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An *atpE*-specific promoter within the coding region of the *atpB* gene in tobacco chloroplast DNA

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Abstract. The *atpB* and *atpE* genes encode β and ε subunits, respectively, of chloroplast ATP synthase and are co-transcribed in the plant species so far studied. In tobacco, an *atpB* gene-specific probe hybridizes to 2.7- and 2.3-kb transcripts. In addition to these, a probe from the *atpE* coding region hybridizes also to a 1.0-kb transcript. The 5′ end of the *atpE*-specific transcript has been mapped 430/431 nt upstream of the *atpE* translation initiation site, within the coding region of the *atpB* gene. In-vitro capping revealed that this transcript results from a primary transcriptional event and is also characterized by –10 and –35 canonical sequences in the 5′ region. It has been found to share a common 3′ end with the bi-cistronic transcripts that has been mapped within the coding region of the divergently transcribed *trnM* gene, approximately 236 nt downstream from the *atpE* termination codon. Interestingly, this transcript accumulates only in leaves and not in proplastid-containing cultured (BY-2) cells, indicating that, unless it is preferentially degraded in BY-2 cells, its expression might be transcriptionally controlled.

Key words: *atpE* – Chloroplast transcript – Internal promoter – *Nicotiana tabacum*

Introduction

Studies on chloroplast genome organization have revealed that most of the genes are organized in complex operons. Moreover, genes with common function have often been found to be grouped together in these operons (Sugiura 1992; Mullet 1993). This is true, also, for the genes encoding polypeptides of the energy-transducing supra-molecular complex, the ATP synthase, of thylakoid membranes,. In land plants and green algae, this complex consists of nine subunits, of which six are encoded in the chloroplast genome, whereas, genes for the rest have been localized in the nucleus. The chloroplast-encoded genes are grouped in two transcriptional units, namely *atpIHFA* and *atpB/E*.

As far as transcriptional activity is concerned, the *atpIHFA* gene cluster produces up to 20 transcripts of lengths varying from 0.5 to 6.0 kb (Bird et al. 1985; Westhoff et al. 1985; Cozens et al. 1986; Hudson et al. 1987). But, the *atpB/E* operon is transcribed into multiple bi-cistronic transcripts ranging from 2.4 to 2.8 kb which are capable of translating both β and ε subunits (Zurawski et al. 1982; Shinozaki et al. 1983; Mullet et al. 1985; Kanno and Hirai 1993). In spinach, multiple transcripts for this operon were found to originate from multiple promoter activities in the upstream region of the *atpB* gene (Chen et al. 1990). In tobacco, however, apart from a 2.7-kb bi-cistronic transcript, a 1.3-kb transcript was reported to hybridize specifically to a probe from the *atpE* coding region (Shinozaki et al. 1983). The 5′ end of the bi-cistronic mRNA was mapped by an S1 protection assay to a position "–255" relative to the *atpB* start codon and this region was also recognized as a promoter by *E. coli* RNA polymerase (Shinozaki and Sugiura 1982). Subsequently, taking a clue from the presence of multiple promoters for the spinach *atpB* gene, Orozco et al. (1990) examined the entire region between the *atpB* and *rbcL* coding sequences in tobacco. They found six different 5′ ends relative to the *atpB* translation initiation site, of which three (viz., –255, –490 and –610) were recognized as in-vitro transcription initiation sites by spinach chloroplast and *E. coli* RNA polymerases. However, no further investigation was undertaken to determine the origin of the 1.3-kb transcript hybridizing to the *atpE*-specific probe.

Studies on translational aspects of maize *atpB* and *atpE* in *E. coli* revealed that translation of these two overlapping genes is coupled, because a frame-shift mutation introduced in the *atpB* coding region, causing immature termination of the polypeptide, greatly hampered the translation of *atpE* (Gatenby et al. 1989). This implies, indirectly, that *atpE*-specific ribosome binding activity is not enough to initiate translation, independent of translation from *atpB*. Hence, if such a transcript (like the *atpE*-spe-

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cific 1.3-kb transcript in tobacco, Shinozaki et al. 1983) exists which lacks a functional *atpB* coding region, it would be interesting to determine its origin and its role in the proper functioning of the ATP synthase complex.

