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RESEARCH ARTICLE

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Sequences upstream of the YRTA core region are essential for transcription of the tobacco *atpB* NEP promoter in chloroplasts in vivo

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Abstract Transcription of the plastid *atpB* gene is accomplished by plastid-encoded (PEP) and nuclear-encoded (NEP) RNA polymerases. In contrast to NEP promoters of many other plastid genes, the tobacco *atpB* NEP promoter exhibits robust activity in chloroplasts in vivo. Previously, in vitro transcription assays using extracts from non-photosynthetic cells identified two elements required for full *atpB* NEP promoter activity, a core sequence and an upstream GAA box. Of these, only the core sequence containing the motif YRTA is conserved in the majority of NEP promoters. We used plastid transformation to examine the requirements for *atpB* NEP promoter activity in chloroplasts. Our results demonstrate that sequences upstream of the core element are essential for promoter activity in vivo, and that transcription of the NEP promoter in chloroplasts is not dependent on activity from an overlapping PEP promoter.

Keywords Plastid promoter · Plastid RNA polymerase · Plastid transformation · *atpB*

Introduction

Plant plastid genomes encode subunits of an *Escherichia coli*-like RNA polymerase (PEP) which initiates transcription from sigma^{70}-type promoters (reviewed in Hess and Börner 1999; Liere and Maliga 2001). PEP promoter recognition is mediated by nuclear-encoded sigma factors (Allison 2000), whose expression or activity is controlled by developmental cues and by light (Baginsky et al. 1997; Lahiri and Allison 2000). In addition to PEP, plastids utilize a nuclear-encoded RNA polymerase (NEP). The complete subunit composition of NEP is not fully determined, although the catalytic subunit is known to resemble the single-subunit RNA polymerases of the T7 bacteriophage and mitochondria (reviewed in Hess and Börner 1999; Liere and Maliga 2001). The similarities between mitochondrial RNA polymerases and NEP extend to their respective promoters (reviewed in Weihe and Börner 1999). Sequence alignments indicate that most NEP promoters are composed of a short ~11-nucleotide consensus element encompassing a YRTA motif, resembling the YRTAT sequence of mitochondrial promoters (Hajdukiewicz et al. 1997; Kapoor et al. 1997; Miyagi et al. 1998; Silhavy and Maliga 1998a; Kapoor and Sugiura 1999; Liere and Maliga 1999). In vitro transcription assays have confirmed the functional importance of the YRTA motif for specifically initiated transcription from two NEP-dependent plastid promoters, PrpoB-345 (Liere and Maliga 1999) and PatpB-289 (Kapoor and Sugiura 1999).

PatpB-289 is an unusual NEP promoter, since it is not only highly active in undifferentiated proplastids, but also in green-leaf chloroplasts (Hajdukiewicz et al. 1997; Kapoor et al. 1997). NEP activity is dramatically enhanced in undifferentiated proplastids, but most NEP promoters are silenced in differentiated chloroplasts (reviewed in Liere and Maliga 2001). Exceptions to this trend have been reported for NEP promoters for the *atpB*, *atpI*, *clpP*, *accD*, and *rpoB* genes (Hajdukiewicz et al. 1997; Kapoor et al. 1997; Miyagi et al. 1998). For *accD* and *rpoB*, which are transcribed solely by NEP, promoter activity is barely detectable in mature chloroplasts. In contrast, utilization of NEP promoters for *atpB*, *clpP*, and *atpI* is readily detectable in green leaves. Therefore, some unique feature of these NEP promoters renders them active in fully differentiated photosynthetic plastids.

Deletion analyses of *clpP* and *atpB* NEP promoters attempted to define the sequences required for their activity. Sequence alignments revealed that the tobacco

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clpP NEP promoter, PclpP-53, bears no resemblance to other NEP recognition elements (Hajdukiewicz et al. 1997). In vivo dissection determined that sequences downstream of the PclpP-53 transcription initiation site (from –5 to +25) are required to support accurately initiated transcription from this promoter in tobacco chloroplasts (Sriraman et al. 1998). Therefore, it is unclear whether tobacco *clpP* is expressed by NEP assembled with a different promoter-selectivity factor, or is expressed by an uncharacterized plastid RNA polymerase. In contrast to *clpP*, the tobacco *atpB* and *atpI* NEP promoters have a perfect match to the consensus YRTA core. In addition, they both contain an upstream sequence element (referred to either as box II, or the GAA box) centered at –35. The functional significance of the GAA box was confirmed in vitro for PatpB-289, using transcription extracts from tobacco BY-2 cultured cells (Kapoor and Sugiura 1999). Therefore, it is possible that the strong activity of this promoter in chloroplasts is also mediated by the GAA-box element. Since the BY-2 cells used to make transcription extracts are non-photosynthetic, it was not possible to test this hypothesis with the in vitro system.

