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Nicotiana chloroplast genome

7. Expression in *E. coli* and *B. subtilis* of tobacco and Chlamydomonas chloroplast DNA sequences coding for the large subunit of RuBP carboxylase

Y. S. Zhu*, P. S. Lovett, D. M. Williams and S. D. Kung
University of Maryland, Baltimore County, Department of Biological Sciences, 5401 Wilkens Avenue, Catonsville, MD 21228, USA

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Summary. RuBPCase, the enzyme responsible for carboxylation and oxidation of RuBP in a wide variety of photosynthetic organisms, is the major protein found in the chloroplast. Here we present the first evidence for direct expression in *E. coli* and *B. subtilis* of tobacco and *Chlamydomonas* ct-DNA sequences coding for the LS of RuBPCase as demonstrated by a simple in situ immunoassay.

Key words: *Nicotiana* – *Chlamydomonas* – Chloroplast DNA – RuBPCase – *E. coli* – *B. subtilis*

Ribulose-1, 5-bisphosphate carboxylase (RuBPCase) (Ec 4.1.1.39), the enzyme responsible for both CO_2 fixation and evolution in a wide variety of photosynthetic organisms, is the major protein found in the chloroplast (Kung 1977). In higher plants it occurs as an oligomer of eight large subunits (LS) (MW 53,000) and eight small subunits (SS) (MW 12,000–15,000) (Jensen and Bahr 1977). The LS, containing the catalytic sites, are coded by the chloroplast genome (Chan and Wildman 1972) and are synthesized in the chloroplast (Gray and Kekwick 1973). The SS, whose function remains uncertain, are coded by the nuclear genome and synthesized in the cytoplasm as a precursor of higher molecular weight (MW 20,000) that is cleaved and transported into the chloroplast (Chua and Schmidt 1978; Highfield and Schmidt 1978). This enzyme provides an attractive model for studying nucleus-chloroplast cooperation and gene regulation in plants. Genes for the LS of RuBPCase from maize (Coen et al. 1977), spinach (Bottomley and Whitfeld 1978; Erion et al. 1981) and *C. reinhardii* (Malnoë et al. 1979) have been expressed in vitro in coupled transcription-translation systems, and maize and wheat LS genes have been expressed in vivo in *E. coli* (Gatenby et al. 1981). Here we present the first evidence for direct expression in *E. coli* and *B. subtilis* of chloroplast DNA (ct-DNA) sequences from tobacco and *Chlamydomonas* coding for the LS of RuBPCase using a simple in situ immunoassay.

A clone library of *Nicotiana otophora* ct-DNA has been constructed and mapped with respect to *Bam*HI and *Sma*I sites (Zhu et al. 1982). Unique restriction fragments generated by cleavage of the ct-DNA with *Sma*I, *Sal*I, *Hind*III or *Eco*RI contain the intact LS gene suitable for expression studies, whereas *Bam*HI and *Pst*I cut within the gene. This was determined by using the spinach LS gene as a probe previously inserted into plasmid pBR322 (pJEA4) by Erion et al. (1981). Figure 1 A shows the identification of the tobacco LS gene by the hybridization of ^{32}P-labeled pJEA4 to *N. otophora* ct-DNA digested with *Sma*I, *Sal*I, *Pst*I, *Hind*III, *Eco*RI and *Bam*HI. The fragments Sal 6 (14 kb) and Hind 2 (11 kb) containing the intact LS gene were isolated from total *N. otophora* ct-DNA and cloned in *E. coli* using pBR325 and pBR322 as the respective vectors (Fig. 1 B). Cloned fragments Sal 6 (in plasmid PRCZ2) and Hind 2 (in plasmid PRCZ1) hybridized with ^{32}P-labeled spinach LS gene probe, confirming that both clones contain the LS gene, although these fragments are considerably larger than the LS gene itself (1.3–1.6 kb).

