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Mapping of promoters for the nucleus-encoded plastid RNA polymerase (NEP) in the *iojap* maize mutant

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Abstract Plastid genes of higher plants may be transcribed by the plastid-encoded or the nucleus-encoded plastid RNA polymerases (PEP or NEP). The objective of this study was to identify NEP promoters in maize. To separate the NEP and PEP transcription activity, NEP promoter mapping was carried out in the *iojap* maize mutant which lacks the PEP. We report here that *atpB*, an ATPase subunit gene has promoters for both NEP and PEP, while *clpP*, a protease subunit gene, and the *rpoB* operon, encoding three PEP subunit genes, are exclusively transcribed from NEP promoters. The maize NEP promoters share sequence homology around the transcription initiation site, including the ATAGAATA/GAA loose consensus identified for tobacco, suggesting conservation of the NEP transcription machinery between monocots and dicots.

Key words Maize *iojap* mutant · Plastid promoter · Plastid RNA polymerase · Transcription

Introduction

In the plastids of photosynthetic higher plants the genes are transcribed by at least two RNA polymerases: the plastid-encoded plastid RNA polymerase (PEP) and the nucleus-encoded plastid RNA polymerase (NEP). The four plastid-encoded PEP subunits (α, β, β' and β'') share homology with the α, β, and β' subunits of *Escherichia coli* RNA polymerase. The σ-factor homologues and PEP regulatory factors are encoded in the nucleus and are imported into the plastids (Igloi and Kossel 1992; Gruissem and Tonkyn 1993; Link 1996; Stern et al. 1997; Tanaka et al. 1997). Much less is known about NEP. The likely candidate for NEP is an approximately 13-kDa protein which has biochemical properties similar to yeast mitochondrial and phage RNA polymerases (Lerbs-Mache 1993; Hedtke et al. 1997).

Dicot promoters for NEP were identified in tobacco plants lacking PEP due to deletion of *rpoB*, encoding the PEP β subunit (Δ*rpoB* plants). The general rule emerging from these studies is that photosystem-I and -II genes are exclusively transcribed by PEP, while most other genes have promoters for both NEP and PEP. An exception is the tobacco *accD* gene, which is transcribed exclusively by the NEP (Allison et al. 1996; Hajdukiewicz et al. 1997). Transcription of housekeeping genes by both RNA polymerases was also described in cultured tobacco cells with enhanced NEP activity (Vera and Sugiura 1995; Vera et al. 1996; Kapoor et al. 1997).

The objective of the present study was to gain insights into NEP function in monocots. As plastid transformation is not available in monocots, Δ*rpoB* plants could not be obtained for maize NEP promoter analysis. However, mutants with a defect in plastid ribosome accumulation are available in barley (*albostrians*; Hess et al. 1993) and maize (*iojap*; Walbot and Coe 1979; Han et al. 1992, 1993). In the absence of plastid ribosomes these mutants cannot synthesize PEP. Both mutants accumulate mRNAs for a subset of plastid genes, indicating NEP activity (Han et al. 1993; Hess et al. 1993). We mapped NEP promoters in white ribosome-less maize *iojap* seedlings.

We report here that the maize *atpB* gene has alternative NEP and PEP promoters while the *clpP* and the *rpoB* genes are transcribed from a NEP promoter only. DNA sequence alignment revealed that the maize NEP promoters share homology directly upstream of the transcription initiation site. The homologous region includes the previously identified tobacco NEP promoter consensus, suggesting conservation of the NEP transcription machinery between monocots and dicots.

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Materials and methods

Plant materials. Iojap is a recessive striped mutant of maize. Maternal white and green seedlings were obtained by crossing a striped ij/ij maternal parent (1404) with pollen from a wild-type male (inbred Oh51a). The seeds were kindly provided by Rob Martienssen and Mary Byrne, Cold Spring Harbor Laboratory. Surface-sterilized seeds were germinated in vitro on 2% MS medium (24 °C, 16-h illumination).

