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Engineering of the *rpl23* gene cluster to replace the plastid RNA polymerase α subunit with the *Escherichia coli* homologue

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Abstract The *Escherichia coli* RNA polymerase (RNAP) α, β, and β′ core subunits are evolutionarily conserved among bacteria and plastids, and the plastid specificity factors form a functional holoenzyme with the *E. coli* core. To investigate whether the *E. coli* core subunits may form a functional hybrid enzyme with the plastid core subunits, we replaced the tobacco plastid RNAP α subunit gene (*rpoA*) with the *E. coli* α subunit gene by targeted gene insertion. The transplastomic tobacco plants look similar to tobacco *rpoA* deletion mutants in that they are chlorophyll-deficient and non-photoautotrophic. In addition, they lack transcripts from promoters recognized by the *E. coli*-like plastid RNA polymerase. These results indicate that evolutionary conservation between the *E. coli* and plastid RNA polymerase α subunits is insufficient to allow substitution of the tobacco α subunit with its bacterial counterpart. Interestingly, the cyanobacterial α subunits are as different as the *E. coli* α subunits; and therefore it is unlikely that replacement of the tobacco α subunit with cyanobacterial α subunits would yield a functional enzyme. Replacement of plastid *rpoA* with the *E. coli* RNA polymerase α subunit gene represents the first engineering of a plastid operon in higher plants.

Key words Plastid RNA polymerase · Plastid transcription · *Escherichia coli* RNA polymerase α subunit · Transplastomic tobacco

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Introduction

During evolution, plastids have maintained a multi-subunit *Escherichia coli*-like RNA polymerase (RNAP) termed the plastid-encoded plastid RNAP (PEP) and, in addition, have acquired a second RNAP related to the mitochondrial RNAP (nucleus-encoded plastid RNAP, or NEP). The PEP core subunits exhibit sequence conservation with counterparts (α, β, β′) found in *E. coli* and other bacteria, although in plastids the β′ subunit N- and C-termini are encoded by separate genes (*rpoC1*, *rpoC2* encoding β′ and β″ subunits, respectively; Sugiura 1992; Link 1996; Stern et al. 1997). There is a division of labor between the two RNAPs: photosynthetic genes are transcribed by the PEP, while most housekeeping genes are transcribed by both RNAPs (Maliga 1998; Hess and Börner 1999). PEP expression is controlled by the NEP since it transcribes the PEP subunit genes (Serino and Maliga 1998; Silhavy and Maliga 1998; Liere and Maliga 1999).

The core subunits of the PEP are absolutely required for normal chloroplast biogenesis and function (Allison et al. 1996; Serino and Maliga 1998; De Santis-Macciossek et al. 1999). PEP is responsible for transcription from plastid promoters, which are similar to *E. coli* σ^{70}-type promoters. The PEP core subunits exhibit sequence conservation with counterparts found in *E. coli* and other bacteria. The similarity is not surprising, since the plastids are thought to have been derived from cyanobacteria-like endosymbiotes which subsequently lost most of their genes to the nucleus (Martin et al. 1998). Plastid promoter recognition is facilitated by nuclear-encoded proteins related to bacterial σ^{70} factors (Liu and Troxler 1996; Isono et al. 1997; Tanaka et al. 1997). Interestingly, the purified plastid specificity factors assemble into a functional holoenzyme with the purified *E. coli* RNAP core and initiate transcription from plastid promoters (Bülow and Link 1988; Tiller et al. 1991; Kestermann et al. 1998), suggesting significant conservation of function between the two RNAPs. We

were interested to find out whether the RNAP α subunits are also interchangeable.

We considered the possibility of replacing the plastid α subunit gene with a cyanobacterial counterpart, since plastids are thought to have evolved from cyanobacteria. However, we calculated that the tobacco and the cyanobacterial α sequences are not significantly more similar than the tobacco and *E. coli* α subunits. The sequence similarity and identity values for *Synechocystis* sp. strain PCC6803 are 50% and 38%; for *Synechococcus* PCC6301 they are 51% and 38%; and for *E. coli* they are only 46% and 32%, respectively. The abundance of information available for the *E. coli* α subunit made it a more attractive choice. Moreover, no direct functional analysis has been performed on the *Synechocystis* α subunit.

In tobacco, the PEP α subunit is encoded by the plastid *rpoA* gene and is expressed independently of the *rpoB* operon, which includes *rpoB*, *rpoC1* and *rpoC2* encoding the β, β′ and β″ subunits. To obtain a hybrid plastid RNAP with the *E. coli* α subunit, we replaced the tobacco plastid *rpoA* gene with the *E. coli rpoA* gene in the plastid genome. We found that transplastomic tobacco plants with the *E. coli rpoA* gene have a mutant phenotype similar to tobacco plants lacking the plastid *rpoA* gene (Serino and Maliga 1998). These results indicate that evolutionary conservation between the *E. coli* and plastid RNAP α subunits is insufficient to allow substitution of the tobacco α subunit with the bacterial counterpart.

