). In the Δ*rpoB* plants only the –289 RNA species was present. The 5′ end of this transcript was capped using guanylyltransferase (Figure 2B). Therefore, we propose that the –289 RNA is transcribed from a NEP promoter, termed *PatpB*–289. Interestingly, the –289 transcript is also present in the wild-type leaves, although it is significantly less abundant than in the Δ*rpoB* plants. The –255, –488, –502 and –611 transcripts are absent in the Δ*rpoB* plants (Figure 2A), suggesting that they are transcribed by PEP in plastids.

The *atpI* operon includes the *atpI–atpH–atpF–atpA* genes (Figure 3C). In wild-type tobacco leaves, we mapped mRNA 5′ ends to the –207 region (–212, –209 and –207) and at nucleotides –130 and –85. Interestingly, in Δ*rpoB* leaves only the –207 transcript is detectable (Figure 3A; data not shown). This transcript could be capped in the

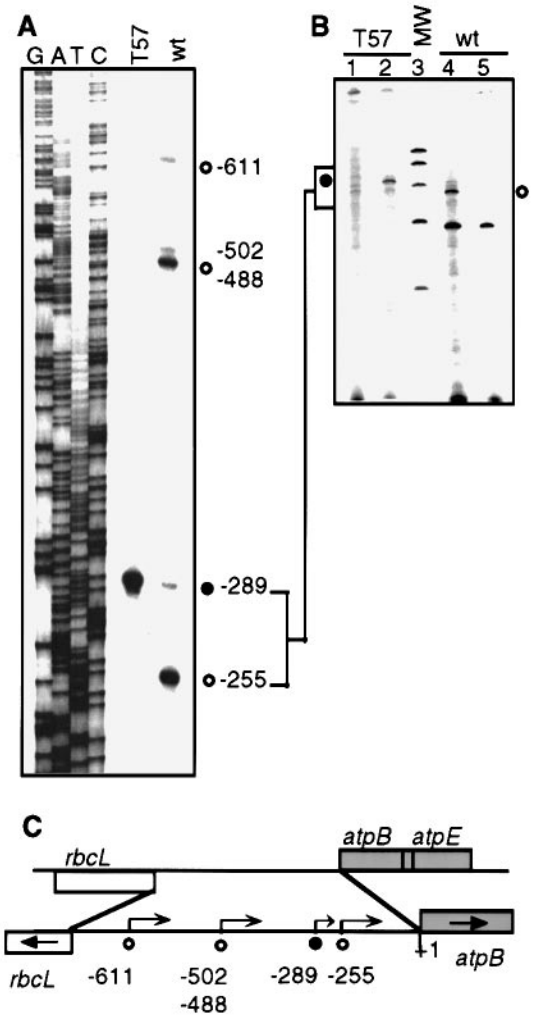

Fig. 2. Mapping *atpB* transcription initiation sites in wild-type and Δ*rpoB* tobacco leaves. (**A**) Primer extension analysis. End-labeled primer extension products from wild-type (wt) and Δ*rpoB* (T57) samples were run alongside the homologous sequence obtained using the same primer. Numbers alongside the sequence refer to the distance from the ATG translation initiation codon. Primary transcripts from NEP and PEP promoters are marked by filled and open circles, respectively. (**B**) *In vitro* capping and RNase protection assay to identify primary transcript 5′ ends. Note that the RNase protection construct is short and protects only the –255 and –289 ends. Lanes were loaded with Δ*rpoB* (T57; lanes 1 and 2) and wild-type (wt; lanes 4 and 5) RNA samples with (lanes 2 and 4) and without (lanes 1 and 5) protecting complementary antisense RNA. Molecular weight (MW) markers (100, 200, 300, 400 and 500 nt) were loaded in lane 3. The transcript 5′ ends in (A) correspond to the protected fragment size in brackets: –255 (277 nt), –289 (311 nt). The size of the protected fragment may be longer (or shorter) than the distance between the translation initiation codon and the transcript 5′ end, and depends on the size of the protecting single-stranded *in vitro* RNA. Note artifact slightly below the 200 nt marker which is present in the unprotected RNA samples. (**C**) Physical map of the *atpB–rbcL* intergenic region. Map position of the primary transcript 5′ ends for the *atpB* NEP and PEP promoters are marked as in (A).