The LS gene from *Chlamydomonas reinhardii* was used for cloning in *Bacillus*. This LS gene was previously cloned in plasmid pBR322 (pLM401) by L. Metz (personal communication, Fig. 2 A). Plasmid PRCZ3 (Fig. 2 B) was constructed by substituting the 0.7 kb *Eco*RI – *Hind*III region of the *B. subtilis* expression plasmid pPL608 (Williams et al. 1981) with the *Eco*RI – *Hind*III fragment (1.15 kb and 0.75 kb) spanning the anterior region of the *Chlamydomonas* LS gene. This substitution is clearly illustrated with the

* Present address: Department of Cellular and Developmental Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

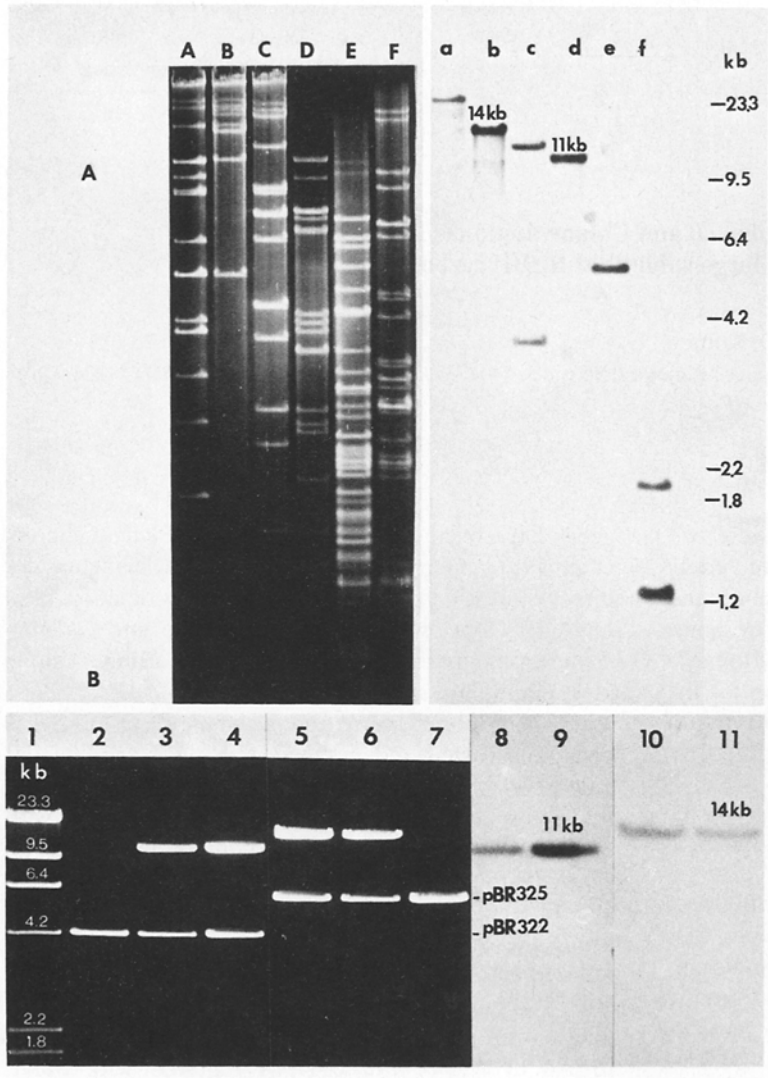

Fig. 1. A Restriction patterns (*left*) and hybridization to ^{32}P-labelled spinach LS gene (*right*) of *N. otophora* ct-DNA. 10 μg of purified ct-DNA (Rhodes and Kung 1981; Kolodner and Tewari 1975) were digested with *Sma*I, *Sal*I, *Pst*I, *Hind*III, *Eco*RI and *Bam*HI and subjected to electrophoresis in a 1% agarose gel (*channels A → F*, respectively). DNA fragments in the gel were transferred by blotting to nitrocellulose filter paper (Smith and Summers 1980). The plasmid pJEA4 DNA was prepared according to Lovett and Keggins (1979), and labelled with [α-^{32}P] dATP by the nick-translation reaction (Maniatis et al. 1975). Hybridization and autoradiography were made according to Southern (1975). On the *right* (*a → f*) with size marker is an autoradiograph of the gel shown on the *left*, after hybridization with the spinach LS gene. **B** Cloning of *Hind 2* and *Sal 6* restriction fragments of *N. otophora* ct-DNA in *E. coli* and hybridization to ^{32}P-labelled spinach LS gene. 10 μg of *N. otophora* ct-DNA was digested with *Hind*III or *Sal*I, and electrophoresed through 0.8% low-gelling-temperature agarose according to Herrmann et al. (1980). The purified ct-DNA fragment *Hind 2* was ligated to *Hind*III-digested pBR322, and fragment *Sal 6* was ligated to *Sal*I-digested pBR325 according to Bolivar and Backman (1979). The hybrid plasmids were transformed into CSR603 or HB101 cells as described by Dagart and Ehrlich (1979). Amp^r Tc^s recombinants were selected and the ct-DNA inserts were analysed. *Channels 1,2,3,* and *4* contain *Hind*III digests of *1* λDNA; *2* pBR322; *3* and *4* PRCZ1 (*Hind 