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Plastid promoter utilization in a rice embryogenic cell culture

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Abstract Plastid promoter utilization was characterized in rice by mapping transcript 5′-ends in samples derived from leaves and cultured embryogenic cells. We have found that *rbcL*, *atpB* and the rRNA operon are transcribed by the plastid-encoded plastid RNA polymerase (PEP), while *clpP* is transcribed by the nucleus-encoded plastid RNA polymerase (NEP) in both chloroplasts and the non-green plastids of embryogenic cultured cells. This finding is in contrast to reports on BY2 tobacco, in which NEP promoter activity in cultured cells was enhanced relative to leaves, facilitating identification of NEP promoters which are undetectable in chloroplasts. Therefore, it appears that activation of plastid NEP promoters in rice is not essential for adaptation to cell culture.

Key words Embryogenic cell culture · Plastid promoter · Plastid RNA polymerase · Rice

Introduction

Plastid genes in higher plants are transcribed by at least two RNA polymerases: the plastid-encoded plastid RNA polymerase (PEP) and the nuclear-encoded plastid RNA polymerase (NEP). The promoters for PEP are reminiscent of the *Echerichia coli* σ^{70}-type promoters, with two conserved hexameric blocks of sequences (TTGACA or '-35' element ; TATAAT or '-10' element) (reviewed in Gruissem and Tonkyn 1993; Link 1996; Stern et al. 1997). NEP promoters share a loose ten-nucleotide consensus ATAGAATA/GAA overlapping the transcription initiation site, which is reminiscent of promoters recognized by the mitochondrial and phage T3/T7 RNA polymerases (Hajdukiewicz et al. 1997). The general rule is that photosystem-I and -II genes are exclusively transcribed by PEP, a few genes are transcribed by NEP, while most other genes have promoters for both NEP and PEP (Vera and Sugiura 1995; Allison et al. 1996; Vera et al. 1996; Hajdukiewicz et al. 1997; Kapoor et al. 1997; Hübschmann and Börner 1998; Silhavy and Maliga 1998; reviewed in Maliga 1998).

Our long-term interest is in plastid transformation in rice. The targets for rice plastid transformation are embryogenic cultured cells from which plant regeneration can be readily obtained (Vasil 1994; Christou 1997). The level of transcripts derived from NEP promoters was shown to be enhanced in cultured tobacco BY2 cells as compared to leaves. Enhanced NEP activity has facilitated the identification of a NEP promoter for the rRNA operon which is silent in leaf chloroplasts (Vera and Sugiura 1995), and increased the abundance of mRNAs derived from a NEP promoter for *atpB* and *rpl32* (Vera et al. 1996; Kapoor et al. 1997).

To learn about plastid promoter utilization in a rice embryogenic cell culture, RNA 5′-ends were mapped for: *rbcL*, encoding the large subunit of the ribulose-1,5-bisphosphate carboxylase; *atpB*, encoding the ATPase β subunit; the rRNA operon promoter upstream of *16SrDNA* ; and *clpP*, a protease subunit gene (Hiratsuka et al. 1989). We have found that in rice the same promoters are utilized in both leaves and cultured embryogenic cells. Therefore, it appears that activation of plastid NEP promoters in rice is not essential for adaptation to cell culture.

Materials and methods

Plant materials. Embryogenic rice calli were initiated from mature seed of cv Taipei 309 on LS2.5 medium (Abdullah et al. 1986). The calli were introduced into liquid AA medium (Muller and Grafe 1978) to establish embryogenic suspension cultures, and subcultured at bi-weekly intervals. DNA and RNA were prepared from 3-month-old cultures 14 days after subculture. Plants were regenerated from embryogenic calli on complete MS medium supplemented with

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2 mg/l of BAP and 3% sucrose (Murashige and Skoog 1962) and transferred onto hormone-free MS medium. Leaves for the isolation of nucleic acids were taken from these plants after 4 weeks.

RNA and DNA gel blots. Total cellular RNA was prepared according to Stiekema et al. (1988). The RNA (5 μg per lane) was separated on a formaldehyde-agarose gels, blotted and hybridized (Hajdukiewicz et al. 1997). 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.