RNA gel blots. Total cellular RNA was prepared (Stiekema et al. 1988) from the leaves of 9-day old seedlings. The RNA (5 µg per lane) was electrophoresed on 1% agarose/formaldehyde gels, then transferred to Hybond N membranes (Amersham) using the Posiblot Transfer apparatus (Stratagene). Hybridization to random-primer labeled fragments was carried out in Rapid Hybridization Buffer (Amersham) overnight at 65 °C. Double-stranded ptDNA probes were prepared by random-primed ^{32}P-labeling of PCR-generated or gel-purified DNA fragments. The sequence of the primers used for PCR, along with their positions within the tobacco (*N.t.*, accession no. Z00044; Shinozaki et al. 1986) or maize (*Z.m.*, accession no. X86563; Maier et al. 1995) ptDNA are as follows:

gene/5′ nt position in ptDNA/sequence,

atpB (*Z.m.*)	55 860 (C)	GAGAGGAATGGAAGTGATTGACA
	55 103	GAGCAGGGTCGGTCAAATC
clpP (*Z.m.*)	69 840	ATCCTAGCGTGAGGGAATGCTA
	70 064 (C)	AGGTCTGATGGTATATCTCAGTAT
psbA (*N.t.*)	1 550 (C)	CGCTTCTGTAACTGG
	667	TGACTGTCAACTACAG.

The following ptDNA fragments were used as probes: *rbcL* (*N.t.*), a *Bam*HI fragment (nucleotides 58 047–59 285 in ptDNA); *16SrDNA* (*N.t.*), a *Eco*RI to *Eco*RV fragment (nucleotides 138 447–140 855); and a *rpoB* (*N.t.*) *Hin*dIII fragment (nucleotides 24 291–24 816). 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.

Primer-extension analysis. Primer-extension reactions were carried out on 10 µg of total leaf RNA as described (Allison and Maliga 1995). The primers are listed below, with the nucleotide position in the published maize plastid genome sequence (Maier et al. 1995). Oligonucleotides added to create cloning sites are underlined; in this case the position of the first nucleotide is given in the genome sequence:

gene/5′ nt position in ptDNA/sequence,

clpP#1	70 182	GGTACTTTTGGAACACCAATGGGCAT
atpB#1	56 095	GAAGTAGTAGGATTGGTTCTCATAAT
atpB#2	56 640	GGTCTAGAATTCCTATCGAATTCCTTC
rpoB#1	21 545 (C)	GAATCTACAAAATCCCTCGAATTG
rpoB#2	21 418 (C)	ACTCTTCATCAATCCCTACG.

Note, that the sequence of *atpB*#2 oligo differs from the maize ptDNA sequence in GenBank. We found that the maize ptDNA we studied has a 15-nt insertion relative to the published sequence.

Identification of primary transcripts by in vitro capping. Total leaf RNA (20 µg for *clpP* or 100 µg for *rpoB* and *atpB* transcripts) from white seedlings was capped in the presence of 0.25 or 1.0 mCi [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 *Xba*I restriction site (underlined) upstream of the amplified fragment. The 3′ primers were designed to add a *Xho*I (*atpB, rpoB*) or *Eco*RI (*clpP*) site (underlined) downstream from the amplified sequence. The amplified product was cloned after digestion with the appropriate restriction enzyme into a pBSKS$^+$ vector (Stratagene). To generate unlabeled RNA complementary to the 5′ end of RNAs, the resulting plasmid was linearized with *Xho*I (*atpB, rpoB*) or *Eco*RI (*clpP*) and transcribed in a Megascript (Ambion) reaction with T7 RNA polymerase. Markers (100, 200, 300, 400, and 500 nucleotides) were prepared with the RNA Century Markers Template Set (Ambion), following the manufacturer's protocol:

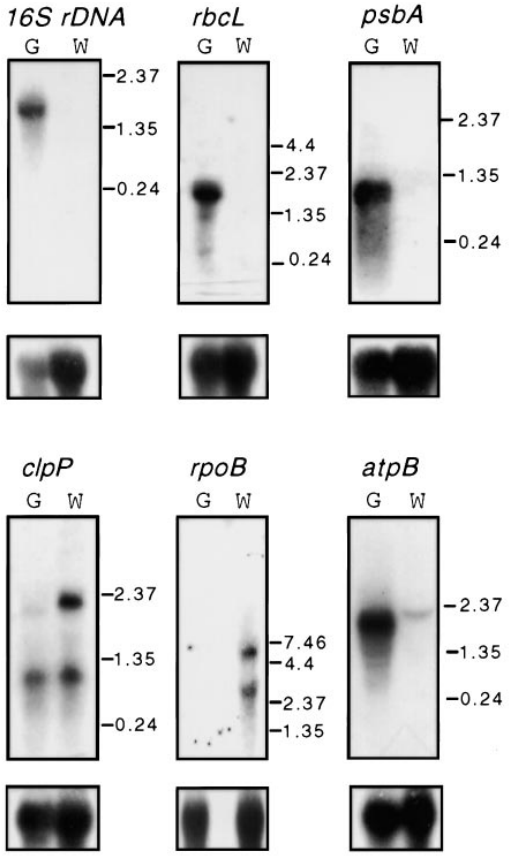

Fig. 1 RNA steady state concentrations in green (*G*) and white (*W*) *iojap* maize leaves. To control for loading, the blots were stripped and probed for cytoplasmic 25S ribosomal RNA (lower panels)

gene/5′ nt position in ptDNA/sequence

clpP#2	70 241	GGTCTAGACTACACTTTAATATGGA
clpP#3	70 549 (C)	GGGAATTCTGTTTGTAAGAAGA
atpB#2	56 640	GGTCTAGAATTCCTATCGAATTCCTTC
atpB#3	56 832 (C)	GGCTCGAGGGACAACTCGATAGGATT-AGG
rpoB#3	21 394 (C)	GGTCTAGAATCTAGCAATCATGGAATC
rpoB#4	21 066	GGCTCGAGCGTGCTATTCTAAATCGT.

Results

Plastid transcript accumulation in the maternal white and green maize plants

Lack of 16S rRNA accumulation in the white maize plants confirmed the lack of plastid ribosomes in the *iojap* seedlings (Fig. 1; Walbot and Coe 1979). The level of *rbcL* and *psbA* mRNAs was barely detectable (Fig. 1), as expected for *iojap* seedlings (Han et al. 1993). Transcript accumulation was analyzed for three additional plastid genes: *clpP, rpoB* and *atpB*. Readily detectable accumulation of mRNA in the ribosome-less *iojap* plants confirmed an active NEP

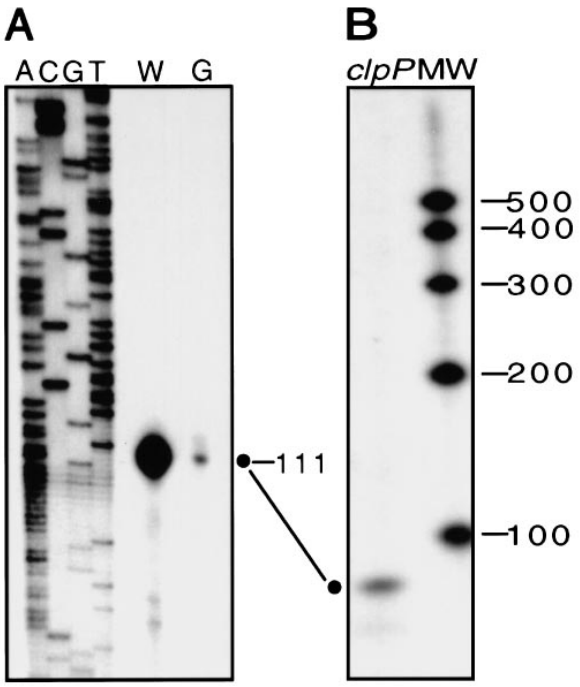

Fig. 2A, B Mapping of *clpP* promoters. **A** primer extension analysis of RNA from green (*G*) and white (*W*) *iojap* maize leaves. The *number* –111 refers to the nucleotide position of the transcript 5′ end relative to the ATG translation initiation codon. The DNA sequence obtained with the same primer is also shown. **B** in vitro capping and RNase protection assay to identify primary transcript 5′ ends. Note that the RNase protection construct protects only a 79-nt fragment. The size of molecular-weight (*MW*) markers (100, 200, 300, 400, and 500 nucleotides) is also shown