Materials and methods

Plasmid construction

Plasmid pGS94 is the precursor of plasmid pGS95 (Serino and Maliga 1998) and is a pBSKS+ vector derivative (Stratagene) with a 3,127-bp *Hin*cII plastid DNA fragment cloned into the *Eco*RV site (*Hin*cII sites at nucleotides 78,993–82,120 of the tobacco chloroplast genome; GenBank Accession No. Z00044; Shinozaki et al. 1986; Wakasugi et al. 1998). The *Hin*cII fragment contains the plastid *rpoA* gene and flanking DNA to serve as targeting sequences in plasmid pJYS54. To facilitate selection of transplastomic lines, a spectinomycin resistance (*aadA*) gene was cloned into the *petD-rpoA* intergenic region in plasmid pJYS54. The *aadA* gene is related to the marker in plastid vector pZS197 (Svab and Maliga 1993): it has a chimeric rRNA operon promoter (Prrn) and the *psbA* 3′ untranslated region (TpsbA). The plastid targeting region of plasmid pJYS54 has been deposited in GenBank under Accession No. AY005806.

Plasmid pJYS60 is identical to plasmid pJYS54, except that an *Xba*I site was introduced upstream of the *rpoA* coding region in a *Sca*I site. The DNA sequence directly upstream of the *rpoA* coding region is 5′-AGTTCTAGAACT**ATG**-3′ and downstream of the coding region is **TAA**GGATCC. Nucleotides introduced during plasmid construction (*Xba*I and *Bam*HI, respectively) are underlined. The *rpoA* translation initiation and termination codons are in bold.

Plasmid pJYS62 is a pJYS60 derivative in which the plastid *rpoA* gene was replaced with the N-terminal 6 × His-tagged *E. coli rpoA* gene from plasmid pHT7f1-NHα (Tang et al. 1995) as a *Xba*I-*Bam*HI fragment. The DNA sequence directly upstream of the *rpoA* coding region is 5′-TCTAGACCAAACAGAGGACACA**ATGCACCACCACCACCACCAC**-3′ and downstream of the coding region is 5′-**TAA** GGATCC-3′. The *Xba*I and *Bam*HI restriction sites are underlined. The start codon (ATG), the histidine codons (CAC) of the 6 × His-tag and the stop codon (TAA) are in bold.

Tobacco plastid transformation

Plastid vectors were introduced into tobacco leaves and transplastomic clones were obtained as previously described (Svab and Maliga 1993). Tentative transplastomic clones were identified by polymerase chain reaction (PCR) analysis. One primer was complementary to the *aadA* N-terminus (codons 15–22; 5′-CGCTCGATGACGCCAACTACC-3′); the second primer was complementary to the 5′ end sequence of the *E. coli rpoA* (5′-CCGGAATTC**ATG** CAGGGTTCTGTGACAGAGTTTC-3′; an added *Bam*HI site is underlined and the *E. coli rpoA* start codon is in bold). Plastid transformation was confirmed by DNA gel blot analysis. Plants transformed with plasmids pJYS54 and pJYS60 are green and grow photoautotrophically in the greenhouse, although the Nt-pJYS60 plants have a transient seedling phenotype. Plants transformed with plasmid pJYS62 are pigment-deficient (white) and can be maintained in sterile culture by growing them on sucrose-containing medium or as grafts in the greenhouse using *Nicotiana tabacum* cv. *xanthi* as rootstock.

DNA and RNA gel blot analysis

Southern analysis was performed on total leaf cellular DNA (1 μg/lane) digested with appropriate restriction endonucleases (Svab and Maliga 1993). DNA fragments were separated in 0.8% agarose gels and transferred to Hybond-N membranes (Amersham) using Posiblot Transfer apparatus (Stratagene). Blots were hybridized at 65 °C in rapid hybridization buffer (NEN) and washed under stringent conditions [0.2 × standard saline citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), 65 °C, 30 min; 0.1 × SSC, 0.1% SDS, 65 °C, 30 min]. Insertion into the targeting region was tested with a 2.4-kb *Xho*I-*Hin*cII plastid DNA fragment (Probe 1; nucleotides 79,717–82,122 of the tobacco plastid genome; GenBank Accession No. Z00044). The *E. coli rpoA* probe was a 1.03-kb *Xba*I-*Bam*HI fragment from plasmid pREIIα (Tang et al. 1995). Probes were ^{32}P-labeled by random primed labeling (Boehringer Mannheim).