Another potential explanation for the strong activity of both *atpI* and *atpB* NEP promoters in green leaves is their physical proximity to PEP promoters (Hajdukiewicz et al. 1997; Kapoor et al. 1997; Miyagi et al. 1998). Transcription from the *atpI* NEP promoter initiates at position –207, just a few nucleotides distant from an upstream PEP promoter, which directs transcription initiation at –209 and –212. Similarly for *atpB*, the NEP transcription initiation site physically overlaps with a downstream PEP promoter. In bacteria, there are several examples of adjacent promoters influencing each other's activity (Gafny et al. 1994; Kubori and Shimamoto 1997). Therefore, it is possible that PEP promoter activity aids transcription of adjacent NEP promoters in mature chloroplasts containing active PEP.

To learn more about the requirements for NEP promoter utilization in green leaf chloroplasts, we used plastid transformation in tobacco to examine the activity of PatpB-289 in the presence and absence of sequences upstream of the core promoter and in the presence and absence of activity from the overlapping PEP promoter. Our in vivo analysis indicates that promoter sequences upstream of the core YRTA element are essential for transcription in chloroplasts and that activity of the adjacent PEP promoter does not contribute to the unusually strong transcription from PatpB-289 in green tissues.

Materials and methods

Plasmid constructs

A 367-base pair DNA fragment encompassing the –255 (PEP) and –289 (NEP) transcription initiation sites (positions 57,239 to 56,873 in the tobacco plastid DNA; numbering according to Shinozaki et al. 1986) was amplified from tobacco using primers UNL73 (5′-gggagctcCCTCTATGAATCTATGAAAGG-3′) and UNL87 (5′-gggctgcagTTAGCACTCGATTTCGTTGG-3′), where the upper case sequence is complementary to the tobacco plastid DNA and the lower case letters include engineered restriction sites. The PCR product was subcloned as a *Sac*I-*Pst*I fragment upstream of the *uidA* reporter gene in plasmid pGX20, to generate plasmid pGX23. Plasmid pGX20 consists of a stuffer fragment of the chloroplast genome (positions 31,918 to 33,810) cloned as a *Sac*I-*Pst*I fragment upstream of a *rbs::uidA::rps16* reporter gene in pPRV112A (Zoubenko et al. 1994). The *uidA* gene was engineered with 33 additional nucleotides at the N-terminus, encoding a myc-FLAG tag (Allison, unpublished). To inactivate the PEP promoter, the –10 box (TATAAT) in pGX23 was mutagenized to GCGAAT, using megaprimer PCR (Smith and Klugman 1997) with primers UNL73, UNL87, and UNL74 (5′-CATTTGTCTATCATT*CGC*GACAATCCCATCCAT-3′; anneals from 57,019 to 57,051; the mutated nucleotides are in italics). The resulting PCR fragment was sequenced to confirm the mutation, then subcloned as a *Sac*I/*Pst*I fragment into plasmid pGX20 to generate pGX26. For the deleted promoter derivative, PCR was done on pGX26, using primers UNL87 and UNL78 (5′-gggagctcTCCATAGAATAGATAATATGG-3′; anneals from 57,068 to 57,048). The resulting promoter derivative was subcloned into the *Sac*I/*Pst*I sites of pGX20 to generate plasmid pGX34.

Plastid transformation

Leaves of sterile-propagated tobacco (*Nicotiana tabacum*) were bombarded with plasmid-coated tungsten particles, using the DuPont PDS1000He biolistic device at 7,600 kPa, according to previously published protocols (Svab and Maliga 1993). Transformed shoots were regenerated from bombarded leaf tissue on RMOP medium (Svab et al. 1990) supplemented with 500 μg spectinomycin dihydrochloride/ml. Transgenic shoots, confirmed by DNA gel blot analysis, were rooted on RM medium (agar-solidified Murashige and Skoog salts with 3% sucrose) prior to transfer to the greenhouse.