In the present study, as a initial step to understanding the significance of the *atpE*-specific transcript, we have analyzed whether it arises directly from transcription initiation or by processing of the bi-cistronic transcripts having their start sites in the 5′ upstream region of the *atpB* coding region (see Orozco et al. 1990). We report here the presence of a new, *atpE*-specific, promoter in the coding region of the *atpB* gene. A strong possibility that this promoter might express in a developmentally-regulated manner has been discussed in the light of the data from tobacco BY-2 cultured cells having undifferentiated plastids (proplastids) instead of chloroplasts.

Materials and methods

Plant material. Seedlings of *Nicotiana tabacum* (var. BY-4) were raised on vermiculite for 25 days at 25 ±1 °C in light/dark cycles of 18 h/6 h daily. Liquid cultures of *N. tabacum* BY-2 cell line (reviewed in Nagata et al. 1992) were grown in MS medium (Murashige and Skoog 1962) supplemented with 2,4-D (2 μg/ml), vitamin B1 (1 μg/ml), inositol (100 μg/ml) and NaH_2PO_4 (3.7 μg/ml) at pH 5.8, for 6 days in an incubator shaker at 100 rpm and 25 °C.

Preparation of nucleic acids. Total cellular RNA from tobacco leaves and BY-2 cells was isolated using the procedure developed by Logemann et al. (1987) and stored in 100-μg batches at −70 °C in 95% ethanol. DNA fragments from pTB27, a recombinant plasmid containing 7 056-bp of tobacco chloroplast DNA comprising the coding regions of *rbcL, atpB, atpE, trnM* and *trnV* (Shinozaki et al. 1986; Sugiura et al. 1986), were purified from agarose-gel pieces using Superec™ 0.2 DNA purification spin columns (TaKaRa Biomedical, Japan) and subcloned into the Bluescript SK^+ expression vector (Stratagene, USA). The resultant plasmids, pTC1, pTH2, pTH3 and pTCB4, were used as templates for in-vitro transcription using T3 or T7 RNA polymerases and [α-^{32}P]UTP, after digestion with appropriate restriction enzymes as described earlier (Vera et al. 1992). A 321-bp *Taq*I fragment was used to prepare a 3′-labelled strand-specific probe for an S1-nuclease protection assay (see Fig. 3). Using a DNA synthesizer (Applied Biosystems USA, Type 380A) a 20-mer oligonucleotide, 5′ TAGCTATAATGTCCTGAAGT 3′, was prepared for primer extension analysis.

Northern-blot analysis. Ten micrograms each of leaf and BY-2 denatured RNA samples were resolved on 1.2% agarose-formaldehyde gels (Ausubel et al. 1989) and transferred to Hybond N^+ nylon membranes according to the manufacturer's specifications. Pre-hybridization and hybridization of the filters was as described earlier (Kapoor et al. 1993). For hybridization, strand-specific riboprobes were synthesized from the plasmids described above.

Ribonuclease protection assays. Fifty micrograms of total leaf and BY-2 RNA were hybridized to gel-purified [α-^{32}P]UTP-labelled riboprobes and digested by a mixture of RNases A and T1 essentially according the instruction manual supplied with the ribonuclease protection assay kit (Ambion, USA).

5′ transcript mapping. Primer extension experiments were carried out using 12 and 24 μg of total RNA from leaves and BY-2 cells, respectively, with 1 pmol of the 5′ [^{32}P]-labelled 20-mer oligonucleotide as primer (described above) and 10 units of AMV reverse transcriptase (BRL, USA), according to the method described by Sambrook et al. 1989. The reaction products were analyzed on a sequencing gel. A sequencing ladder, generated by a PCR ds-sequencing kit (BRL, USA), using pTB27 plasmid and the same primer, was used as a reference to determine the length of the extended products.

S1 nuclease transcript mapping of the 3′ end. The S1-nuclease protection assay was carried out essentially as described (Shinozaki and Sugiura 1982). The 3′-labelled probe of the coding strand was annealed to 15 μg of leaf RNA and treated with S1 nuclease. The protected fragment was electrophoresed on a 6% polyacrylamide urea gel along with a sequence ladder generated by using the Maxam-Gilbert method (Sambrook et al. 1989).

In-vitro capping coupled with a ribonuclease protection assay. Total leaf RNA (100 μg) was capped using 20 units of guanylyltransferase (BRL, USA) according to the method of Kennell and Pring (1989). Hybridization with a non-radioactive riboprobe and ribonuclease protection assays were done as described by Vera and Sugiura 1992.