RNA gel blot analysis was performed on 3.0 μg RNA isolated from fully expanded leaves of plants grown under sterile conditions (Serino and Maliga 1998). RNA was electrophoresed in 1.2% agarose-formaldehyde gels. Blots were treated as described for Southern analysis. Plastid DNA probes were as follows: *psbD* (nucleotides 34,341–35,280; 0.9-kb fragment); *rbcL* (57,557–58,855; 1.3-kb fragment); *rrn16* (138,540–139,822; 1.3-kb fragment); *psbA* (534–1,595; 1.1-kb fragment); *atpB* (55,759–56,520; 0.76-kb fragment); *clpP* (73,343–73,595; 0.25-kb fragment). The cytoplasmic 25S rDNA probe was PCR-amplified using primers 5′-TCACCTGCCGAATCAACTAGC-3′ and 5′-GACTTCCCTTGCCTACATTG-3′ and total tobacco cellular DNA as template (Dempsey et al. 1993). Radioactive probes were prepared by random primed labeling (Boehringer Mannheim). Relative hybridization intensities were determined by PhosphorImager using the ImageQuant program (Molecular Dynamics). Values for Northern analysis were normalized for signals obtained for the 25S probe and were expressed relative to the WT signal, which was designated as 1.0.

Primer extension analysis

Primer extension analysis was performed as previously described, using 7.5 μg total RNA as template (Allison and Maliga 1995). The DNA sequence of the *atpB* primer was 5′-CCCCAGAACCAGAAGTAGTAGGATTGA-3′ (5′ nucleotide position in tobacco plastid genome: 56,744).

RNAP α subunit amino acid sequence analysis

Alignment was made using the ClustalW program in the Sequence Interpretation tools section of GenomeNetWWW. RNAP α subunit sequences were obtained from NCBI GenPept for *Agrobacterium tumefaciens* (Accession no. AAD47422), *Bordetella pertussis* (P37368), *E. coli* (P00574), *Synechococcus* sp. strain PCC 6301 (BAA22472), *Synechocystis* sp. strain PCC6803 (P73297) and *N. tabacum*, (P06269). Sequence identity was calculated using the bestfit program of the GCG sequence analysis software package with a gap weight of 8 and a length weight of 2.

Results

Tobacco plants with the *E. coli rpoA* gene

The tobacco *rpoA* gene is the last open reading frame of the *rpl23* gene cluster. To investigate whether or not the *E. coli rpoA* gene could functionally replace the endogenous tobacco *rpoA* gene, we designed plastid vector pJYS62 in which the tobacco *rpoA* coding region is replaced by the *E. coli rpoA* coding region. In the pJYS62 construct, *rpoA* transcription is dependent on upstream promoters as in the wild-type operon (Fig. 1A). To facilitate recovery of transplastomic clones with the *rpoA* replacement, a selectable spectinomycin resistance (*aadA*) gene was cloned downstream of the *E. coli rpoA* gene. Plasmid pJYS62 was introduced into chloroplasts on the surface of tungsten particles by the biolistic process, followed by selection on spectinomycin medium. During initial screening, five transplastomic clones were identified by PCR and DNA gel blot analysis. Plants regenerated from the five transformed clones were chimeric and had leaves with white and green sectors. DNA gel blot analysis of plants regenerated from the white sectors confirmed replacement of the plastid *rpoA* gene with the *E. coli rpoA* gene. White plants were regenerated from each of the five transplastomic clones, indicating that the pigment-deficient phenotype was not the consequence of an accidental, independent mutation. We chose to study one line further: Nt-pJYS62-13. DNA gel blot analysis was carried out to confirm replacement of the wild-type plastid genome region with the plastid-targeting region of the transformation vectors and to verify absence of wild-type plastid genome copies (Fig. 1B). Probing the total cellular DNA after digestion with various combinations of *Xho*I, *Xba*I, *Nde*I, *Sac*I and *Pst*I restriction enzymes and probing with the wild-type *Xho*I-*Hin*cII targeting region fragment (Probe P1) confirmed the predicted transformed genomic structure. The only exception was the lack of a predicted 0.65-kb *E. coli rpoA* fragment in *Xho*I/*Pst*I-digested DNA, which did not hybridize with the tobacco *rpoA* sequence under the conditions used (DNA sequence identity ~45%). This 0.65-kb fragment and an 1.54-kb fragment containing *E. coli rpoA* sequences could be detected on the same blot by the *E. coli rpoA* gene probe.

Two types of control transgenic plants were obtained in which the predicted plastid genome structure was confirmed by DNA gel blot analysis (Fig. 1 A, B). Plants transformed with plastid vector pJYS54 were constructed to verify that insertion of *aadA* in the *petD-rpoA* intergenic region does not interfere with plastid function. Plasmid pJYS54-transformed plants exhibit a normal green phenotype, indicating that insertion of *aadA* had no deleterious consequence. Plants representing line Nt-pJYS54-7 were used as controls. Plants transformed with plasmid pJYS60 were used to test that mutations upstream of the *rpoA* are neutral. The Nt-pJYS60-11 plants have a distinct, leaf pigment phenotype when the seedlings are young. The phenotype is transient and older leaves are normal green, thus the Nt-pJYS60-11 plants can be grown photoautotrophically in the greenhouse. Apparently, the introduced *Xba*I site interferes with *rpoA* expression at a specific developmental stage, but the phenotype is subsequently normalized (Fig. 2).