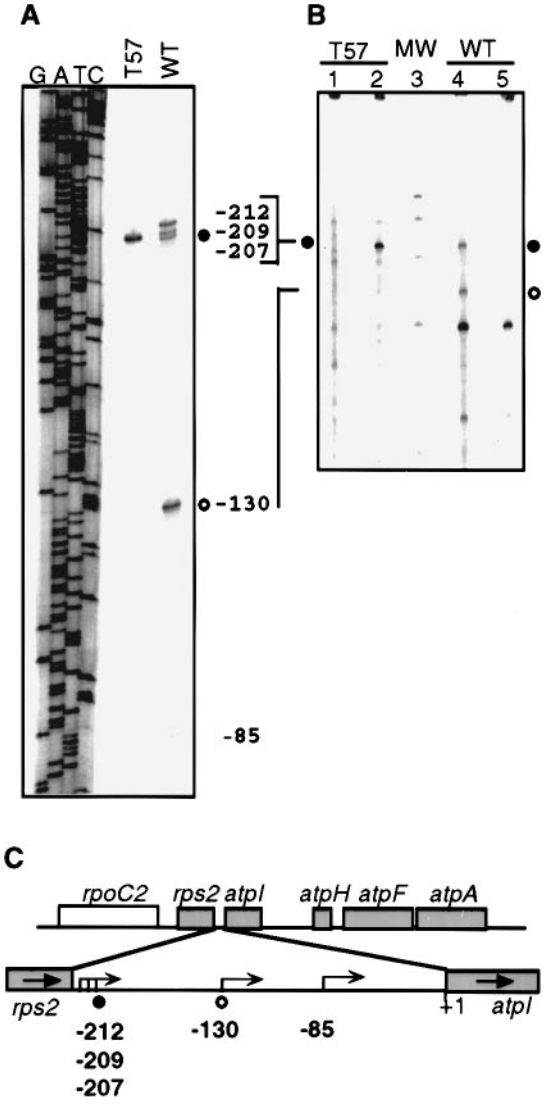

Fig. 3. Mapping *atpI* transcription initiation sites in wild-type and Δ*rpoB* tobacco leaves. (**A**) Primer extension analysis. End-labeled primer extension products from wild-type (wt) and Δ*rpoB* (T57) samples were run alongside the homologous sequence obtained using the same primer. Numbers alongside the sequence refer to the distance from the ATG translation initiation codon. Primary transcripts from NEP and PEP promoters are marked by filled and open circles, respectively. (**B**) *In vitro* capping and RNase protection assay to identify primary transcript 5′ ends. Lanes were loaded with Δ*rpoB* (T57; lanes 1 and 2) and wild-type (wt; lanes 4 and 5) RNA samples with (lanes 2 and 4) and without (lanes 1 and 5) protecting complementary antisense RNA. Molecular weight (MW) markers (100, 200, 300, 400 and 500 nt) were loaded in lane 3. The transcript 5′ ends in (A) correspond to the protected fragment size in brackets: –130 (235 nt), –207, 209, 212 (303, 305, 309; not resolved). Note artifact slightly below the 200 nt marker which is present in the unprotected RNA samples. (**C**) Partial map of the tobacco plastid genome containing the *atpI* operon. Map position of the primary transcript 5′ ends for the *atpI* NEP and PEP promoters are marked as in (A).

Δ*rpoB* RNA sample (Figure 3B), demonstrating that it originates directly from a NEP promoter. A signal at the same position was obtained in the *in vitro* capping reaction of wild-type RNA samples, corresponding to the –207, –209 and –212 transcripts which were not resolved in the assay. We could also cap the –130 transcript which is present only in wild-type leaf (Figure 3A and B). Thus, it is likely that *PatpI*–130 is recognized by PEP.

A clpP NEP promoter is highly active in chloroplasts

Primer extension analysis with wild-type plants identified *clpP* RNA 5′ ends at nucleotide positions –53, –95 and