Results

Transcripts of the atpB/E gene cluster in leaf and BY-2 cells

To confirm the presence of an *atpE*-specific transcript and its relative abundance in leaves and BY-2 cells. Northern-blot analysis was performed using strand-specific ribo-

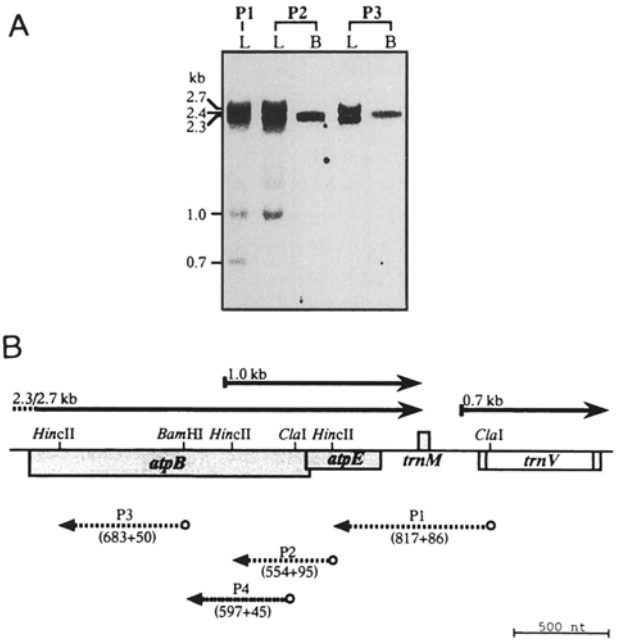

Fig. 1A, B. Northern-blot analysis of the *atpB/E* operon. **A** autoradiogram of the transcript pattern. Total RNA from tobacco leaves (*L*) and BY-2 cells (*B*) was hybridized to in-vitro transcribed [^{32}P]-labelled RNA probes as marked on the top of each lane. **B** schematic representation of the region between 53 598 and 56 816 nt of the total tobacco chloroplast DNA sequence (Shinozaki et al. 1986). Protein-coding regions are shown as *shaded boxes* and the intron within *trn*V as an *empty box*. *Long arrows* indicate the length and direction of in-vivo transcripts. *Broken arrows* marked P1 to P4 represent the cloned DNA fragments and the direction of in-vitro transcribed RNA probes from the respective clones, also utilized in subsequent experiments. *Figures in brackets* are lengths of fragment and vector sequences in the in-vitro transcribed RNA probes, respectively

probes (Fig. 1). Panel A shows RNA gel blots of transcripts complementary to probes P1, P2 and P3, which accumulate in leaves and BY-2 cultured cells (L and B lanes, respectively). The diagram in panel B shows the approximate sizes and location of four transcript species detected in this experiment. The 2.7- and 2.3-kb transcripts are the most abundant, hybridizing to all three probes in leaves, suggesting that they have their 5′ ends upstream of the *atpB* translation initiation site. An approximately 1.0-kb transcript, described as the 1.3-kb transcript by Shinozaki et al. (1983), hybridizes to probes P1 and P2. Therefore, this transcript, having the coding potential for the ε subunit, was thought to have its 5′ end in the 3′ coding region of the *atpB* gene. The levels of this transcript were only 10% as compared to the individual levels of the 2.3- and 2.7-kb transcripts. Another 0.7-kb transcript, hybridizing only to probe P1, represents the intron-containing pre-tRNA^{Val} (see Deno et al. 1982).

As far as the expression of the *atpB/E*-hybridizing transcripts in BY-2 cells is concerned, instead of two bi-cistronic transcripts only one transcript of 2.4 kb was detected in significant amounts (Fig. 1, lanes B for P2 and P3). Interestingly, however, no detectable signal from the 1.0-kb transcript was obtained. This led us to a more detailed analysis of this *atpE*-specific transcript with respect to its origin.