Accumulation of plastid mRNAs in plants with the *E. coli rpoA* gene is similar to plants lacking the *rpoA* gene

The mRNA for the *rpoA* gene in tobacco leaves accumulates to a very low level. Also, the RNAP is present at very low levels. Thus, we decided to test PEP promoter activity as the most sensitive assay to characterize PEP expression in the transgenic plants.

Replacement of the plastid *rpoA* with the *E. coli rpoA* yielded pigment-deficient (white) plants similar in phenotype to RNAP α subunit knock-out (Δ*rpoA*) plants (Serino and Maliga 1998). We assumed therefore that the *E. coli* α subunit is unable to substitute for the tobacco α subunit. To confirm this, we characterized mRNA accumulation in the Nt-pJYS62 plants, since lack of PEP function is associated with a distinct mRNA accumulation pattern. The mRNAs for photosynthetic genes transcribed by the PEP are absent or greatly reduced in Δ*rpoA* plants, because mRNA accumulation for these genes is dependent on read-through transcription from NEP promoters. However, accumulation of mRNAs for housekeeping genes is elevated, due to the activation of NEP promoters (Allison et al. 1996; Hajdukiewicz et al. 1997). We tested plants carrying *the E. coli rpoA* gene (Nt-pJYS62; white), plants with the engineered tobacco *rpoA* upstream region (Nt-pJYS60-11; green), Δ*rpoA* plants (Nt-pGS95; white) and wild-type plants.

The pattern of mRNA accumulation in tobacco plants with the *E. coli rpoA* gene (Nt-pJYS62) was similar to Δ*rpoA* plants (Fig. 3). The level of plastid rRNAs was significantly lower than wild-type, as judged by ethidium bromide staining (Fig. 3 A) and Northern analysis using the 16S rRNA (*rrn16*) gene probe (Fig. 3B). No mRNA was detectable for the photosynthetic genes *psbD*, *rbcL* and *psbA* (Fig. 3 B). However, significant mRNA accumulation was observed for *clpP* (encoding the proteolytic subunit of the Clp ATP-dependent protease) and *atpB* (encoding the β subunit of the ATP synthase), as expected. Both genes are