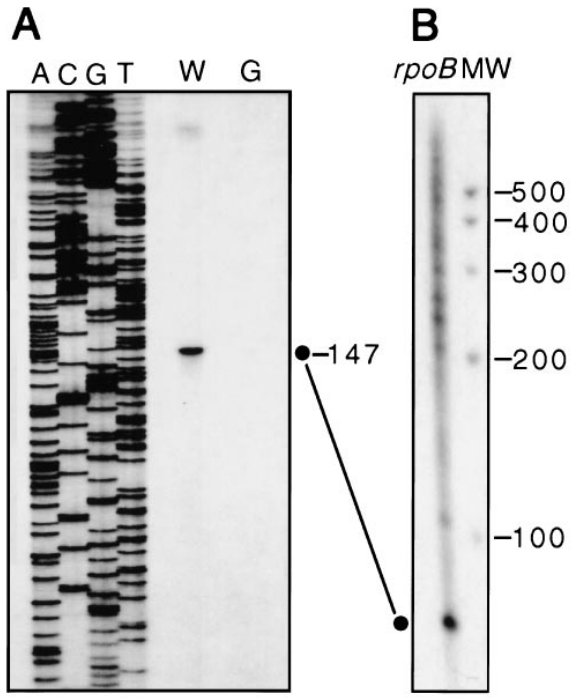

Fig. 3A, B Mapping of *rpoB* promoters. **A** primer extension analysis of RNA from green (*G*) and white (*W*) *iojap* maize leaves. The *number* –147 refers to the transcript 5′ end relative to ATG. The DNA sequence obtained with the same primer is also shown. **B** in vitro capping and RNase protection assay to identify primary transcript 5′ ends. Note that the RNase protection construct protects only a 74-nt fragment. The size of molecular-weight (*MW*) markers (100, 200, 300, 400, and 500 nucleotides) is also shown

promoter for each of these genes (Fig. 1; Han et al. 1993; Hess et al. 1993; Hajdukiewicz et al. 1997).) The *rpoB* mRNA in the wild-type leaf is below the level of detection.

The *clpP* NEP promoter is efficiently transcribed in white and green seedlings

To identify the NEP promoters, transcript 5′-ends were mapped by primer extension analysis. To distinguish between 5′-ends that represent transcripts from NEP promoters and those generated by RNA processing, the 5′-ends were capped using guanylyltransferase.

For *clpP*, significant mRNA accumulation was found in both white and green seedlings. Primer-extension analysis identified only one major *clpP* 5′ end at nucleotide position –111 (the nucleotide upstream of the ATG being at –1 position). The *clpP* –111-transcript could be capped in vitro (Fig. 2B), confirming that this 5′ end is a primary transcript and identifies the maize NEP promoter P*clpP*-111. The same 5′-end was found in both white and green maize seedlings indicating that the same *clpP* promoter is active in chloroplasts as well as in the nonphotosynthetic *iojap* plastids (Fig. 2 A). Therefore, P*clpP* –111 is considered constitutive. The same *clpP* promoter is also active in rice (data not shown).

The *rpoB* NEP promoter activity is enhanced in *iojap* plastids

RNA gel-blot analysis shows that *rpoB* mRNA accumulates to a detectable level only in white seedlings (Fig. 1). Two 5′-ends could be identified, a major band at nucleotide position –147, and a minor band at position –285 (Fig. 3A). The in vitro capping assay confirmed that the –147 RNA species is a primary transcript (Fig. 3B), and therefore the product of the P*rpoB*-147 promoter.