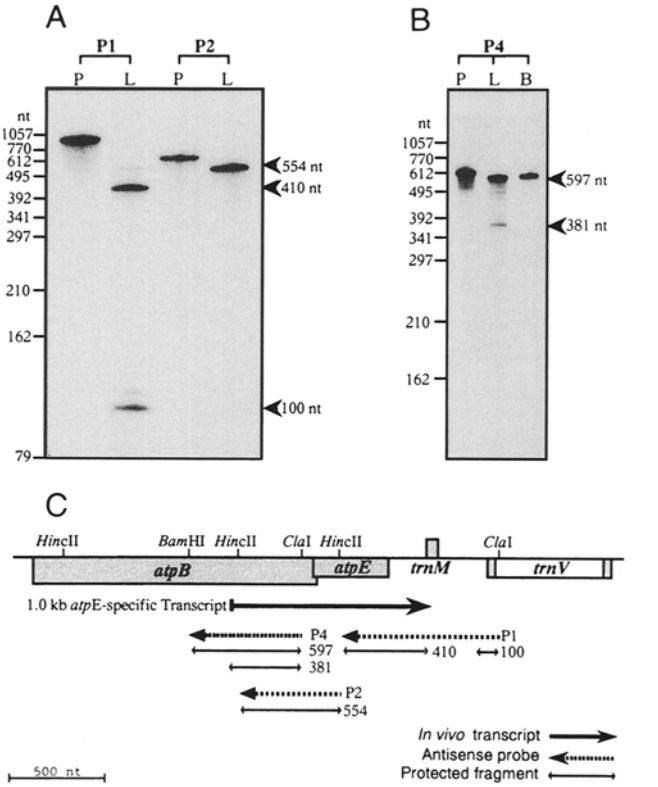

Fig. 2A–C. Determination of 3′ and 5′ ends of the 1.0-kb *atpE*-specific transcript by ribonuclease protection assays. **A, B** *P1*, *P2* and *P4* are the in-vitro transcribed [^{32}P]-labelled RNA probes, loaded as such (*P*) or hybridized to leaf (*L*) or BY-2 (*B*) total RNA and digested with RNases before separating on a denaturing polyacrylamide gel. Ribonuclease-protected fragments and their sizes are marked on the right. **C** schematic representation of the RNA probes P1, P2 and P4 and ribonuclease-protected fragments. For details also see text and Fig. 1

Determination of the 3′ and 5′ boundaries of the 1.0-kb transcript

Figure 2 represents the data on ribonuclease protection assays using probes P1, P2 and P4, for the fine mapping of the 1.0-kb transcript. Starting from the 3′ end, a ribonuclease protection assay with P1 (817+86 nt) and total-leaf RNA results in two protected bands of approximately 400 and 100 nt, respectively. Taking into consideration, also, the data of Northern-blot analysis, the 100-nt band seems to be due to the protection of the intron-containing pre-tRNA^{Val}, whereas the 400-nt band represents the protected region of 3′ end of the *atpB* and *atpE* transcripts. No band for the completely-protected probe P1 (817 nt) was detected, thereby suggesting that the *atpB/E* and *trnV* genes constitute separate transcriptional units. Furthermore, the presence of only one protected band (400 nt) for the *atpB/E* 3′ region indicates that the 2.7/2.3-kb and the 1.0-kb transcripts share a common termination site.

For precise mapping of the 3′ end, an S1-nuclease protection assay was performed using total-leaf RNA and a 321-bp *Taq*I fragment (positions 14 and 335 relative to the *atpE* termination codon) labelled at its 3′ end with ^{32}P (Fig. 3). The result shows the presence of three bands, corresponding to positions 236-, 237- and 238-nt 3′ of the *atpE* termination codon, within the coding region of the divergently-transcribed *trnM* gene. The untranslated region between *atpE* and *trnM* is characterized by three inverted repeat sequences that can potentially form stable stem-loop structures, and the third putative stem-loop structure also includes 8 nt of the *trnM* coding sequence (Fig. 3B). The position of the mapped 3′ end, only 7 nt downstream from the third putative stem-loop structure, is suggestive of its involvement in the stabilization of *atpB/E* transcripts.

Protection assays with probe P2 resulted only in a single band representing the fully-protected P2 probe (554 nt), suggesting that the 5′ end of the 1.0-kb transcript was situated either very near to, or further upstream from, the *Hin*cII site (Fig. 2C). Therefore, another probe, P4 (a 597-nt *Cla*I-*Bam*HI fragment, covering 269 nt more 5′ of the gene) was used for the ribonuclease protection analysis with leaf (L) and BY-2 (B) total RNA (Fig. 2B). This time, apart from a fully-protected band of 597 nt – representing protection of the 2.7- and 2.3-kb transcripts initiating 5′ to the *atpB* translation initiation site – a smaller band of 381 nt was also observed. This indicates that the 1.0-kb transcript has its 5′ end approximately 431 nt upstream of the translation initiation site. To verify the results obtained from Northern hybridizations, BY-2 total RNA was also used for a ribonuclease protection assay with the P4 probe. As can be seen in lane B of Fig. 2B, only one protected band of 597 nt was present and no protected signal from the 1.0-kb *atpB*-specific transcript (expected size 381 nt) was obtained.