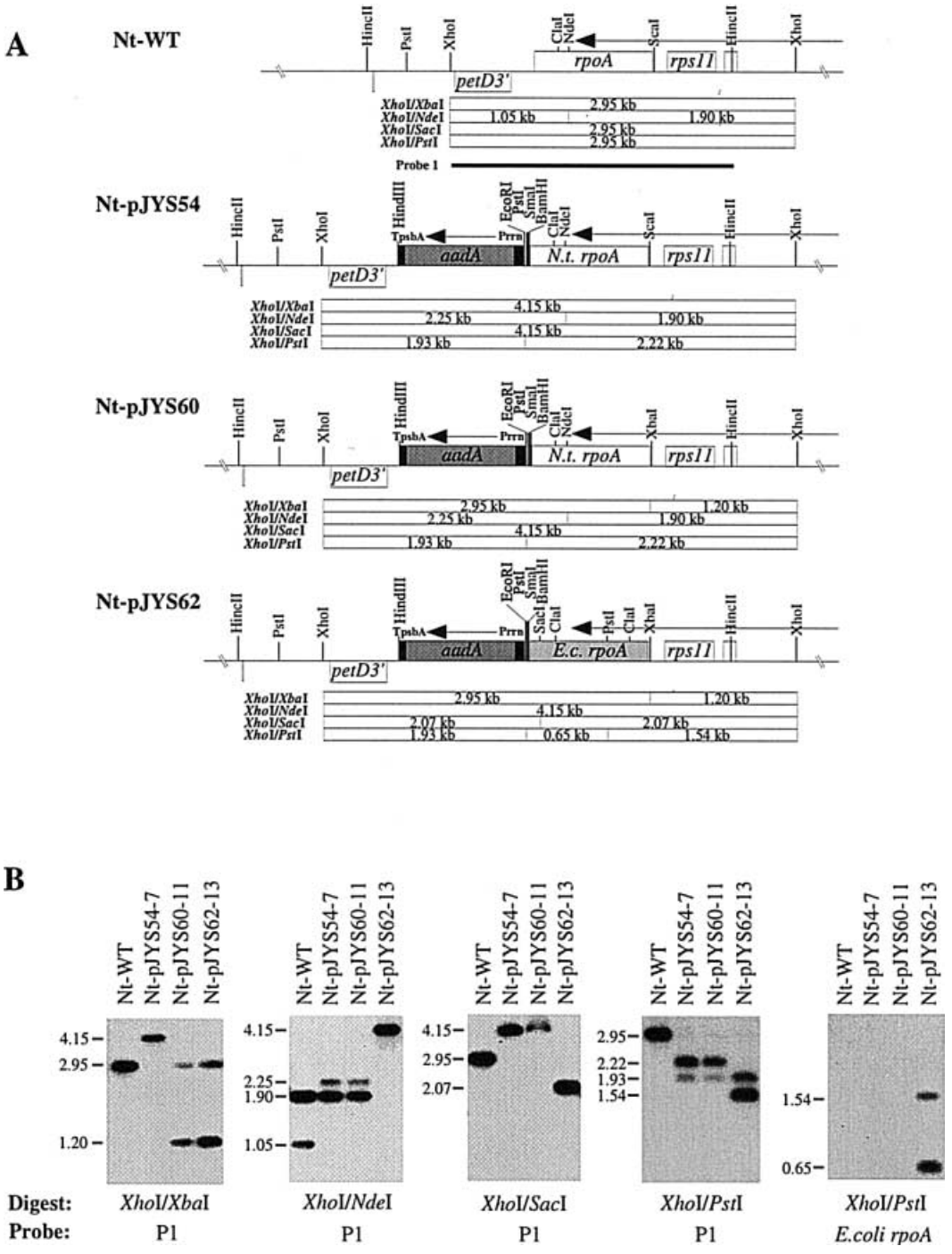

Fig. 1 A, B DNA gel blot analysis confirms predicted plastid genome structure. **A** Map of the plastid genome containing the *rpoA* gene in the wild-type (*Nt-WT*) and transplastomic tobacco plants. *Nt-pJYS54* is a control plant carrying *aadA* in the *petD-rpoA* intergenic region, *Nt-pJYS60* is a control plant with engineered *Xba*I site upstream of tobacco *rpoA* and *Nt-pJYS62* is a tobacco plant with the *Escherichia coli rpoA* gene. Vectors pJYS54, pJYS60 and pJYS62 carry targeting regions between the *Hin*cII sites. Plastid DNA fragments detected by *Probe 1* (*Xho*I-*Hin*cII fragment) are shown below the maps. *aadA* is the chimeric spectinomycin resistance gene, *E.c. rpoA* is the *E. coli rpoA* gene, *N.t. rpoA*, *petD*, *rpoA* and *rps11* are tobacco plastid genes; and *arrows* indicate the direction of transcription **B** DNA gel blot analysis of transplastomic clones. Probes were *P1*, the wild-type plastid targeting region (2.4-kb *Xho*I-*Hin*cII fragment; Fig. 1A) and the *E. coli rpoA* gene (*Bam*HI-*Xba*I fragment)

transcribed from NEP and PEP promoters. In the absence of PEP, transcription is enhanced from the NEP promoters (Hajdukiewicz et al. 1997). The control tobacco plants with the engineered *Xba*I site (Nt-pJYS60) had an essentially wild-type expression profile (Fig. 3).

Primer extension analysis confirms lack of PEP promoter activity

The most sensitive assay for PEP activity in vivo is detection of transcription from individual promoters by

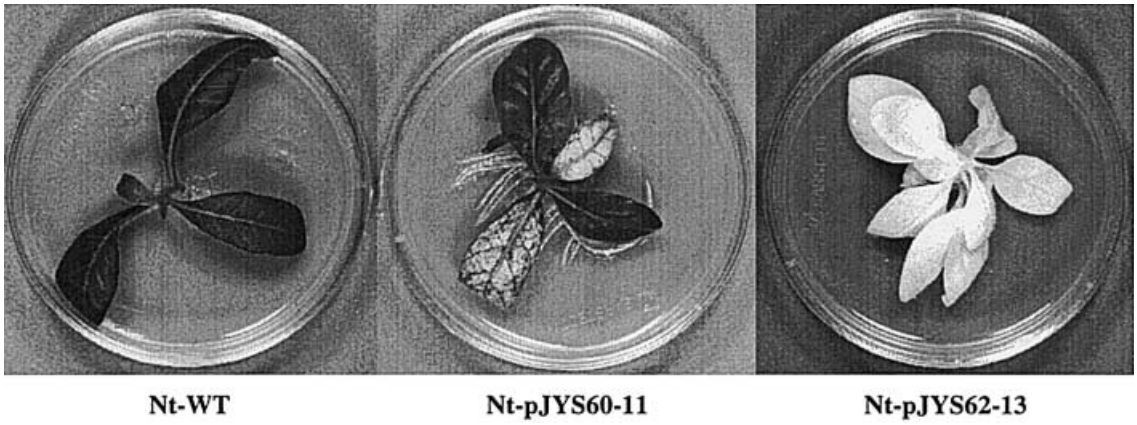

Fig. 2 Phenotype of wild-type tobacco (*Nt-WT*) and engineered, transplastomic plants expressing the tobacco *rpoA* (*Nt-pJYS60-11*) and *E. coli rpoA* (*Nt-pJYS62-13*)

primer extension analysis. The *atpB* gene is transcribed by five major promoters initiating transcription 611, 502, 488, 289 and 255 nucleotides upstream of the translation initiation codon. With one exception (−289), all promoters are recognized by the PEP. The relative contribution of PEP and NEP promoters to *atpB* transcript accumulation has been well characterized in wild-type (Orozco et al. 1990) and Δ*rpoB* plants (Hajdukiewicz et al. 1997). Primer extension analysis therefore simultaneously tested the activity of three of the PEP promoters (−255, −488 and −502), using the NEP promoter as the reference (−289). Data in Fig. 4 indicate that in the Nt-pJYS62 plants with the *E. coli rpoA* all PEP promoters are silent, supporting the conclusion that the *E. coli* α subunit does not functionally substitute for the tobacco plastid α subunit. In contrast, in the control plant with the altered sequence upstream of the tobacco *rpoA* (Nt-pJYS60) transcription occurs from all PEP promoters. The Nt-pJYS60 RNA sample in Fig. 4 was taken from a green leaf.