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.*), an *Eco*RI to *Eco*RV fragment (nucleotides 138 447–140 855). The probe for tobacco 25 S rRNA was from plasmid pKDR1 (Dempsey et al. 1993) containing a 3.75-kb *Eco*RI fragment from a tobacco 25 S/18 S locus cloned in plasmid pBR325. Total-leaf DNA for relative plastid genome copy number determination was prepared (Mettler 1987), digested with the *Eco*RI restriction endonuclease, separated on 0.7% agarose gels, blotted and hybridized with the plastid 16SrDNA and cytoplasmic 25SrDNA probes (Allison et al. 1996).

Primer extension analysis. Primer extension reactions were carried out on total-leaf RNA as described (Allison and Maliga 1995). The primers are listed below, with the nucleotide position as given in the published rice plastid genome sequence (Hiratsuka et al. 1989).

Gene	5′ nt position in ptDNA	Sequence
rbcL	54 124	ACTTGCTTTAGTTTCTGTTTGTGGTG-ACAT
atpB	53 287	AGAAGTAGTAGGATTGGTTCTCATAAT
16S rRNA	123 777	CCGCCAGCGTTCATCCTGAGC
clpP	68 263	GGTACTTTTGGAACACCAATGGGCAT.

Primer extension reactions were carried out with 1 μg of RNA from leaves, and 10 μg (*clpP*, *16SrDNA*) and 30 μg (*rbcL*, *atpB*) of RNA from embryogenic cells.

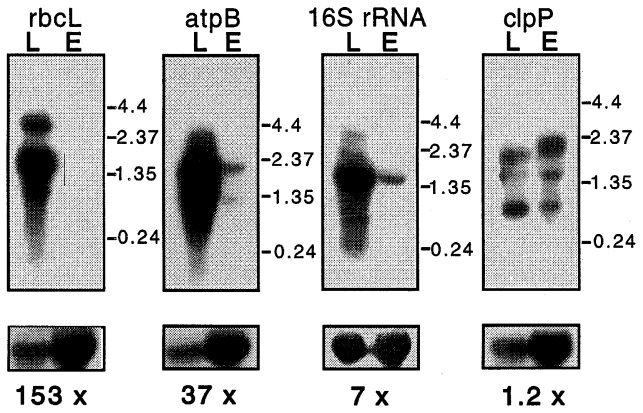

Fig. 1 RNA steady state concentrations in leaf (*L*) and in embryogenic cultured cells (*E*) of rice. RNA accumulation was studied for the *rbcL*, *atpB*, *16SrDNA* and *clpP* genes (*upper panels*). To control for loading, the blots were stripped and probed for cytoplasmic 25 S ribosomal RNA (*lower panels*). Hybridization signals in the upper panels were quantified with a Molecular Dynamics PhosphorImager and normalized to the 25S rRNA signal. The fold-excesses of leaf over cultured embryogenic-cell signals for each probe is shown below the lanes

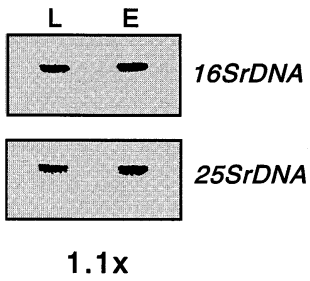

Fig. 2 Relative plastid genome copy number in leaves (*L*) and in cultured embryogenic cells (*E*) of rice. *Eco*RI-digested total cellular DNA (2 μg per lane) was probed for plastid *16SrDNA* (upper) and nuclear-encoded *25SrDNA* (lower). Hybridization signals were quantified with a Molecular Dynamics PhosphorImager. The relative signal intensity (*16SrDNA/25SrDNA*) in leaves to embryogenic cells was 1.1

Results

Plastid transcript accumulation in leaves and in cultured embryogenic cells

Transcript accumulation was studied in genes for which promoters have been identified in the leaves of rice and other cereals. The *rbcL* gene in rice and maize is transcribed from a PEP promoter (Mullet et al. 1985; Nishizawa and Hirai 1987). The *atpB* gene in maize chloroplasts is transcribed from a PEP promoter (Mullet et al. 1985), whereas in maternal white *iojap* seedlings lacking PEP it is transcribed from an alternative NEP promoter (Silhavy and Maliga 1998). The *16SrDNA* gene (the first gene of the plastid ribosomal RNA operon) in barley chloroplasts is transcribed from a PEP promoter (Reinbothe et al. 1993), whereas in the white *albostrians* seedlings lacking PEP it is transcribed from an uncharacterized NEP promoter (Hess et al. 1993; Hübschmann and Börner 1998). The *clpP* gene in wild-type and *iojap* maize plastids is transcribed from a NEP promoter (Silhavy and Maliga 1998).