DNA gel blots

Total leaf DNA was prepared according to Mettler (1987) and digested with *Bam*HI. Digestion products were separated on 0.7% agarose gels, transferred to Hybond-N+ Nylon membranes (Amersham Life Science), using the Posiblot transfer apparatus (Stratagene), and hybridized overnight at 65 °C in RapidHyb buffer (Amersham Life Science) with a radioactive probe. The probe was amplified from tobacco plastid DNA, using primers that spanned the transgene insertion site (from nucleotide 139,675 to 140,829). The purified PCR product was ^{32}P-labeled, using the Prime-It II random primer labeling kit (Stratagene).

Primer extension analysis

Total leaf RNA was prepared using TRIzol (Gibco BRL), following the manufacturer's protocol. Primer extension reactions were performed on 5 μg or 15 μg of total leaf RNA, as described by Allison and Maliga (1995). Primers used were either an *atpB* primer (5′-CCCCAGAACCAGAAGTAGTAGGATTGA-3′), which is complementary to the *atpB* coding region from nucleotide 56,736 to 56,762, or a *uidA* primer (5′-TCCAGACTGAATGCCCACAGGCCGT-3′), which anneals to the *uidA* gene at nucleotides 47–71 in the native coding region. We note that, since the *uidA* gene in this study contains an additional 33 nucleotides of engineered sequence after the ATG, the primer actually anneals between nucleotides 80 and 104 of the engineered reporter gene. Sequence ladders were generated with the *uidA* primer on plasmid pGX23 using the Sequenase II kit (USB).

Results

A 367-base pair plastid genome fragment directs transcription initiation of a reporter mRNA at PatpB-255 and PatpB-289 in vivo

The *atpB* gene is located adjacent to the divergently transcribed *rbcL* gene in the chloroplast genome and is transcribed from several different promoters (Fig. 1). Initiation sites for *atpB* transcripts (bent arrows in Fig. 1) have been mapped at positions –611, –502, –488, –289, and –255, with respect to the translation initiation codon (Orozco et al. 1990; Hajdukiewicz et al. 1997; Kapoor et al. 1997). Only one of these primary transcripts, –289, is generated through the activity of NEP (black arrows) and all other transcripts are produced by PEP (gray arrows). The core sequence of the NEP promoter, PatpB-289, overlaps with the –35 sequence motif of a downstream PEP promoter, PatpB-255 (Fig. 1). To isolate these two overlapping promoters from other transcriptional regulatory sequences in the *rbcL*/*atpB* intergenic region, we amplified a 367-base pair fragment, diagrammed in Fig. 1, which encompassed both PatpB-289 (the NEP promoter) and PatpB-255 (the PEP promoter). This fragment was fused to the reporter gene, *uidA*, in a transformation vector containing the spectinomycin resistance gene, *aadA*; and the linked genes were introduced into the tobacco plastid genome by biolistic transformation. A spectinomycin-resistant transgenic tobacco line (labeled GX23) was regenerated and verified for the presence of the transgenes in the plastid genome by DNA gel blot analysis (Fig. 2B, lane 2). The gel blots confirmed that the transgene was targeted to the expected site in the plastid DNA and that no wild-type genomes were apparent in the transformed line, indicating that the majority of plastid genomes were transformed (Fig. 2B, lane 1 vs lane 2).