2* fragment in pBR322 cloned in HB101 and CSR603 respectively). *Channels 5,6,* and *7* contain *Sal*I digests of *5* and *6*, PRCZ2 (*Sal 6* fragment in pBR325 cloned in HB101 and CSR603, respectively); *7* pBR325. *Channels 8,9, 10* and *11* are autoradiograms demonstrating the hybridization of ^{32}P-labelled spinach LS gene to gel *channels 3,4,5* and *6*

dotted lines in Fig. 2 between A and B. Two contiguous *Hind*III fragments (0.75 kb and 1.25 kb) of pLM401 (Fig. 2 A), which contain the major posterior region of the LS gene, were inserted into the *Hind*III site of plasmid pPL608, generating pRCZ4. This insertion is also marked with the dotted lines in Fig. 2 between A and C. Both recombinant plasmids were transformed into *B. subtilis* strain IS53 with selection for the neomycin-resistance trait specified by pPL608.

For detection of the LS gene product a simple in situ immunoassay was employed (Anderson et al. 1980). The clones CSR603 (PRCZ1) and CSR603 (PRCZ2) containing the *N. otophora* LS gene were inoculated and incubated on a N-Z bottom agarose plate overnight at 37 °C. CSR603 cells containing pBR322 and pBR325 were used as controls. After incubation, the cells in the plates were lysed with chloroform vapor and lysozyme. A channel was made in the center of the plate into which was added antiserum against the LS of RuBPCase. Incubation was continued for another day at room temperature. A sharp immunoprecipitation line was formed between CSR603 (PRCZ1) and the central channel demonstrating that this clone has produced the LS polypeptide (Fig. 3 A). *B. subtilis* cells carrying PRCZ3 and PRCZ4 and control cells containing the vector pPL608 were similarly cultured on N-Z bottom agarose plate, lysed with chloroform vapor and lysozyme, and overlayered with molten agarose mixed with the antiserum against LS. Incubation continued for one week at 4 °C. Colonies harboring both recombinant plasmids showed an immunoprecipitation ring, whereas the control cells exhibited no immunoreaction (data not shown). This result was confirmed by immunoprecipitation of extracts of these clones with antiserum against LS in an Ouchterlony double diffusion test (Fig. 3 B). Figure 3 B shows expression in *B. subtilis* of anterior portion of the LS gene from *Chlamydomonas* and in *E. coli* of the LS