The *atpB* gene is transcribed from a NEP promoter in white plants and from a PEP promoter in green seedlings

There is substantial *atpB* mRNA accumulation in green leaves, while much less is found in the white *iojap* leaves (Fig. 1). Primer-extension analysis of mRNA from green leaves identified a transcript with a 5′-end at nucleotide position –298 (Fig. 4A) confirming an earlier report (Mullet et al. 1985). This –298 mRNA species was absent in leaf RNA isolated from white plants, indicating that the –298 mRNA is a PEP transcript. Instead, another *atpB* transcript was mapped to nucleotide position –601 (Fig. 4A). The difference in the size of the two mRNAs is apparent on the RNA gel blot shown in Fig. 1. The –601 transcript

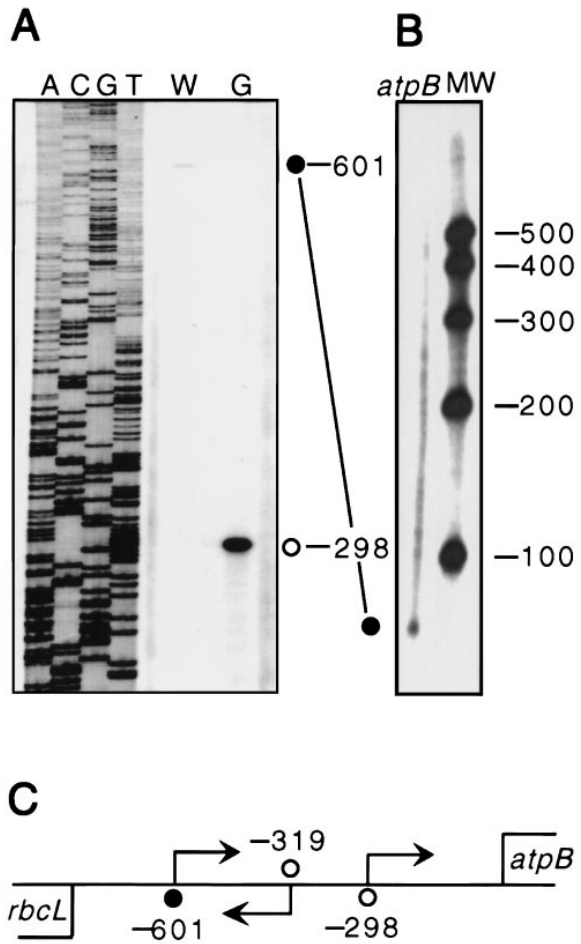

Fig. 4A, B Mapping of *atpB* promoters. **A** primer extension analysis of RNA from green (*G*) and white (*W*) *iojap* maize leaves. The numbers –298 and –601 refer to transcript 5′ ends. The DNA sequence obtained with the same primer is also shown. **B** in vitro capping and RNase protection assay to identify primary transcript 5′ ends. Note that the RNase protection construct protects only a 79-nt fragment. The size of molecular-weight (*MW*) markers (100, 200, 300, 400, and 500 nucleotides) is also shown. **C** physical map of the *atpB–rbcL* intergenic region. The map positions of the primary transcript 5′ ends for the NEP and PEP promoters are marked with *filled and open circles*, respectively