To determine whether the fused *atpB* fragment could support accurately initiated transcription from PatpB-289 (NEP) and PatpB-255 (PEP) promoters, total leaf RNA from the transgenic GX23 plant and from a wild-type plant was analyzed by primer extension (Fig. 3). The primer extension reactions used either an *atpB* coding sequence primer to detect endogenous *atpB* transcripts (lanes 1, 4, filled circles), or a *uidA* coding sequence primer to detect transgene transcripts (lanes 2, 3, asterisks). The endogenous *atpB* –289 and –255 transcripts were readily detected in both wild-type plants (lane 1) and in the transgenic GX23 line (lane 4). As previously reported (Orozco et al. 1990; Hajdukiewicz et al. 1997; Kapoor et al. 1997), the PEP-derived transcript (–255) accumulated to higher levels than the NEP-derived transcript (–289) in green leaf tissue. Primer extension with the *uidA* primer yielded no extension products in the wild-type RNA, as expected (lane 2). In contrast, in the GX23 RNA sample, *uidA* extension products were detected of the size predicted for transgenic transcripts initiated within the fused *atpB* fragment at the NEP and PEP promoters (lane 3, asterisks). Although the transgenic –289 band is faint, it was not detected at all in the wild-type RNA with this primer and therefore represents a *uidA* transcript 5′ end. As was observed for the endogenous *atpB* transcripts, the transgenic transcripts derived from PatpB-255 accumulated to a higher level than those derived from PatpB-289, indicating that the fused *atpB* fragment reproduced the transcription activity of the endogenous *atpB* regulatory region in green chloroplasts in vivo. This *atpB* fragment was therefore used as the starting point to ask two specific questions: is the activity of the PatpB-289 NEP promoter in chloroplasts dependent on activity from the adjacent PatpB-255 PEP promoter; and is the core YRTA NEP consensus sequence sufficient for transcription of the PatpB-289 NEP promoter in chloroplasts?

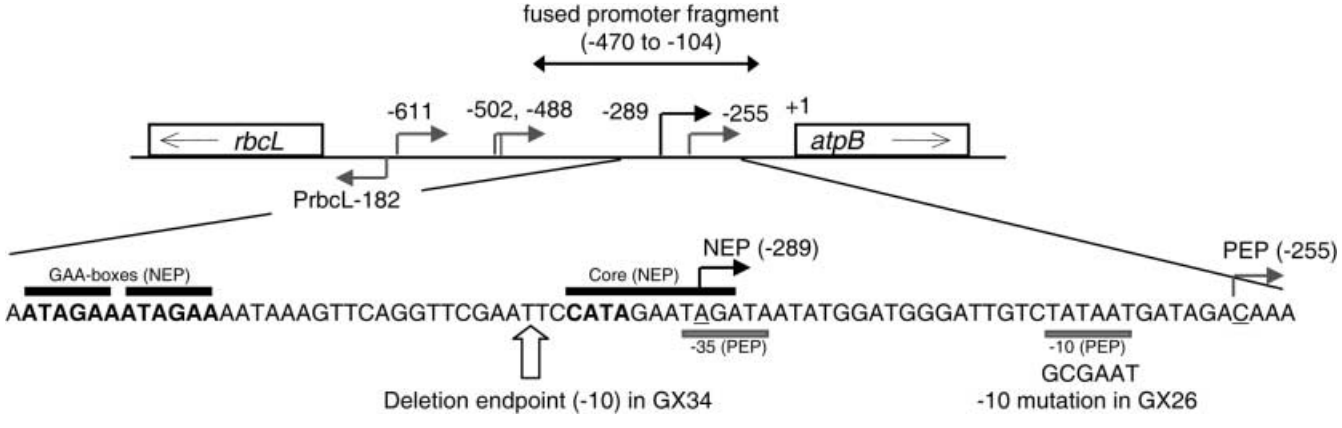

Fig. 1. Diagram depicting features of the *atpB* regulatory region. The intergenic region between tobacco *rbcL* and *atpB* genes is shown, with transcription initiation sites (*bent arrows*) numbered with respect to the translation initiation nucleotide of their cognate gene. Plastid-encoded RNA polymerase (PEP) promoters and their consensus elements are underlined by *gray boxes*, while nuclear-encoded RNA polymerase (NEP) promoters and their elements are delineated by *black boxes*. The overlap between NEP-289 and PEP-255 promoters is indicated in the sequence shown *below the line drawing*. Transcription start sites are *underlined* and important NEP promoter sequences are shown *in bold*. The *double-headed arrow* indicates the portion of the *atpB* regulatory region that was fused to the *uidA* reporter gene in this study