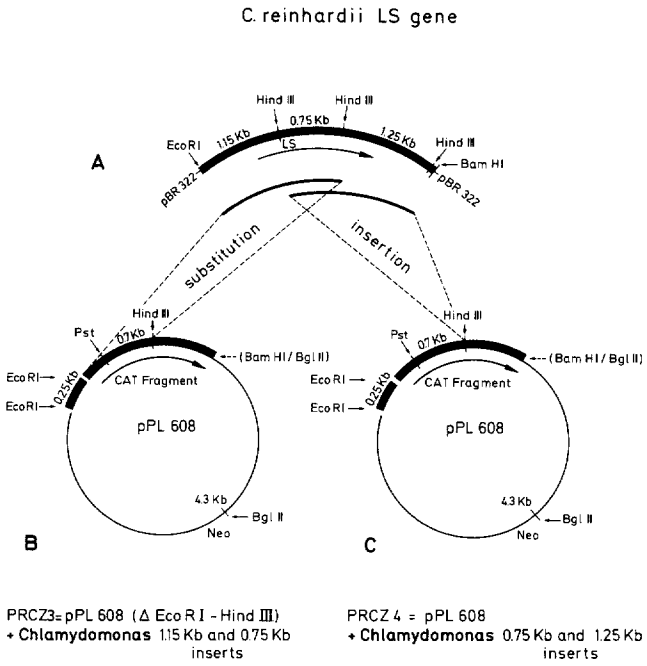

Fig. 2A–C. Cloning of the *C. reinhardii* LS gene in *B. subtilis.* **A** *C. reinhardii* ct-DNA fragment (3.2 kb) was inserted into *Eco*RI and *Bam*HI sites of pBR322 and cloned in HB101 as recombinant plasmid pLM401 by L. Metz. It contains the LS gene as marked by the *arrow* indicating the direction of transcription according to Metz's data. The central region of this gene is located in a 0.75 kb *Hind*III fragment. The 5′ and 3′ ends are located within the 1.15 kb *Eco*RI-*Hind*III fragment and 1.25 kb *Hind*III fragment respectively. **B** Construction of PRCZ3: 10 μg of pPL608 DNA was digested with *Hind*III for 2 h at 37 °C, then partially digested with *Eco*RI for 5 min at room temperature. A plasmid fragment containing the 4.3 kb *Eco*RI-*Hind*III region plus the 0.25 kb *Eco*RI promoter fragment as marked by the split bar, was separated and isolated by low-gelling-temperature agarose gel electrophoresis. The anterior portion of the *Chlamydomonas* LS gene (1.15 kb and 0.75 kb) in Fig. 2A was obtained by partial *Hind*III digestion of the *Eco*RI linear form of PLM401. The deleted form of pPL608 and anterior portion of the *Chlamydomonas* LS gene were ligated and introduced into *B. subtilis* IS53 as described by Lovett and Keggins (1979). **C** Construction of PRCZ4: Plasmids pLM401 and pPL608 were digested with *Hind*III and ligated, and transformed into *B. subtilis* IS53. Neo^r, Cm^s recombinants were selected and the inserts were analysed. A clone PRCZ4 containing 0.75 kb and 1.25 kb *Hind*III fragments was obtained