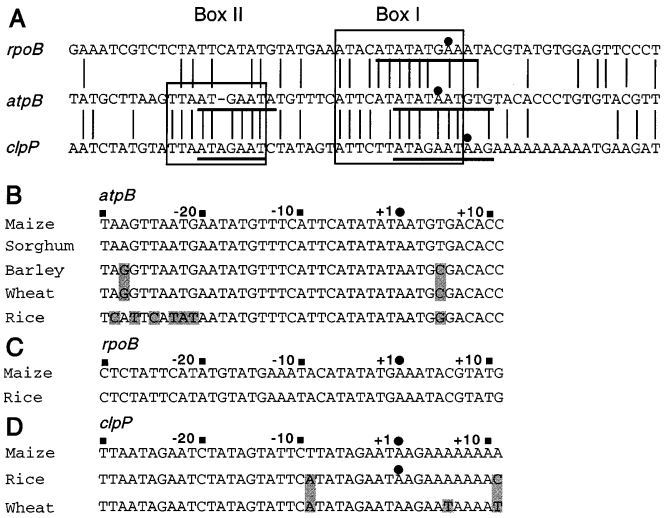

Fig. 5A–D Conservation of DNA sequences around the cereal NEP promoter transcription initiation sites. **A** alignment of maize *rpoB, atpB* and *clpP* NEP promoter regions. Regions with significant homology are *boxed*. Sequences with homology to the loose 10-nt dicot NEP consensus are *underlined*. NEP transcription initiation sites are marked with *filled circles*. Alignment of promoter regions in monocots for: **B** *atpB*; **C** *rpoB*; **D** *clpP*. DNA sequence information for rice and maize are based on the plastid genome sequence (Hiratsuka et al. 1989, accession no. X15901; Maier et al. 1995, accession no. X86563, respectively). For the DNA sequence of *atpB* in sorghum, barley and wheat see Golenberg et al. 1993 (S72305), Zurawski et al. 1984 (X00630) and Howe et al. 1985 (M16843), respectively. For the wheat *clpP* sequence see Gray et al. 1990 (X54484)

could be capped in vitro by guanylyltransferase, indicating that it is a primary transcript (Fig. 4B). Therefore, it is the product of the P*atpB*-601 NEP promoter, with readily detectable activity only in white *iojap* leaves.

Discussion

Alignment of the maize *atpB, rpoB* and *clpP* NEP promoters revealed significant homology upstream of the transcription initiation sites (Box I; Fig. 5A). In addition, *atpB* and *clpP* share eight out of nine nucleotides further upstream (Box II in Fig. 5A). These NEP promoter regions are highly conserved in the monocots, including those of *atpB*, *rpoB* and *clpP* (Fig. 5B–D). Interestingly, each maize NEP promoter has sequence similarity around the transcription initiation site with the loose dicot NEP promoter consensus (ATAGAATA/GAA ; Hajdukiewicz et al. 1997; underlined in Fig. 5A). An additional interesting feature is the duplication of the NEP consensus in a truncated form in Box-II of the maize *clpP* and *atpB* NEP promoters. These tandem repeats may play a role in regulating NEP promoter activity. It should be noted that the Box-I and Box-II designation is based solely on sequence conservation. The function of these boxes needs be confirmed experimentally.

The *rpoB* operon is one of few genes known to be expressed by NEP only. The P*rpoB*-147 is a NEP promoter but, unlike *clpP* and *atpB*, lacks Box II (Fig. 5A). Accumulation of mRNA from this promoter is low in mature leaves due to down-regulation of transcription rates (Baumgartner et al. 1993). The P*rpoB*-147 promoter identified in this study probably plays a central role in plastid development since it regulates the expression of three of the four plastid-encoded PEP subunits (Shimada et al. 1990). According to one model, the two RNA polymerases form a developmental cascade during chloroplast differentiation. During the early stages of plastid development, plastid genes encoding the plastid's transcription and translation apparatus would be transcribed by the NEP. Once PEP is made, it would initiate the transcription of photosynthetic genes from PEP promoters, and take over the transcription of housekeeping genes from alternative PEP promoters (Hess et al. 1993; Lerbs-Mache 1993; Mullet 1993; Hajdu-

kiewicz et al. 1997). Consistent with this model is the transcription of *rpoB* from a NEP promoter. However, in maize at least one gene, *clpP*, is exclusively and efficiently transcribed by NEP in mature chloroplasts, indicating that NEP remains active and essential for cellular functions even if PEP is present. We therefore prefer an alternative model, which assumes that NEP and PEP are constitutively present all the time and selective transcription is mediated by promoter-specific transcription factors. Cloning of the putative NEP catalytic subunit gene (Hedtke et al. 1997) and identification of NEP promoters for dicots (Vera and Sugiura 1995; Allison et al. 1996; Vera et al. 1996; Hajdukiewicz et al. 1997; Kapoor et al. 1997) and monocots (this paper) are the first step to experimentally define the role of the NEP in plastid function and development.

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