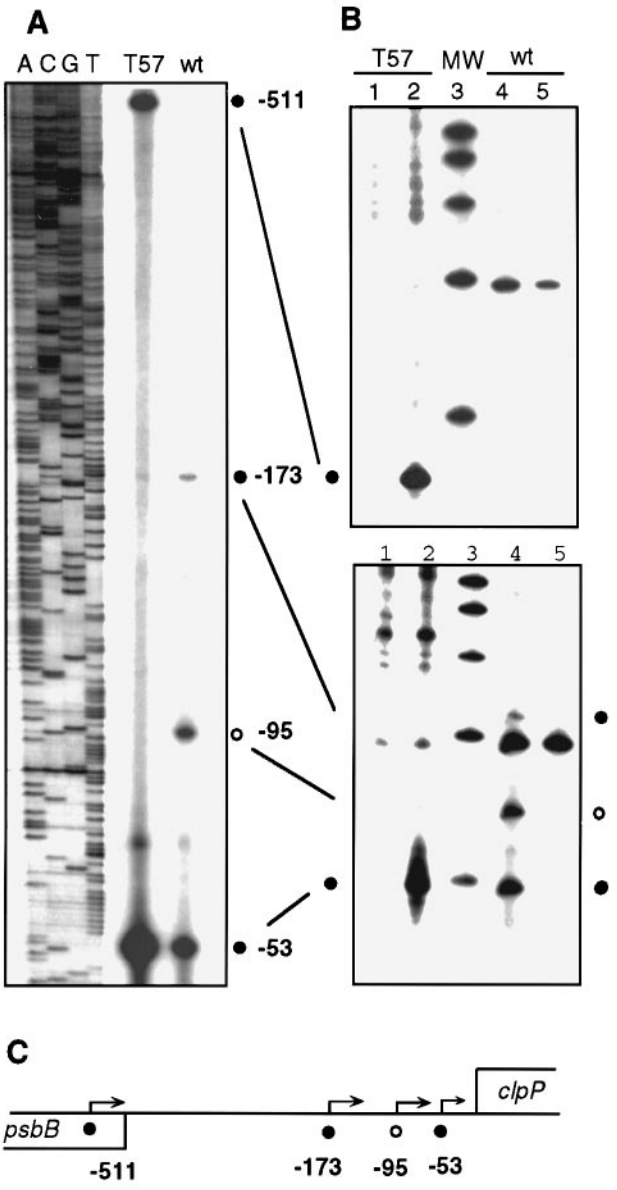

Fig. 4. Mapping *clpP* transcription initiation sites in wild-type and Δ*rpoB* tobacco leaves. (**A**) Primer extension analysis. End-labeled primer extension products from wild-type (wt) and Δ*rpoB* (T57) samples were run alongside the homologous sequence obtained using the same primer. Numbers alongside the sequence refer to the distance from the ATG translation initiation codon. Primary transcripts from NEP and PEP promoters are marked by filled and open circles, respectively. (**B**) *In vitro* capping and RNase protection assay to identify primary transcript 5′ ends. Lanes were loaded with Δ*rpoB* (T57; lanes 1 and 2) and wild-type (wt; lanes 4 and 5) RNA samples with (lanes 2 and 4) and without (lanes 1 and 5) protecting complementary antisense RNA. Molecular weight (MW) markers (100, 200, 300, 400 and 500 nt) were loaded in lane 3. The transcript 5′ ends in (A) correspond to the protected fragment size in brackets: –53 (96 nt), –95 (138 nt), –173 (216 nt) and –511 (69 nt). Note artifact slightly below the 200 nt marker which is present in the unprotected RNA samples. (**C**) Physical map of the *clpP–psbB* intergenic region. Map position of the primary transcript 5′ ends for the *clpP* NEP and PEP promoters are marked as in (A).

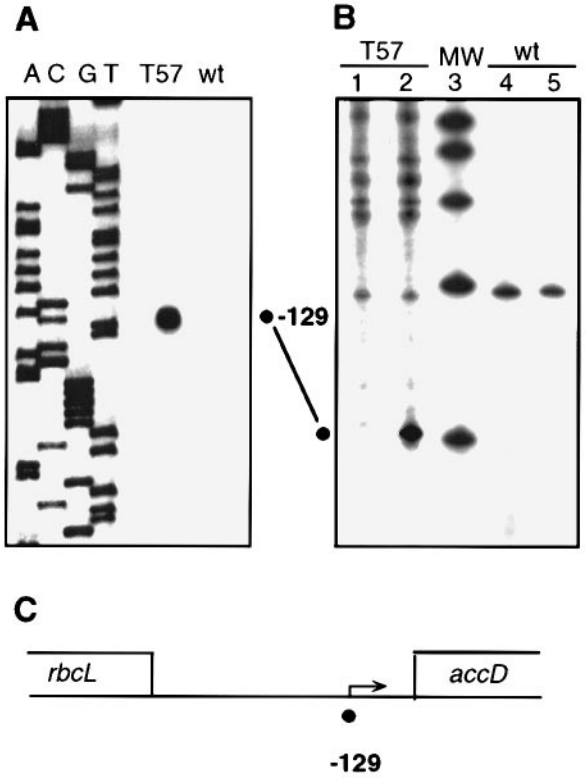

Fig. 5. Mapping *accD* transcription initiation sites in wild-type and Δ*rpoB* tobacco leaves. (**A**) Primer extension analysis. End-labeled primer extension products from wild-type (wt) and Δ*rpoB* (T57) samples were run alongside the homologous sequence obtained using the same primer. Numbers alongside the sequence refer to the distance from the ATG translation initiation codon. Primary transcript for the *PaccD*–129 NEP promoter is marked by a filled circle. (**B**) *In vitro* capping and RNase protection assay to identify primary transcript 5′ ends. Lanes were loaded with Δ*rpoB* (T57; lanes 1 and 2) and wild-type (wt; lanes 4 and 5) RNA samples with (lanes 2 and 4) and without (lanes 1 and 5) protecting complementary antisense RNA. Molecular weight (MW) markers (100, 200, 300, 400 and 500 nt) were loaded in lane 3. The –129 transcript 5′ end in (A) corresponds to the protected 103 nt fragment. Note artifact slightly below the 200 nt marker which is present in the unprotected RNA samples. (**C**) Transcription initiation site of the *PaccD*–129 NEP promoter in the *accD–rbcL* intergenic region.

–173. In contrast, in Δ*rpoB* leaves, the 5′ ends mapped to nucleotides –53, –173 and –511 (Figure 4A). Since *in vitro* capping reaction verified that each of these are primary transcripts (Figure 4B), it would seem likely that these transcripts derive from NEP promoters. The *PclpP*–53 promoter is highly expressed in both wild-type-and Δ*rpoB* plants; thus, it is a NEP promoter with a potential for high-level expression in different tissue types. The *PclpP*–53 promoter is well conserved in spinach, in which it is the only promoter transcribing the *clpP* gene (Westhoff, 1985). Additional NEP promoters are *PclpP*–173 and *PclpP*–511. The *PclpP*–511 transcript accumulates only in Δ*rpoB* plants (Figure 4A). Note also, that *PclpP*–511 is located within the *psbB* coding region (Figure 4C); therefore, its activity in wild-type plastids may be affected by the convergent *psbB* PEP promoter (not marked).

Transcripts from *PclpP*–95 accumulate only in wild-type leaves. Therefore, it is likely that this promoter is recognized by PEP.