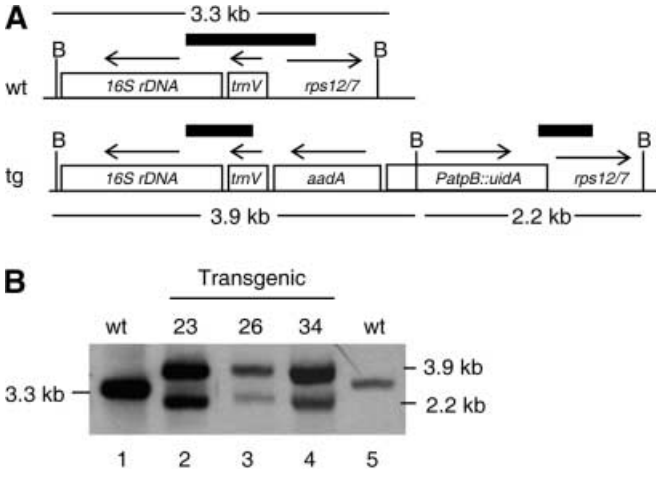

Fig. 2A, B. Analysis of plastid transformants by DNA gel blots. **A** Diagram of wild-type plastid genomes (*wt*) around the transgene insertion site and transgenic plastid genomes (*tg*) with the *aadA* and *uidA* transgenes inserted. Gene names and direction of transcription (*arrows*) are shown. Sizes of *Bam*HI (*B*) restriction fragments are indicated. Probe annealing sites are depicted by *black bars*. **B** DNA gel blots to analyze transgenic lines. Plant DNA digested with *Bam*HI was hybridized with a probe spanning the transgene insertion site. Wild-type (*wt*) DNA samples (*lanes 1, 5*) generated a 3.3-kb band while transgenic lines (GX23, GX26, GX34) generated two bands at 3.9 kb and 2.2 kb (*lanes 2–4*). Note the apparent absence of wild-type genomes in the transgenic samples

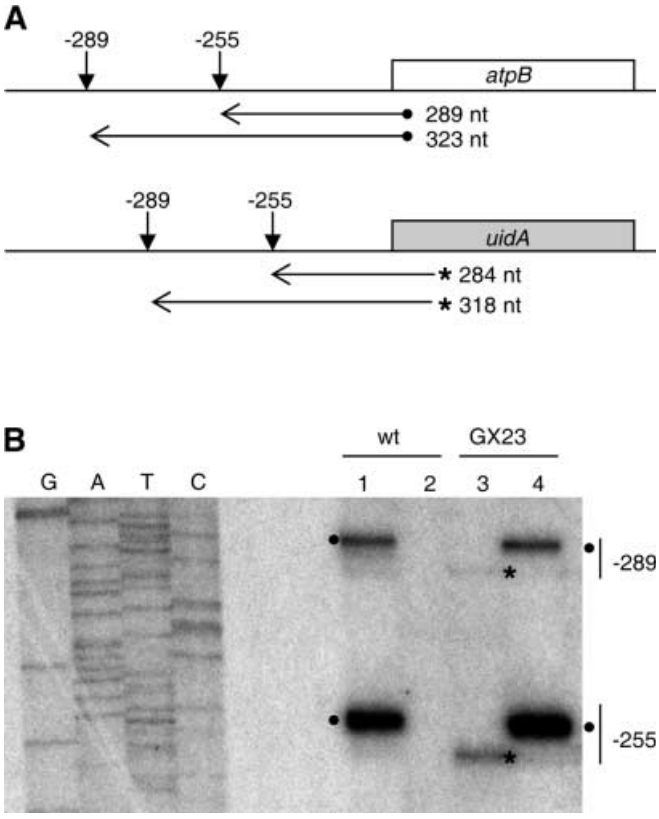

Fig. 3A, B. Analysis of transcript 5′ ends derived from *atpB* NEP and PEP promoters. **A** Diagram showing expected sizes of primer extension products. The *atpB* primer anneals to endogenous *atpB* transcripts initiated from the NEP (*–289*) or PEP (*–255*) promoters (*upper figure*). The *uidA* primer generates transgene extension products initiating from within the fused *atpB* regulatory fragment at NEP (*–289*) and PEP (*–255*) promoters (*lower figure*). Product sizes (nucleotides; *nt*) reflect where the primers anneal in the *atpB* or *uidA* coding sequences and, for *uidA* extension products, reflect the amount of *atpB* sequence downstream of PatpB-255 that was fused to the reporter gene. **B** Primer extension products derived from *atpB* (*circles*) and *uidA* (*asterisks*) transcripts in 5 μg of RNA from wild-type (*wt*) or transformed (*GX23*) tobacco lines. The *adjacent sequence ladder* was generated with the *uidA* primer on the transforming plasmid, pGX23