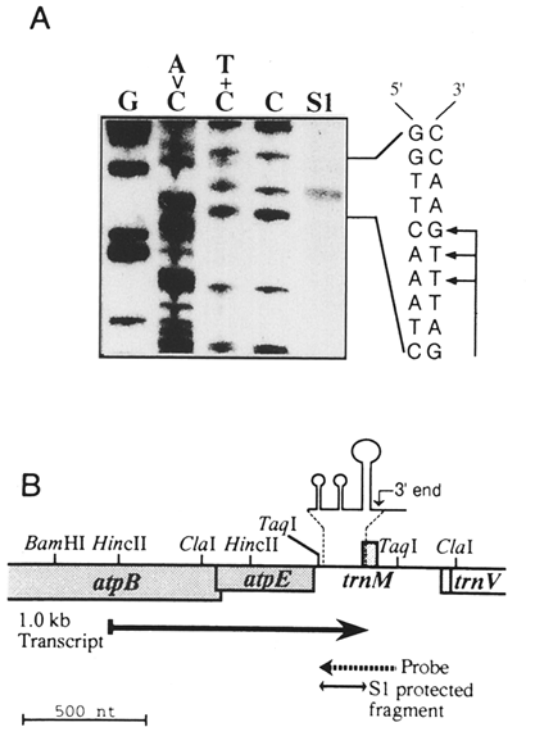

Fig. 3A, B. Determination of the 3′ end by S1-nuclease mapping. **A** autoradiogram showing an S1-protected fragment (*lane S1*) and the DNA sequence ladder of the same region. **B** schematic representation of the procedure and position of putative secondary structures in the 3′ untranslated region of the *atpB/E* transcripts

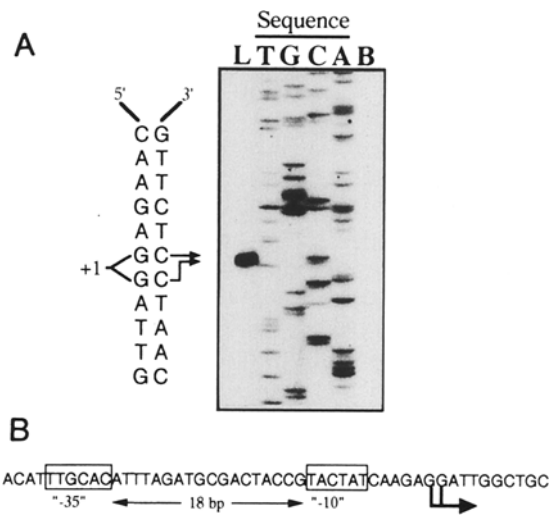

Fig. 4A, B. Precise mapping of the 5′ end of the 1.0-kb *atpE*-specific transcript by primer extension. **A** *lanes T, G, C and A* are the result of the di-deoxy sequencing reaction. *Lanes L and B* show the extension products from leaf and BY-2 cells, respectively. **B** sequence showing the position of the transcription start site (*arrow*) with respect to the –10 and –35 canonical sequences