The accD gene is transcribed exclusively from a NEP promoter

RNA for the lipid biosynthetic gene *accD* accumulates to high levels only in Δ*rpoB* plants. A major transcript initiating at nucleotide position –129 (Figure 5A) could

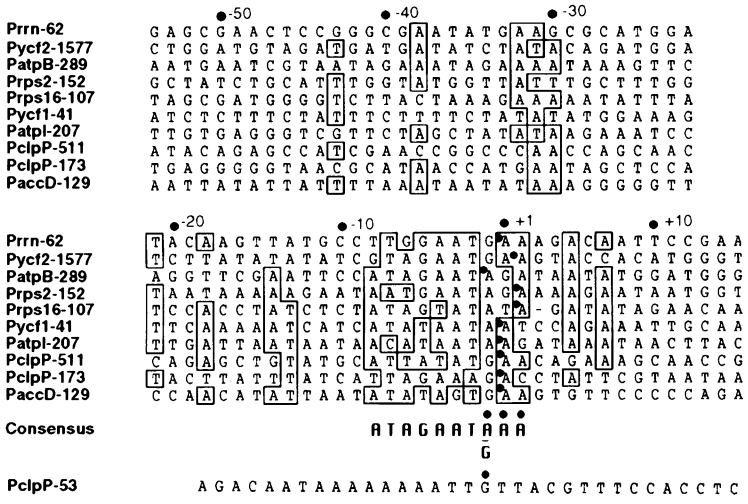

Fig. 6. Alignment of DNA sequences flanking the NEP promoter transcription initiation sites. Nucleotides with more than six matches are boxed. Consensus sequence adjacent to the transcription initiation site is shown below. Position of 5′ ends are marked by filled circles.

be capped *in vitro* (Figure 5B). Therefore, this RNA is transcribed from a NEP promoter. The mRNA from *PaccD*–129 accumulates to very low levels in the photosynthetically active leaf mesophyll cells, whereas it is abundant in the non-photosynthetic plastids of Δ*rpoB* plants.

NEP promoters share a loose consensus adjacent to the transcription initiation site

Sequences flanking 10 transcription initiation sites were aligned to identify conserved NEP promoter elements (Figure 6). Included in the sequence alignment are nine promoters identified in this study and *Prrn*–62, the NEP promoter described in Allison *et al.* (1996) and Vera and Sugiura (1995). We have included sequences for *Pycf2*–1577 and *Pycf1*–41 for which the 5′ ends were shown to be primary transcripts by capping *in vitro* (data not shown). Both of these promoters are active in Δ*rpoB* leaves, but not in the leaves of wild-type plants. Included in the sequence alignment are tentative NEP promoters for *rps2* and *rps16*, for which there is mRNA in Δ*rpoB* leaves. Transcripts for these tentative NEP promoters were mapped by primer extension analysis. However, the *in vitro* capping assays were inconclusive due to low abundance of the mRNAs (data not shown).

Multiple sequence alignment of the regions immediately flanking the NEP 5′ ends identified a loose 10 nucleotide consensus around the transcription initiation site (Figure 6). Clustering of conserved sequences around the transcription initiation site is reminiscent of the promoters of the single-subunit mitochondrial and phage T3/T7 RNA polymerases (Raskin *et al.*, 1993; Tracy and Stern, 1995). Conservation of additional nucleotides upstream and downstream of the NEP transcription initiation site is also apparent. Clustering of conserved sequences around the NEP transcription initiation site contrasts PEP promoters for which –35/–10 elements are localized upstream of the transcribed region (Igloi and Kössel, 1992; Gruissem and Tonkyn, 1993; Link, 1994, 1996). All these NEP promoters are inactive in chloroplasts, and are utilized only in the proplastid-like organelles of the Δ*rpoB* plants. Interestingly, the region around the transcription initiation site is less well conserved for *PclpP*–53, the only NEP promoter highly active in chloroplasts (bottom of Figure 6). Identification of functionally relevant sequences for *PclpP*–53 and the other NEP promoters will require dissection of the regions containing the transcription initiation sites.

Discussion

Based on their promoters, plastid genes and operons can be assigned to three classes: those with PEP (Class I), NEP and PEP (Class II), and NEP promoters only (Class III). Class I mainly includes photosynthetic genes which are transcribed from σ^{70}-type PEP promoters, including genes shown in Figure 1A and *rbcL*, *psbA* and *psbD* (Allison *et al.*, 1996). RNA steady-state concentrations for these genes are high in wild-type leaves, and very low—if at all detectable—in the leaves of Δ*rpoB* plants (Group I). Class II transcription units *rrn*, *atpB*, *atpI* and *clpP* have both NEP and PEP promoters (Allison *et al.*, 1996; also see Results). The mRNAs for these operons accumulate to significant levels in both wild-type and Δ*rpoB* plants (Figure 1B; see also Figure 4B in Allison *et al.*, 1996). Although no attempt was made to fully characterize *rps16*, *rpl33–rps18*, *ndhB*, *ndhF* and *ycf1* transcription, it is likely that high transcript levels for these genes in both leaf types is also due to transcription by both the plastid-encoded and nucleus-encoded RNA polymerases. Class II genes encode diverse functions, although none is a photosystem I or II polypeptide. Class III is small, and includes *accD* and *ycf2* transcribed from the *PaccD*–129 and *Pycf2*–1577 NEP promoters. *rpoB* is also likely to be a Class III gene (Hess *et al.*, 1993). With respect to mRNA steady-state concentrations, these genes belong to Group III: there is significantly more mRNA in the leaves of Δ*rpoB* plants than in those of wild-type plants (Figure 1C).