Disruption of PatpB-255 promoter activity does not influence activity from the overlapping NEP promoter

To eliminate activity from the PEP PatpB-255 promoter, we introduced mutations into the sequence of the –10 promoter element, changing it from TATAAT to GCGAAT (Fig. 1). In PEP promoters, as in eubacterial promoters, the –10 sequence is an essential element for promoter recognition and activity (Nakahira et al. 1998; Satoh et al. 1999; Thum et al. 2001). Mutagenesis of the first three nucleotides of this element would be expected to dramatically reduce expression from PatpB-255. A transformation vector containing *uidA* linked to the 367-base pair *atpB* promoter fragment bearing the –10 mutation was introduced into the plastid genome of tobacco, generating fully transformed plant line GX26 (Fig. 2, lane 3). Leaf RNA from GX26 was subjected to primer extension with the *uidA* primer and the transgene extension products were compared to those generated by the same primer from GX23 RNA (Fig. 4B, lane 1 vs lane 2). To enhance the *uidA* primer extension signals, three times more input RNA was used than in the experiment shown in Fig. 2. This analysis showed that mutagenesis of the –10 promoter element dramatically reduced transcription from the PEP promoter, as evidenced by almost undetectable levels of the –255 transcript. In contrast, there was no obvious change in the levels of the NEP transcript derived from PatpB-289. This result clearly demonstrates that activity from the overlapping PatpB-255 promoter does not influence the relatively high levels of transcription from PatpB-289 in chloroplasts.

PatpB-289 core YRTA consensus element alone cannot support chloroplast transcription by NEP

The second question we addressed is whether 11 nucleotides of the core NEP consensus (as defined by Hajdukiewicz et al. 1997), encompassing the YRTA motif, are sufficient for transcription of the PatpB-289 NEP promoter in chloroplasts. A deletion derivative of the *atpB* promoter fragment was generated, lacking all PatpB-289 sequences upstream of position –10 (with respect to the transcription initiation site) but retaining the core promoter YRTA motif (Fig. 1). This derivative was made in the *atpB* fragment bearing the PEP –10 mutation, so that only the effect of the deletion on NEP promoter activity would be tested. The deletion derivative, linked to *uidA* in a plastid transformation vector, was introduced into the plastid genome of tobacco to generate two plastid transformants, GX34-1

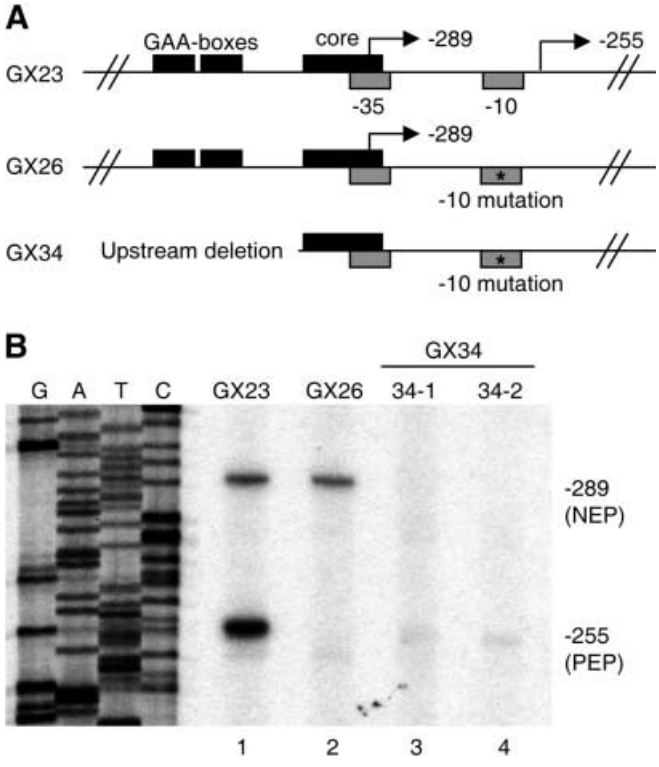

Fig. 4A, B. Effect of PEP activity and upstream sequences on transgenic PatpB-289 activity. **A** Diagram of fused *atpB* promoter derivatives used in this experiment. NEP promoter elements are depicted as *black boxes*, PEP elements as *gray boxes*. Transcription initiation from the NEP (*–289*) and PEP (*–255*) promoters is indicated by *bent arrows*. **B** Primer extension done with the *uidA* primer on 15 μg of RNA from plants transformed with promoter derivatives shown in **A**. A single transformant for each of GX23 (*lane 1*) and GX26 (*lane 2*) was analyzed, while two independently transformed GX34 lines are shown (*lanes 3, 4*). The *adjacent sequence ladder* was generated with the *uidA* primer on the transforming plasmid, pGX23