To determine which of these genes are highly expressed in embryogenic rice cells, accumulation of mRNAs was tested on Northern blots (Fig. 1). We have found that the transcript levels in embryogenic cells relative to leaves were barely detectable for *rbcL* (153-fold lower), reduced for *atpB* (37-fold lower) and *16SrDNA* (7-fold lower), and similar for *clpP*. Differences in transcript levels were not due to differences in the number of plastid genome copies per cell, since the number of plastid genome copies per cell is about the same in embryogenic cells and leaves (Fig. 2).

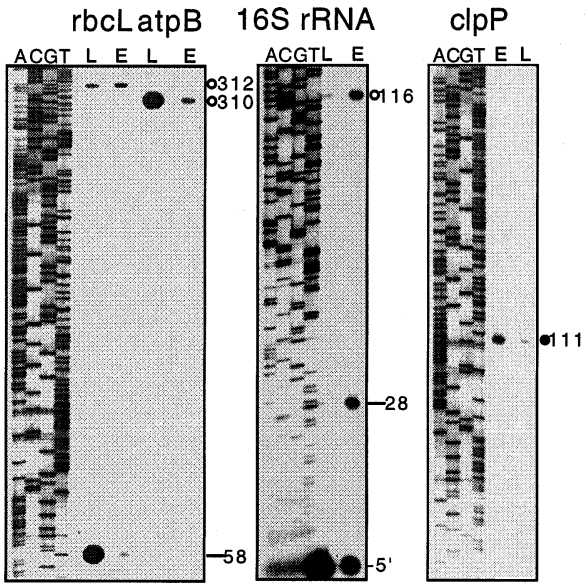

Fig. 3 Mapping of plastid mRNA 5′-ends from rice leaves (*L*) and embryogenic cultured cells (*E*) by primer extension. *Numbers* on the right indicate the distance between the translation initiation codon (ATG) and 5′-ends of primary transcripts (PEP, ○; NEP, ●), or of processed mRNAs (–). DNA sequences on the left are size markers. The sequence ladders shown for *16 S rDNA* and *clpP* were obtained with an homologous template and the oligonucleotides used for primer extension analysis

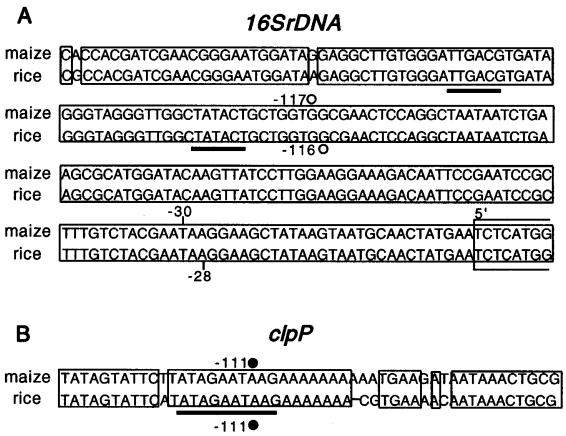

Fig. 4 Alignment of the rice plastid *rrn* operon (**A**) and *clpP* (**B**) promoter regions with the cognate regions in maize. PEP (○) and NEP (●) transcription initiation sites and processed 5′-ends (|) are marked. The conserved PEP '-35' (TTGACG) and '-10' (TATACT) promoter elements, and the tentative NEP promoter consensus (ATAGAATA/GAA; Hajdukiewicz et al. 1997) are *underlined*. Transcription initiation sites for the maize *16SrDNA* (Strittmatter et al. 1985) and maize and rice *clpP* have already been described (Silhavy and Maliga 1998)

Identification of plastid promoters in leaves and in cultured embryogenic cells

To identify active plastid promoters, transcript 5′-ends were mapped by primer extension in leaves and in cultured embryogenic cells. For *rbcL*, we mapped two 5′-ends 312 and 58 nucleotides upstream of the translation initiation codon (Fig. 3). The same two 5′-ends were present in leaf chloroplasts, and the plastids of embryogenic cells. Two 5′-ends were mapped to similar positions by S1 nuclease analysis in rice chloroplasts (Nishizawa and Hirai 1987). The *rbcL*-312-end is downstream from the -10/-35 σ^{70}-type promoter elements which are conserved in monocots; the -58-end is generated by RNA processing (Mullet et al. 1985; Reinbothe et al. 1993).