It appears that genes with similar functions are transcribed by PEP, or by both PEP and NEP as a group. A good example is transcription of all tested photosystem I (*psa*) and photosystem II (*psb*) genes by PEP (see above). Also, the mRNAs for most ribosomal protein genes accumulate to relatively high levels in both leaf types,

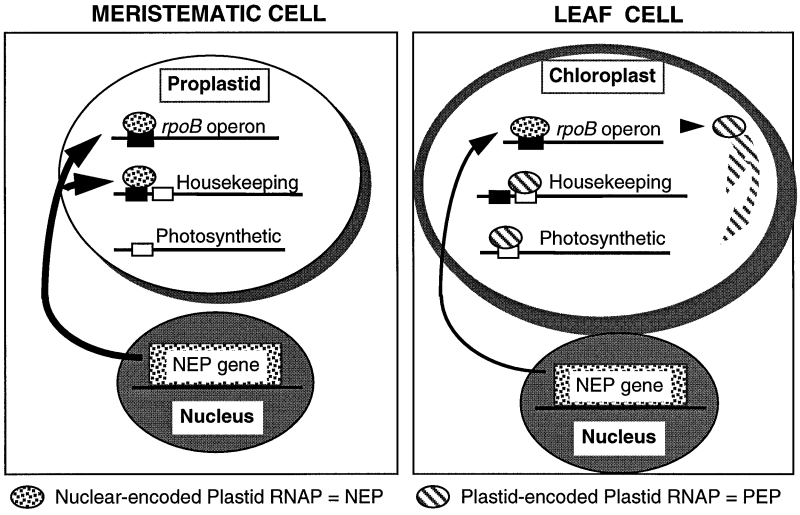

Fig. 7. Proposed scheme for differential gene expression during chloroplast development based on recognition of distinct promoters by NEP and PEP (based on Mullet, 1993; Hess *et al.*, 1993; this paper). Note that in proplastids, NEP transcribes housekeeping genes and the *rpoB* operon encoding the β, β′ and β′′ subunits of PEP, whereas in chloroplasts both housekeeping and photosynthetic genes are transcribed by PEP.

including those of *rps16* and *rpl33–rps18* (Figure 1). An exception is *rps14*, for which there is very little mRNA in the Δ*rpoB* leaves (Figure 1A). The *rps14* gene is transcribed as part of the *psaA* operon which contains two photosystem I genes (*psaA*, *psaB*; Meng *et al.*, 1988) and apparently has no dedicated NEP promoter. Puzzling is the differential accumulation of the mRNAs for the *ndh* genes, since they are assumed to be components of the same complex (Matsubayashi *et al.*, 1987): *ndhB* and *ndhF* accumulate to high levels in both wild-type and Δ*rpoB* plants, whereas *ndhA* mRNA is present only in the wild-type leaves. Observed differential accumulation may indicate complementation of some, but not all plastid *ndh* genes by mitochondrial copies of the same gene (Matsubayashi *et al.*, 1987).

Given the general need for lipid biosynthesis, *accD* mRNA should be present in all cell types. However, in wild-type leaves, the level of *accD* mRNA is low (Figure 1C). The plastid gene *accD* encodes a subunit of the prokaryotic-type acetyl-CoA carboxylase. It is feasible that, in chloroplasts, lipid biosynthesis may depend on the nucleus-encoded eukaryotic enzyme (Sasaki *et al.*, 1995). It remains to be seen in which cell types the plastid *accD* gene is highly expressed from the newly identified NEP promoter (Figure 5).

Interestingly, *clpP* is transcribed from the *PclpP*–53 NEP promoter in wild-type leaves while most other NEP promoters are inactive in the same tissue. Differential accumulation of mRNAs from NEP promoters is possibly due to regulation by nuclear-encoded factors via gene-specific promoter elements, as reported for σ^{70}-type PEP promoters (Sun *et al.*, 1989; Iratni *et al.*, 1994; Allison and Maliga, 1995; Kim and Mullet, 1995).

The general rule emerging from the data is that photosystem I and II genes are transcribed by the PEP polymerase, whereas most other genes have both PEP and NEP promoters. This is in agreement with the observed major role for PEP in the transcription of all plastid genes in chloroplasts, including photosynthetic and housekeeping genes (reviewed in Igloi and Kössel, 1992; Gruissem and Tonkyn, 1993; Link, 1994, 1996). As to the role of NEP, we assume that it plays an important role in non-green plastids, in tissues in which PEP is absent or is present only in limited amounts. A similar role for the two plastid RNA polymerases was proposed in the expression of plastid housekeeping genes from alternative promoters in photosynthetic and non-photosynthetic tissues based on the study of tobacco tissue culture cells (Vera and Sugiura, 1995; Vera *et al.*, 1996). Tissue- and cell type-specific transcription from NEP promoters will have to be determined individually, by following accumulation of RNA and proteins for reporter genes in transgenic plastids.