and GX34-2 (Fig. 2, lane 4 shows GX34-1). Leaf RNA from both lines was subjected to primer extension with the *uidA* primer and transgene extension products were compared with those generated by the same primer from GX26 RNA (Fig. 4, lanes 3, 4 vs lane 2). In contrast to the results seen with the full-length promoter fragment (GX26), there was no NEP-289 transcript detected in RNA from lines with the promoter deletion derivative. The presence in all four lanes of equal levels of a non-specific primer extension product, migrating at a higher position on the gel, indicated that the same amount of RNA had been added for each plant sample (data not shown). These data demonstrate that sequences upstream of the core YRTA promoter element are required for the high-level transcription of the PatpB-289 NEP promoter in chloroplasts in vivo.

Discussion

This study presents the only in vivo analysis of a YRTA-type NEP promoter to date. The majority of NEP promoters are highly active in tissues with undifferentiated proplastids, but are silenced in chloroplasts. This property makes their in vivo dissection impractical in tobacco plastid transformants, due to difficulties in isolating sufficient proplastid-containing tissues from the transgenic lines for promoter analysis. For the few NEP promoters exhibiting robust transcription in chloroplasts (e.g. *clpP*, *atpI*, *atpB*), transgenic approaches to in vivo analysis are possible. For example, the non-YRTA-containing *clpP* NEP promoter, PclpP-53, was dissected in vivo by following its activity in leaf chloroplasts of transformed tobacco (Sriraman et al. 1998). Unfortunately, the results of this analysis could not be extrapolated to other NEP promoters, since PclpP-53 is the only NEP promoter defined to date which does not have a YRTA consensus core sequence and thus contains an atypical NEP recognition element.

We chose to focus on the NEP promoter, PatpB-289, since it is a YRTA-containing promoter with robust chloroplast expression and is therefore amenable to in vivo analysis using plastid reporter genes. Previously, an in vitro analysis of this promoter was accomplished using transcription extracts prepared from proplastid-containing tobacco BY-2 cells (Kapoor and Sugiura 1999). This informative study used promoter deletions to demonstrate that sequences downstream of –41 (with respect to the NEP transcription initiation site) were sufficient for full promoter activity in proplastids. Mutagenesis of the YRTA box reduced promoter activity to ~10% of the wild type in the in vitro system, while mutagenesis of additional sequences within the core region, or of the GAA box centered at ~–35, reduced promoter activity to ~25% of the wild type. These data provided strong evidence for the functional importance of both the core sequences and the GAA box for promoter activity in proplastids. Since BY-2 cells do not contain chloroplasts, the in vitro transcription assays could not address the NEP promoter sequence requirements for transcription in differentiated photosynthetic organelles. Furthermore, in vitro transcription extracts from wild-type tobacco chloroplasts failed to produce any transcripts from the PatpB-289 promoter template (Kapoor and Sugiura 1999). Therefore, to extend the results of the in vitro studies, we used tobacco plastid transformation to study the transcription activity of two PatpB-289 promoter derivatives in chloroplasts in vivo.