For *atpB*, a single 5′-end was mapped 310 nucleotides upstream of the translation initiation codon. As for *rbcL*, the same 5′-ends were identified in embryogenic cells and leaves (Fig. 3). The -310-end is associated with PEP promoter elements, and has been reported earlier in rice and maize chloroplasts (Nishizawa and Hirai 1989; Mullet et al. 1985).

For the rRNA operon, the same two 5′-ends were mapped upstream of the mature 16S rRNA in embryogenic cells and in leaves (Fig. 3). Based on DNA sequence conservation, the -116-end is derived from a PEP promoter whereas the -28-end derives from RNA processing (Fig. 4A; Strittmatter et al. 1985; Vera and Sugiura 1995; Allison et al. 1996).

For *clpP*, the same 5′-end was mapped in embryogenic tissue-culture cells and in leaves (Fig. 3). The transcript initiates 111 nucleotides upstream of the translation initiation codon within the 10-nucleotide NEP consensus (Fig. 4B, underlined). Note that nine out of the ten nucleotides (ATAGAATA/GAA) are conserved. The rice *clpP* mRNA is therefore initiated from a NEP promoter which is conserved between rice and maize (Silhavy and Maliga 1998).

Discussion

Mapping of RNA 5′-ends upstream of *rbcL*, *atpB*, *16SrDNA* and *clpP* identified the same promoters in the leaves and in cultured embryogenic cells of rice, including the *atpB* gene which has both NEP and PEP promoters in maize (Silhavy and Maliga 1998). Our findings in rice contrast with the results reported for BY2 tobacco in which activation of NEP promoters was found in cultured cells relative to leaves. Therefore, it appears that activation of NEP promoters in rice is not essential for adaptation to cell culture.

While PEP promoters are active in the photosynthetically active tobacco chloroplast, most NEP promoters are inactive or weak (Hajdukiewicz et al. 1997; Maliga 1998). Easy availability of leaves containing chloroplasts and a biased interest in the expression of photosynthesis-related genes may explain why the significance of the nucleus-encoded plastid transcription machinery has gone unrecognized for so long. Unambiguous identification of NEP promoters was possible only in non-green plastids which lack PEP due to deletion of a gene for one of its subunits in tobacco (Allison et al. 1996; Hajdukiewicz et al. 1997). A second system for unambiguous identification of NEP promoters is plastid ribosome-less barley, and maize mutants which lack PEP due to the lack of ability to translate the mRNAs (Hübschmann and Börner 1998; Silhavy and Maliga; 1998).

Due to the elevated level of NEP activity relative to PEP, the tobacco BY2 cells were also useful to identify NEP promoters. Based on the report on BY2 cells, we expected that rice tissue-culture calli will also have elevated NEP levels. This, however, is apparently not the case.

There are two important differences between the rice and tobacco cultures that may explain the lack of NEP activation in rice plastids. First, rice and tobacco are taxonomically distant, rice being a monocot and tobacco a dicot species. The second important difference is that our short-term rice cultures faithfully maintain the biology of the species, as indicated by the ability to regenerate plants, while during the many years in culture the BY2 cell line accumulated genetic changes that led to the loss of regeneration ability (Yasuda et al. 1988). Therefore, BY2 plastid gene expression may be significantly different from plastid gene expression in short-term tobacco cultures or in a tobacco plant. In contrast, short-term cultures, such as the rice culture used in this experiment, provide a reproducible source of cells in which the biology of non-green plastids can be studied.

Plastid transformation in rice requires promoters which are active in the non-green plastids of embryogenic cells. Good candidates appear to be the *clpP* NEP and *rrn* PEP promoters, for which relatively high levels of steady state mRNAs are present.

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