During chloroplast development, early transcription of plastid genes encoding the plastid's transcription and translation apparatus relative to genes encoding proteins involved in photosynthesis was documented in barley (Baumgartner *et al.*, 1993) and pea (DuBell and Mullet, 1995). Selective transcription of the gene groups by the nucleus-encoded and plastid-encoded RNA polymerases was proposed as one possible mechanism of differential gene expression (Hess *et al.*, 1993; Lerbs-Mache, 1993; Mullet, 1993). Distinct NEP and PEP promoters reported here for a large number of transcription units provide a general mechanism for developmentally-timed expression of groups of plastid genes by the two plastid RNA polymerases (Figure 7).

Materials and methods

RNA gel blots

Total leaf RNA was prepared from fully-expanded leaves using TRIzol (Gibco-BRL), following the manufacturer's protocol. The RNA was electrophoresed on 1% agarose–formaldehyde gels, then transferred to Hybond N (Amersham) using the Posiblot Transfer apparatus (Stratagene). Hybridization to random-primer labeled fragment was carried out in Rapid Hybridization Buffer (Amersham) overnight at 65°C.

Double-stranded DNA probes were prepared by random-primed ^{32}P-labeling of PCR-generated DNA fragments. The sequence of the primers used for PCR, along with their positions within the tobacco ptDNA (Shinozaki *et al.*, 1986b) are as follows:

Gene	5′ nt position in ptDNA	Sequence
accD	60211	GGATTTAGGGGCGAA
	60875(C)	GTGATTTTCTCTCCG
atpB	56370(C)	AGATCTGCGCCCGCC
	55623	CCTCACCAACGATCC
atpI	15985(C)	GTTCCATCAATACTC
	15292	GCCGCGGCTAAAGTT
clpP	73621(C)	GACTTTATCGAGAAAG
	73340	GAGGGAATGCTAGACG
ndhA	122115(C)	GATATAGTGGAAGCG
	121602	GTGAAAGAAGTTGGG
ndhB	97792(C)	CAGTCGTTGCTTTTC
	97057	CTATCCTGAGCAATT
ndhF	113366(C)	CTCGGCTTCTTCCTC
	112749	CTCCGTTTTTACCCC
ycf1	129496(C)	GTGACTATCAAGAGG
	128895	GACTAACATACGCCCG
ycf2	92881	GCTCGGGAGTTCCTC
	93552(C)	TGCTCCCGGTTGTTC
petB	78221	GGTTCGAAGAACGTC
	78842(C)	GGCCCAGAAATACCT
psaA	43467(C)	TTCGTTCGCCGGAACC
	42743	GATCTCGATTCAAGAT
psbB	75241	GGAGCACATATTGTG
	75905(C)	GGATTATTGCCGATG
psbE	66772(C)	CAATATCAGCAATGCAGTTCATCC
	66452	GGAATCCTTCCAGTAGTATCGGCC
rpl33/*rpsl8*	70133	GGAAAGATGTCCGAG
	70636(C)	GTTCACTAATAAATCGAC

The *rps16* mRNA was probed with an *Eco*RI fragment isolated from plasmid pJS40, containing sequences between nucleotides 4938–5363 and 6149–6656 of the tobacco ptDNA (Shinozaki *et al.*, 1986b). The *rps14* mRNA was probed with the end-labeled oligonucleotide 5′-CACGAAGTATGTGTCCGGATAGTCC-3′ (5′ end at nt 38621 in the plastid genome; Shinozaki *et al*., 1986b).

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-labeled double-stranded DNA probe was mixed with unlabeled plasmid pKDR1 corresponding to a 2-fold excess over the amount of RNA present on the filter.

Primer extension analysis

Primer extension reactions were carried out on 10 mg (wild-type) or 10 mg (Δ*rpoB*) of total leaf RNA as described (Allison and Maliga, 1995). The primers are listed below. Underlined oligonucleotides were also used to generate the capping constructs.

Gene	5′ nt position in ptDNA	Sequence
accD	59758	CCGAGCTCTTATTTCCTATCAGACTAAGC
atpB	56736	CCCCAGAACCAGAAGTAGTAGGATTGA
atpI	15973	GTATTGATGGAACATGATAGAACAT
clpP#1	74479	GGGACTTTTGGAACACCAATAGGCAT
clpP#2	74947	GGGAGCTCCATGGGTTTGCCTTGG
ORF1901	31451	CTTCATGCATAAGGATACTAGATTACC
ORF2280	87419	GGGAGCTCTACATGAAGAACATAAGCC
rps2	16921	CCAATATCTTCTTGTCATTTCTCTC
rps16	6185	CATCGTTTCAAACGAAGTTTTACCAT

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). Labeled RNAs were detected by ribonuclease protection (Vera and Sugiura, 1992) using the RPAII kit (Ambion). To prepare the protecting complementary RNA, an appropriate segment of the plastid genome was PCR-amplified using the primers listed below. The 5′ primers were designed to add a *Sac*I restriction site (underlined) upstream of the amplified fragment. The 3′ primers were designed to add a *Kpn*I site (underlined) downstream of the amplified sequence. The amplified product was cloned as a *Sac*I–*Kpn*I fragment into *Sac*I- and *Kpn*I-restricted pBSKS+ vector (Stratagene). To generate unlabeled RNA complementary to the 5′ end of RNAs, the resulting plasmid was linearized with *Acc*65I (*Kpn*I isoschizomer), and transcribed in a Megascript (Ambion) reaction with T7 RNA polymerase. The only exception was the *atpI* gene, for which an *Eco*RI site was used for cloning at the 3′ end, and for the linearization of the plasmid. Markers (100, 200, 300, 400 and 500 nt) were prepared with the RNA Century Markers Template Set (Ambion), following the manufacturer's protocol.