One explanation for enhanced chloroplast transcription from PatpB-289 was its close physical linkage to a downstream PEP promoter, PatpB-255. There are several documented cases of adjacent promoters in bacteria that influence each other's activity. For example, the rRNA operons in *E. coli* are transcribed from tandem promoters, P1 and P2, separated by ~120 base pairs. Transcription from P1 has been shown to diminish transcription from the downstream P2 promoter, presumably by a mechanism of promoter occlusion (Gafny et al. 1994). In the case of the overlapping PatpB-289 and PatpB-255 promoter elements, we rationalized that activity of the downstream PatpB-255 promoter in

chloroplasts, where PEP RNA polymerase is highly active, might influence the recognition of PatpB-289 by the NEP RNA polymerase. A previous attempt was made to test this model using in vitro transcription with BY-2 cell extracts (Kapoor and Sugiura 1999). However, treatment of the extracts with the PEP transcription inhibitor tagetitoxin resulted in only partial inhibition of PEP activity, as did mutagenesis of the PEP –35 promoter sequence, thereby leading to inconclusive results. Our test of this model involved inactivating the PatpB-255 PEP promoter by mutagenizing its –10 element, leaving all upstream NEP promoter sequences intact. The results clearly showed that the *atpB* NEP promoter functions completely independently of the overlapping PEP promoter, arguing that the placement of these two promoters in close proximity has no functional significance in chloroplasts.

Another possible explanation for PatpB-289 expression in chloroplasts was the presence of additional sequences upstream of the YRTA core, including the GAA box. The GAA box element, while not essential for promoter function in BY-2 cell extracts in vitro, was required for full levels of promoter activity. In addition, the GAA box sequence is conserved in other NEP promoters exhibiting chloroplast activity (Hajdukiewicz et al. 1997; Kapoor and Sugiura 1999). Interestingly, the maize *atpB* NEP promoter contains a consensus core sequence, including the YRTA motif, but lacks a consensus GAA box at –35. This NEP promoter is not expressed in chloroplasts (Silhavy and Maliga 1998a), consistent with a putative role for the GAA box in robust chloroplast expression of YRTA-containing NEP promoters.

We tested whether, in the absence of upstream sequences, the YRTA-containing core promoter of tobacco PatpB-289 could drive transcription of a reporter gene in transgenic tobacco chloroplasts. An equivalent deletion of the tobacco *rpoB* NEP promoter was capable of readily detectable transcription in vitro, albeit at a reduced level (~25%) compared with the full-length promoter (Liere and Maliga 1999). However, in the present study, no *uidA* PatpB-289 transcripts were detected from this deleted promoter in vivo, using an assay in which 25% promoter activity should have been readily detected. This lack of detectable promoter activity may be due to the removal of nucleotides –14 through –11, or the removal of the upstream GAA boxes, or a combination of both. In an in vitro dissection of the tobacco *rpoB* NEP promoter, it was observed that removal of sequences between –14 and –10 reduced promoter activity to 24% of full-length activity (Liere and Maliga 1999). Similarly, the *atpB* NEP promoter with three point mutations introduced at nucleotides –11, –12, and –13, exhibited reduced promoter activity in vitro to ~25% of the wild type (Kapoor and Sugiura 1999). Based on these in vitro data, we expected reduced but detectable primer extension product from the deletion derivative of the PatpB-289 promoter we tested in vivo. The fact that transcript synthesis was reduced to below detectable levels in the deletion derivative may be due to the absence of the GAA boxes upstream of the NEP promoter core, causing additional depletion of activity from the transgene NEP promoter. We note that the *atpB* NEP promoter actually has two tandem copies of the GAA box (Fig. 1), although only one was tested for function by mutagenesis in the in vitro studies.

The overlapping NEP and PEP promoters of *atpB* may provide a compact transcriptional regulatory fragment for monocot plastid transformation vectors. Since transformation of several monocot species is routinely done with embryogenic tissues, it is important that plastid transformation vectors drive selectable marker expression from promoters active in embryonic cells as well as in leaf chloroplasts. NEP promoters are strongly transcribed in plants with either nuclear mutations or engineered plastid deletions that disrupt chloroplast differentiation, thereby supporting an important role for NEP in proplastid gene expression (Hajdukiewicz et al. 1997; Hübschmann and Börner 1998; Silhavy and Maliga 1998a). Although plastid promoter utilization was shown to be similar in rice embryogenic cell cultures and leaves (Silhavy and Maliga 1998b), it seems prudent to include both NEP and PEP promoters in a monocot plastid transformation vector, since the complete regeneration process from transformed embryogenic tissues may involve proplastids at some stage. We anticipate that an 81-base pair tobacco genome fragment (sequence shown in Fig. 1), encompassing PatpB-289 (with two intact GAA boxes) and PatpB-255, will provide a useful addition to the arsenal of tools being developed for plastid transformation of monocots.

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