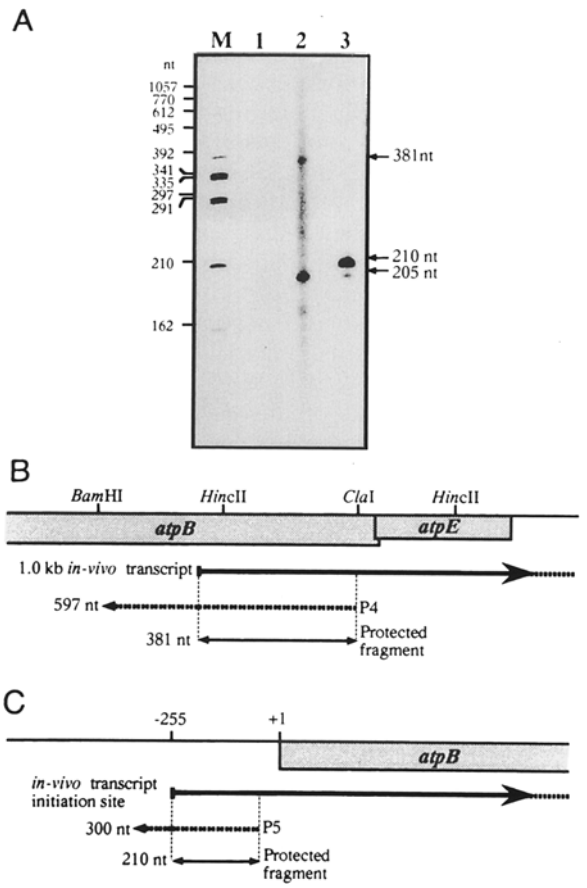

Fig. 5A–C. Determination of the initiation site of the *atpE*-specific transcript by in-vitro capping and ribonuclease protection assays. **A** autoradiogram of ribonuclease-protected capped RNA fragments. *Lane 1* is the negative control with 20 µg of capped leaf RNA without the cold antisense probe. In *lane 2*, 50 µg of 5′-capped leaf RNA was hybridized to cold antisense probe, P4 (see Fig. 3B) prior to digestion with RNases. *Lane 3* is the positive control in which 20 µg of capped leaf RNA was hybridized to a cold antisense probe, P5 (see Fig. 3C), already known to contain the transcript initiation site (255 bp 5′ of the *atpB* ATG codon). "*M*" refers to the ØX174-*Hin*cII marker DNA. Schematic representation of the experimental design for *lanes 2 and 3* is shown in **B** and **C**, respectively

Fine mapping of the 5′ end

Ribonuclease protection analysis suggested that the 5′ end of the *atpE*-specific 1.0-kb transcript was present within the 3′ coding region of the *atpB* gene. To map the 5′ end of this transcript more precisely, we carried out primer extension of a 5′ [^{32}P]-labelled primer (275 nt upstream of the *atpE* translation start site) using total-leaf and BY-2 RNA as templates. The extended products were analyzed on a sequencing gel in parallel to a sequencing ladder obtained by the di-deoxy method using the same primer (Fig. 4). Primer extension using leaf RNA (lane L) shows two bands corresponding to the –430 and –431 positions 5′ to the translation start site of *atpE*. This 5′ end is also characterized by ctp1 (–35, TTGCAC) and ctp2(–10, TACTAT) canonical sequences, separated by 18 bp, in the upstream flanking region, suggesting strongly that the 5′ end determined by primer extension might be the in-vivo transcription initiation site for this leaf-specific 1.0-kb transcript. Taken together the results of both 3′ and 5′ mapping experiments indicate a length of 1 072 nt for the longest *atpE*-specific transcript. As expected, no extension product was obtained with BY-2 total RNA (Fig. 4, lane B) in the corresponding region.

Confirmation of the transcription initiation site

In-vitro capping of a 5′ triphosphate-containing organellar RNA with [α-^{32}P]-GTP and guanylyltransferase, coupled with ribonuclease protection assays using cold antisense riboprobes, has proven useful for distinguishing less abundant primary transcripts from those resulting from the processing of the transcripts (Vera and Sugiura 1992). Following this approach, total leaf RNA was capped, hybridized to non-radioactive riboprobe P4, and subsequently digested with a mixture of RNase A and T1 (Fig. 5). Lane 2 in Fig. 5A shows a protected band of 381 nt, corresponding to a similar band from the ribonuclease protection assay using total-leaf RNA and a radioactive P4 RNA probe (Fig. 2B). This band also corresponds to the 5′ end determined by primer extension (see Fig. 4).

Probe P5, including the –255 promoter region of the *atpB* gene (Orozco et al. 1990), was used as a positive control. As expected, a 210-nt capped and protected band was detected (Fig. 5, panel A, lane 3 and panel C). Another 205-nt band appeared in all three lanes regardless of the probe used. The nature of this band, which appears only in capped RNA samples from leaves, but not from BY-2, is still a mystery (Vera et al. 1992, and unpublished data).

Taken together the above data clearly prove that expression of the 1.0-kb transcript starting –430/–431 nt upstream of the *atpE* translation initiation site is a result of promoter activity in the 3′ coding region of the *atpB* gene. Ribonuclease protection assays and primer extension analysis also suggest that the *atpE*-specific transcript is either not synthesized or else is rapidly degraded in the proplastids of BY-2 cells. About a ten-fold less expression of the 1.0-kb transcript, as compared to a longer bi-cistronic transcript, can be assessed by the Northern hybridization and ribonuclease protection experiments.