Gene	5′ nt position in ptDNA	Sequence
accD	59758(C)	CCGAGCTCTTATTTCCTATCAGACTAAGC
	59576	CCGGTACCATAGGAGAAGCCGCCC
atpB	56750	CCGAGCTCGTAGTAGGATTGATTCTCA
	57131(C)	CCGGTACCGGAGCCAATTAGATACAAA
atpI	15895	CCGAGCTCTGACTTGGAAACCCCC
	16277(C)	CCGAATTCTAGTATTCGCAATTTGT
clpP	74462	GGGAGCTCCAGGACTTCGGAAAGG
	74752(C)	GGGGTACCAATACGCAATGGGG
	74947	GGGAGCTCCATGGGTTTGCCTTGG
	75080(C)	GGGGTACCGCTAATTCATACAGAG
ORF1901	31424	GGGAGCTCCGACCACAACGACCG
	31724(C)	GGGGTACCCTTACATGCCTCATTTC
ORF2280	87419(C)	GGGAGCTCTACATGAAGAACATAAGCC
	87154	GGGGTACCGTGCCTAAGGGCATATCGG

DNA sequence analysis

DNA sequence analysis was carried out utilizing the Wisconsin Sequence Analysis Package (Genetics Computer Group, Inc.).

Acknowledgements

These studies were supported by the National Science Foundation Grants MCB 93-05037 and MCB 96-30763 to P.M. L.A.A. was a recipient of a Charles and Johanna Busch Memorial Fund Postdoctoral Fellowship.

References

Allison,L.A. and Maliga,P. (1995) Light-responsive and transcription-enhancing elements regulate the plastid *psbD* core promoter. *EMBO J.*, **14**, 3721–3730.

Allison,L.A., Simon,L.D. and Maliga,P. (1996) Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants. *EMBO J.*, **15**, 2802–2809.

Baumgartner,B.J., Rapp,J.C. and Mullet,J.E. (1993) Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in barley (*Hordeum vulgare*) chloroplast development. *Plant Physiol*., **101**, 781–791.

Carrillo,N., Seyer,P., Tyagi,A. and Herrmann,R.G. (1986) Cytochrome b-559 genes from *Oenothera hookeri* and *Nicotiana tabacum* show a remarkably high degree of conservation as compared to spinach. *Curr. Genet*., **10**, 619–624.

Dempsey,D., Wobbe,K.W. and Klessig,D.F. (1993) Resistance and susceptible responses of *Arabidopsis thaliana* to turnip crinkle virus. *Mol. Plant Pathol*., **83**, 1021–1029.

DuBell,A.N. and Mullet,J.E. (1995) Differential transcription of pea chloroplast genes during light-induced leaf development. *Plant Physiol*., **109**, 105–112.

Gray,J.C., Hird,S.M. and Dyer,T.A. (1990) Nucleotide sequence of a wheat chloroplast gene encoding the proteolytic subunit of an ATP-dependent protease. *Plant. Mol. Biol*. **15**, 947–950.

Gruissem,W. and Tonkyn,J.C. (1993) Control mechanisms of plastid gene expression. *Crit. Rev. Plant Sci*., **12**, 19–55.

Hallick,R.B. and Bairoch,A. (1994) Proposals for the naming of chloroplast genes. III. Nomenclature for open reading frames encoded in chloroplast genomes. *Plant Mol. Biol. Reporter*, **12**, S29–S30.

Hess,W.R., Prombona,A., Fieder,B., Subramanian,A.R. and Börner,T. (1993) Chloroplast *rps15* and the *rpoB/C1/C2* gene cluster are strongly transcribed in ribosome-deficient plastids: evidence for a functioning non-chloroplast-encoded RNA polymerase. *EMBO J*., **12**, 563–571.

Igloi,G.L. and Kössel,H. (1992) The transcriptional apparatus of chloroplasts. *Crit. Rev. Plant Sci*., **10**, 525–558.

Iratni,R., Baeza,L., Andreeva,A., Mache,R. and Lerbs-Mache,S. (1994) Regulation of *rDNA* transcription in chloroplasts: promoter exclusion by constitutive repression. *Genes Dev.*, **8**, 2928–2938.

Kapoor,S., Wakasugi,T., Deno,H. and Sugiura,M. (1994) An *atpE*-specific promoter within the coding region of the *atpB* gene in tobacco chloroplast DNA. *Curr. Genet.*, **26**, 263–268.

Kennell,J.C. and Pring,D.R. (1989) Initiation and processing of *atp6*, *T-urf13* and *ORF221* transcripts from mitochondria of T cytoplasm maize. *Mol. Gen. Genet.*, **216**, 16–24.

Kim,M. and Mullet,J.E. (1995) Identification of a sequence-specific DNA binding factor required for transcription of the barley chloroplast blue light-responsive *psbD-psbC* promoter. *Plant Cell*, **7**, 1445–1457.