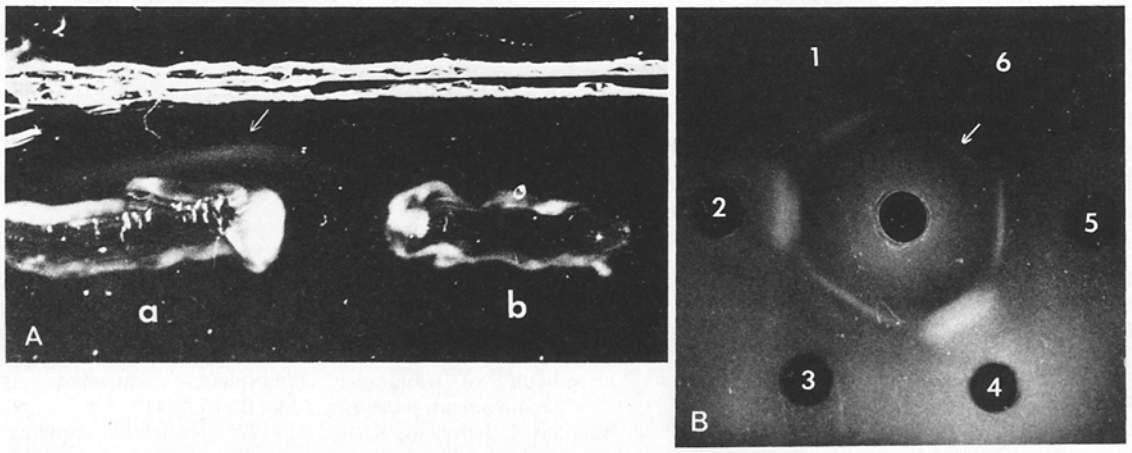

Fig. 3. A Detection of the *N. otophora* LS polypeptide synthesis in *E. coli* by in situ immunoprecipitation reaction. Clones containing different ct-DNA inserts were cultured on N-Z bottom agarose plate containing ampicillin (20 μg/ml) in a 50 mm petri dish. After lysis and washing, a channel was made in the center of the plate and 100 μl of an antiserum against LS was loaded. Overnight incubation at room temperature allowed development of a zone of immunoprecipitation (marked by *arrow*) around CSR603 (PRCZ1) (*a*), whereas control cells (*b*) containing plasmid pBR322 exhibited no reaction. **B** Detection of the synthesis of LS polypeptide of *N. otophora, N. tabacum,* spinach and *C. reinhardii* in *E. coli* or *B. subtilis* by Ouchterlony double diffusion. Five ml of cultures were grown in L broth at 37 °C for 4 h. Cell pellets were washed with TES buffer (Lovett and Keggins 1979) and lysed by boiling in 0.1 ml of 2% SDS for 2 min. After removal of free SDS by acetone treatment, twenty μl of lysates were added to the outer holes. Twenty μl of antiserum against LS was loaded into the central hole. After incubation for one day at room temperature, the immunoprecipitates were observed in all clones containing LS gene except in control cells.

1. PRCZ1: Hind 2 (*N. otophora*)/pBR322/CSR603
2. PRCZ2: Sal 6 (*N. otophora*)/pBR325/CSR603
3. pLM401: *Chlamydomonas* LS gene/pBR322/HB101
4. pJEA4: Spinach LS gene/pBR322/HB101
5. pHPE4: *Eco*R4 (*N. tabacum*)/pBR325/HB101
6. PRCZ3: anterior *Chlamydomonas* LS gene/pPL608/IS53

genes from spinach, tobacco, and *Chlamydomonas.* As it is expected, the smaller incomplete polypeptide made by the anterior portion of the LS gene from *Chlamydomonas* (well 6) migrated faster as compared to the complete LS polypeptide made by the entire LS genes from spinach, tobacco, and *Chlamydomonas* (wells 1–5) in *E. coli.* The sizes of the complete LS of tobacco and spinach are similar (52–53,00 daltons, Erion et al. 1981). Based on the intensity of immunoprecipitation line of a standard, the amount of LS produced in each cell was approximated to be 0.1 pg.

The results presented here demonstrate direct expression in *E. coli* and *B. subtilis* of the LS genes from tobacco and *Chlamydomonas.* Previous studies demonstrated that expression of the maize LS gene in *E. coli* was initiated within its own promoter (Gatenby 1981). Our results also indicate that the expression in *E. coli* of tobacco LS gene was directed by its own promoter, since the LS gene and its promoter are located in the middle of the Hind 2 fragment (11 kb). Furthermore, this promoter is able to initiate the expression in *E. coli* of the galactokinase gene on the promoter-less plasmid pK01 (C. M. Lin and X. F. Kong, unpublished data).

This is the first demonstration of expression of a plant gene in *Bacillus. Hind*III fragments (0.75 and 1.25 kb) cloned in PRCZ4 represent a large posterior part of the LS gene of *C. reinhardii* presumably lacking its own promoter. Its expression in *Bacillus,* which is stimulated by chloramphenicol (0.1 μg/ml) (data not shown), is apparently initiated by a strong promoter located within the 0.25 kb *Eco*RI fragment in pPL608 (Williams et al. 1981) (Fig. 2C). The unique *Hind*III site in pPL608 is located within a structural gene specifying a chloramphenicol-inducible, chloramphenicol acetyltransferase (CAT). Based on the data of Williams et al. (1981) it is expected that the LS polypeptide in PRCZ4 is synthesized as a hybrid with the amino terminal portion of chloramphenicol acetyltransferase. Transcription of the LS gene in the clone PRCZ3, which contains the anterior portion of the LS gene (Fig. 2B) is either initiated within its own promoter or within the plasmid promoter.

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