Discussion

Evolutionary conservation between the *E. coli* and plastid RNA polymerase α subunits was found to be insufficient to allow substitution of the tobacco α subunit with the bacterial counterpart. This finding contrasts assembly and function of the plastid σ factors with the *E. coli* core (Bulow and Link 1988; Tiller et al. 1991; Kestermann et al. 1998). The bacterial σ factors are about as related to the plastid σ-like factors as the bacterial and plastid α subunits (Hakimi et al. 2000). Thus, the requirements for functional complementation with core subunits are more stringent than for specificity factors.

The introduced *E. coli* α subunit carried a His-tag at its N-terminus. We believe that the His-tag did not interfere with plastid RNAP assembly or function, since replacement of the tobacco α subunit with a non-tagged

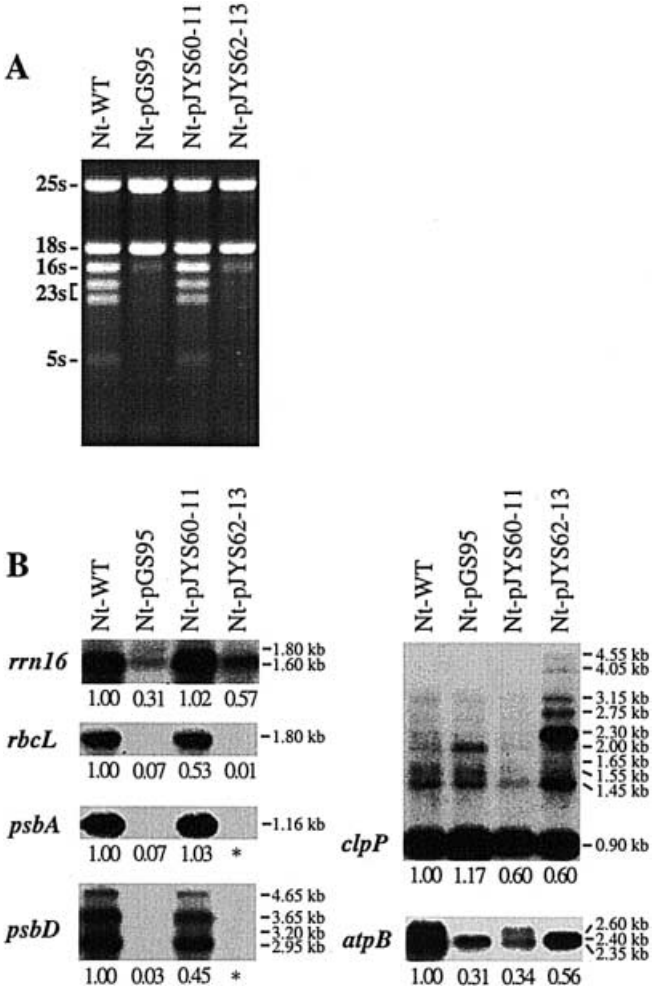

Fig. 3 A, B Plastid mRNA accumulation pattern is similar in tobacco plants expressing the *E. coli rpoA* (Nt-pJYS62-13) and plants lacking the *rpoA* gene (Δ*rpoA*; Nt-pGS95). Data are also shown for wild-type (*Nt-WT*) and transplastomic tobacco plants with mutations upstream of the tobacco *rpoA* (*Nt-pJYS60-11*). **A** Ethidium bromide stained RNA gel. Positions of cytoplasmic (*18S* and *25S*) and plastid (*16S*, processed *23S* and *5S*) RNAs are marked. **B** Accumulation of mRNA for the plastid *psbD*, *rbcL*, *psbA*, *rrn16*, *clpP* and *atpB* genes. Values for Northern analysis were normalized for signals obtained for the cytoplasmic 25S rRNA probe and are expressed relative to the WT signal (1.0; *numbers under lanes*)