Lerbs-Mache,S. (1993) The 110-kDa polypeptide of spinach plastid DNA-dependent RNA polymerase: single-subunit enzyme or catalytic core of multimeric enzyme complexes? *Proc. Natl Acad. Sci. USA*, **90**, 5509–5513.

Link,G. (1994) Plastid differentiation: organelle promoters and transcription factors. In Nover,L. (ed.), *Plant Promoters and Transcription Factors*. Springer Verlag, Heidelberg, pp. 63–83.

Link,G. (1996) Green Life: control of chloroplast gene transcription. *BioEssays*, **18**, 465–471.

Matsubayashi,T. *et al*. (1987) Six chloroplast genes (*ndhA-F*) homologous to human mitochondrial genes encoding components of the respiratory chain NADH dehydrogenase are actively expressed: determination of the splice sites in *ndhA* and *ndhB* pre-mRNAs. *Mol. Gen. Genet.*, **210**, 385–393.

Maurizi,M.R., Clark,W.P., Kim,S.H. and Gottesman,S. (1990) Clp P represents a unique family of serine proteases. *J. Biol. Chem.*, **265**, 12546–12552.

Meng,B.Y., Tanaka,M., Wakasugi,T., Ohme,M., Shinozaki,K. and Sugiura,M. (1988) Cotranscription of the genes encoding two P700 chlorophyll a apoproteins with the gene for ribosomal protein CS14: determination of the transcription initiation site by *in vitro* capping. *Curr. Genet.*, **14**, 395–400.

Mullet,J.E. (1993) Dynamic regulation of chloroplast transcription. *Plant Physiol.*, **103**, 309–313.

Orozco,E.M., Chen,L.J. and Eilers,R.J. (1990) The divergently transcribed *rbcL* and *atpB* genes of tobacco plastid DNA are separated by nineteen base pairs. *Curr. Genet.*, **17**, 65–71.

Raskin,C.A., Diaz,G.A. and McAllister,W.T. (1993) T7 RNA polymerase mutants with altered promoter specificities. *Proc. Natl Acad. Sci. USA*, **90**, 3147–3151.

Sasaki,Y., Hakamada,K., Suama,Y., Nagano,Y., Furusawa,I. and Matsuno,R. (1993) Chloroplast-encoded protein as a subunit of acetyl-CoA-carboxylase in pea plant. *J. Biol. Chem.*, **268**, 25118–25123.

Sasaki,Y.H., Konishi,T. and Nagano,Y. (1995) The compartmentation of acetyl-coenzyme A carboxylase in plants. *Plant Physiol.*, **108**, 445–449.

Shinozaki,K., Deno,H., Sugita,M., Kuramitsu,S. and Sugiura,M. (1986a) Intron in the gene for the ribosomal protein S16 of the tobacco chloroplast and its conserved boundary sequences. *Mol. Gen. Genet.*, **202**, 1–5.

Shinozaki,K. *et al*. (1986b) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. *EMBO J.*, **5**, 2043–2049.

Sun,E., Wu,B.W. and Tewari,K.K. (1989) *In vitro* analysis of the pea chloroplast 16S rRNA gene promoter. *Mol. Cell. Biol.*, **9**, 5650–5659.

Tanaka,M., Obokata,J., Chunwongse,J., Shinozaki,K. and Sugiura,M. (1987) Rapid splicing and stepwise processing of a transcript from the *psbB* operon in tobacco chloroplasts: determination of the intron sites in *petB* and *petD*. *Mol. Gen. Genet.*, **209**, 427–431.

Tracy,R.L. and Stern,D.B. (1995) Mitochondrial transcription initiation: promoter structures and RNA polymerases. *Curr. Genet.*, **28**, 205–216.

Vera,A. and Sugiura,M. (1992) Combination of *in vitro* capping and ribonuclease protection improves the detection of transcription start sites in chloroplasts. *Plant Mol. Biol.*, **19**, 309–311.

Vera,A. and Sugiura,M. (1995) Chloroplast rRNA transcription from structurally different tandem promoters: an additional novel-type promoter. *Curr. Genet.*, **27**, 280–284.

Vera,A., Hirose,T. and Sugiura,M. (1996) A ribosomal protein gene (*rpl32*) from tobacco chloroplast DNA is transcribed from alternative promoters: similarities in promoter region organization in plastid housekeeping genes. *Mol. Gen. Genet.*, **251**, 518–525.

Westhoff,P. (1985) Transcription of the gene encoding the 51 kDa chlorophyll *a*-apoprotein of the photosystem II reaction center from spinach. *Mol. Gen. Genet.*, **201**, 115–123.

Wolfe,K.H. (1994) Similarity between putative ATP-binding sites in land plant plastid ORF2280 proteins and the FtsH/CDC48 family of ATPases. *Curr. Genet.*, **25**, 379–383.

Wolfe,K.H., Morden,C.W. and Palmer,J.D. (1992) Small single-copy region of plastid DNA in the non-photosynthetic angiosperm *Epifagus virginiana* contains only two genes. Differences among dicots, monocots and bryophytes in gene organization at a non-bioenergetic locus. *J. Mol. Biol.*, **223**, 95–104.

Received on November 14, 1996; revised on March 3, 1997