Discussion

Our results suggest, for the first time, that the general assumption about ATP synthase β and ε subunits being translated from a bi-cistronic transcript (without any further processing of the primary transcript) is not absolutely true, at least in the case of *N. tabacum*. In terms of a 10-year-old observation relating to a 1.3-kb transcript hybridizing specifically to a probe from the *atpE* coding region (Shinozaki et al. 1983), we carried out a Northern-blot analysis using strand-specific RNA probes from specific as well as overlapping regions of both genes. We confirmed that in addition to two major bi-cistronic transcripts of 2.7 and 2.3 kb (described as one band of 2.7 kb by Shinozaki et al. 1983), a 1.0-kb mono-cistronic transcript (described as 1.3 kb earlier) is also detected using a gene-specific probe for *atpE*. The latter transcript is potentially translatable to produce a ε subunit without being interfered by *atpB* translation. The absence of this transcript in proplastid-containing BY-2 cells indicates its differential regulation at transcriptional and/or post-transcriptional level(s) in a development-dependent manner. Ribonuclease protection assays, in-vitro capping, and primer extension experiments

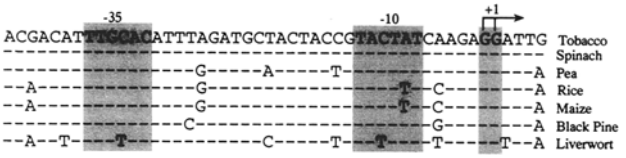

Fig. 6. Comparison of the promoter region of the *atpE*-specific transcript. Common names of the plants sources are on the right. Sequences identical to that of tobacco are denoted with *hyphens*. The transcription start site (marked with an *arrow*), and –10 and –35 motifs (*shaded*) are indicated

confirm that this transcript is the result of a primary transcriptional event. Because it is present in the coding region of *atpB,* the *atpE*-specific promoter region is highly conserved among monocots, dicots, a gymnosperm (black pine) and a liverwort, suggesting that this promoter activity may not be unique to tobacco (Fig. 6). On the other hand, the undetectability of this transcript can be explained either by (1) lower levels of the transcript or (2) the absence of some *trans*-factor essential for initiating transcription from this site in plant species other than tobacco.

Similar promoter activities within the operons have already been reported for tobacco and barley *psbD-C* (Yao et al. 1989; Sexton et al. 1990), maize ORF31-*petG*-ORF42 (Haley and Bogorad 1990), and tobacco *psbK-psbI-trnG* (Meng et al. 1991). The internal promoters in barley *psbD-C* and maize ORF31-*petG*-ORF42 also confer light-responsiveness to the respective transcripts but are located in the intercistronic regions (Haley and Bogorad 1990; Sexton et al. 1990). On the other hand, the *psbC*-specific primary transcript, initiating from a promoter found in the coding region of the *psbD* gene, does not seem to express in a regulated manner (Gamble et al. 1988; Yao et al. 1989). The *atpE*-specific transcript described in the present report, however, besides being initiated from a promoter present in the coding region of an adjacent gene (*atpB*) on the same strand, accumulates in a development-dependent manner. Further work to analyze the level of control for such an expression pattern is in progress.

Light- and developmentally-regulated primary transcripts for barley *psbD-C* and maize ORF31-*petG*-ORF42 operons have been predicted to account for the higher levels of the respective proteins required during light-dependent biogenesis (Gamble et al 1989; Haley and Bogorad 1990; Sexton et al 1990). However, development-dependent accumulation of the *atpE*-specific transcript cannot be explained by a similar reasoning. Because the ε subunit is required only in 1/3 molar equivalents as compared to the β subunit, and provided that the ε subunit is also translated from the 2.7- and 2.3-kb bi-cistronic transcripts, it should not be difficult to achieve the desired level of this polypeptide (Gatenby et al. 1989). Moreover, the *atpE* coding sequences have been shown to be only poorly capable of initiating translation, independent of translation from the *atpB* coding region, at least when expressed in *E. coli* (Gatenby et al. 1989). If such a situation also prevails in tobacco chloroplasts then why this smaller transcript is required, and what role it plays in the proper functioning of chloroplast ATP synthase, are questions for which we do not yet have any suitable explanation.

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