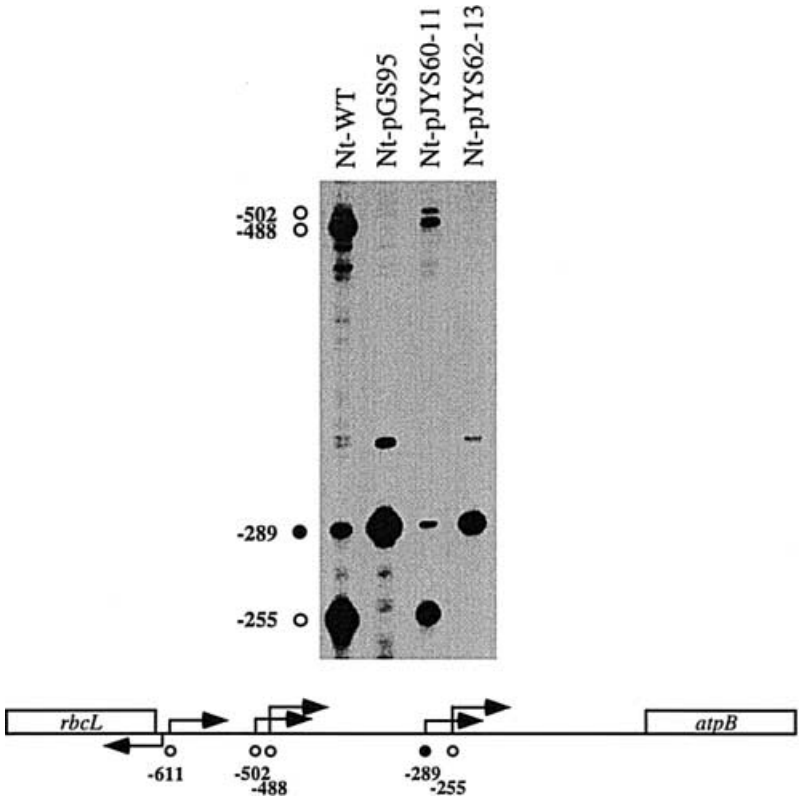

Fig. 4 Primer extension analysis to detect transcription from the plastid *atpB* PEP and NEP promoters. PEP promoters (*open circles*) yield transcripts at −502, −488 and −255; −289 derives from a NEP promoter (*filled circle*). Data are shown for: wild-type (*Nt-WT*), plants lacking the *rpoA* gene (*Nt-pGS95*), plants with mutations upstream of the tobacco *rpoA* (*Nt-pJYS60-11*) and plants with *E. coli rpoA* (*Nt-pJYS62-13*)

E. coli α subunit also yielded pigment-deficient (white) plants (data not shown). Furthermore, His-tagging of the tobacco α subunit C-terminus does not affect plastid RNAP function either (unpublished). Engineering of sequences upstream of the *E. coli rpoA* could prevent its expression. Since the control Nt-pJYS60-11 plants (which carry the mutant sequence upstream of the tobacco *rpoA*) have PEP activity, we believe this is not the case. In addition, insertion of *aadA* at the *petD-rpoA* intergenic region could affect expression of the downstream *psbB* operon. This possibility was excluded by confirming that the Nt-pJYS54 plants, carrying *aadA* in the *petD-rpoA* intergenic region, have a wild-type phenotype (data not shown). *E. coli rpoA* expression was tested by testing PEP promoter activity using primer extension analysis and mRNA gel blots, as other means of testing gene expression are not readily applicable to *rpoA* (see above).

The *E. coli* RNAP α subunit has two functional domains, an N-terminal domain (NTD) required for core subunit assembly and a C-terminal domain (CTD) required for transcription activation at specific highly expressed genes (Igarashi and Ishihama 1991; Busby and Ebright 1994; Fig. 5). The αCTD is not utilized for transcriptional activation at all *E. coli* promoters, but is still required for viability (Hayward et al. 1991). We recently determined that the αCTD of tobacco is dispensable for PEP transcription, chloroplast biogenesis and photosynthesis (unpublished). The αCTD is dispensable, but not the αNTD (αNTD1 and αNTD2), as deletion of *rpoA* yields mutant plants (Serino and Maliga 1998).

The *B. pertussis* α subunit has been shown to substitute for the *E. coli* α subunit in vitro (Lohrke et al. 1999). Also, the *A. tumefaciens* α subunit could substitute for the *E. coli* α subunit in vitro (Steffen and Ullmann 1998). Apparently, the bacterial α N-terminal domains (αNTDs) can interact with heterologous β and β′ subunits to assemble into a core enzyme with proper basal transcription activity, albeit with species-specific αCTD transcription activation properties. The *B. pertussis* and *E. coli* α subunits share a 62% overall identity; the *A. tumefaciens* and *E. coli* α subunits share 53% overall identity; and the tobacco plastid and *E. coli* α subunits share 32% identity and 46% similarity. The identities of the bacterial α subunits reflect the close relationship of *B. pertussis*, *A. tumefaciens* and *E. coli*, which respectively are representatives of the β, α and γ subdivisions of the same (Proteobacteria) group. Apparently, the *E. coli* α subunit is not close enough to the tobacco α subunit to functionally replace it. Sequence conservation between the tobacco plastid α subunit and cyanobacterial α subunits is not significantly higher; and therefore it is unlikely that replacement of the tobacco plastid RNAP α subunit with its cyanobacterial homologue would yield a functional plastid RNAP.

The lack of complementation of the plastid *rpoA* by the *E. coli rpoA* gene could be due to the lack of assembly of a core consisting of the *E. coli* RNAP α sub-

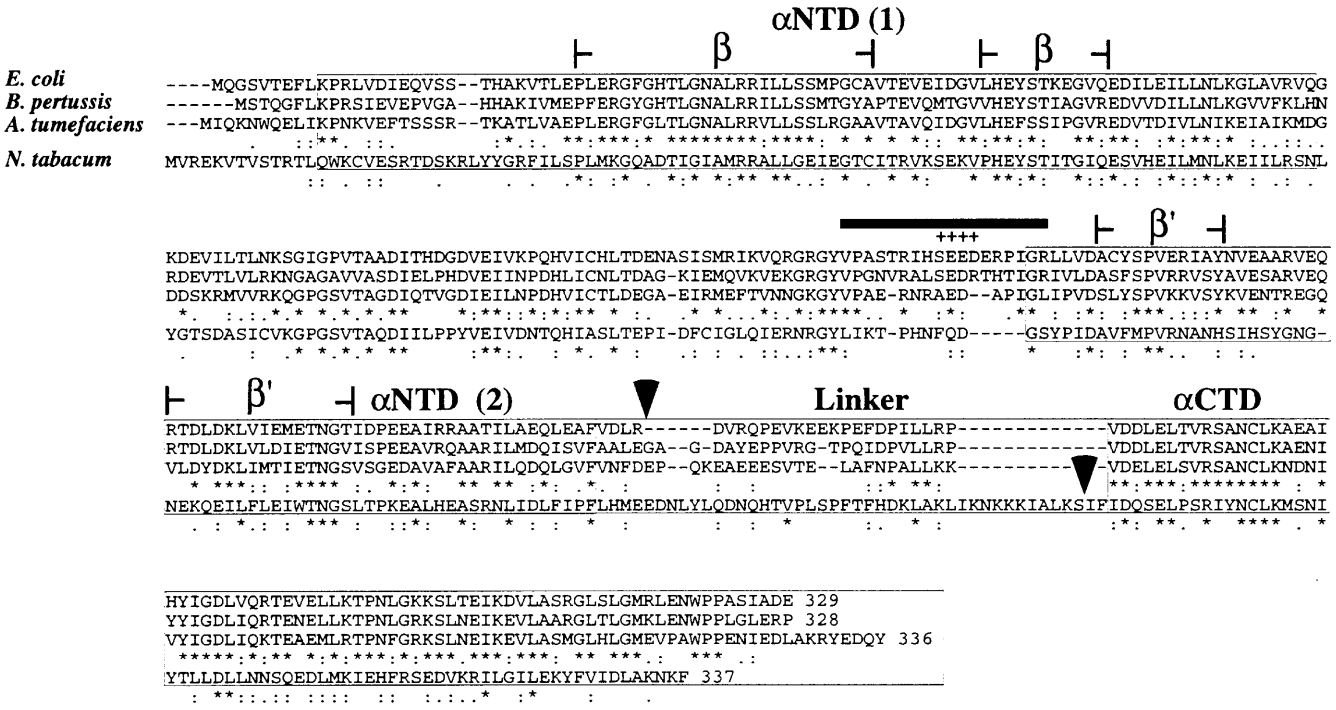

Fig. 5 Amino acid alignment of the bacterial and tobacco plastid RNA polymerase α subunits. *Asterisks* indicate identity, *colons* similarity and *dots* indicate partial conservation. Two domains of the *E. coli* αNTD (*numbers 1 and 2 in parentheses*; Zhang and Darst 1998) and regions important for *β* or *β′* subunit interactions are marked (Heyduk et al. 1996). The *E. coli* αNTD and αCTD are connected by a non-conserved region containing a *Linker* (Jeon et al. 1997). The *vertical arrow* in the *Linker* region marks the endpoint of the αCTD deletion that in *E. coli* is sufficient for core assembly in vitro, but is lethal in vivo (Hayward et al. 1991). The *vertical arrow* in the tobacco sequence marks the endpoint of a plastid αCTD deletion that allows wild-type activity (unpublished). The region between *E. coli* *β* sheet S9 and *β* sheet S10 (Zhang and Darst 1998), which is variable and distinct in length in plastid α subunits, is marked by a *heavy bar*. *E. coli* residues important for CAP-dependent transcription activation are marked by *pluses* (Niu et al. 1996)

unit and the plastid *β*, *β′* and *β″* subunits. Alternatively, lack of complementation of the plastid *rpoA* by the *E. coli rpoA* could be due to the lack of ability to substitute for newly acquired functions such as light regulation, developmental control (Sexton et al. 1990; Baumgartner et al. 1993; Pfannschmidt et al. 1999) and interaction with additional, ancillary proteins associated with the plastid RNAP core (Boyer and Hallick 1998; Pfannschmidt et al. 2000). So far, replacement of coding regions in plastids has been limited to monocistronic transcription units. Replacement of the plastid *rpoA* gene with the *E. coli* RNAP α subunit represents the first engineering of a plastid operon in higher plants.

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