Expression of amyloplast DNA in suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.)

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Amyloplast DNA isolated from heterotrophically grown cells of sycamore (*Acer pseudoplatanus* L.) contains homologous sequences to genes for the large subunit of ribulosebisphosphate carboxylase/oxygenase (RuBisCO), three subunits (α, β and ε) of coupling factor of chloroplasts (CF_1), 32 kDa protein, apoprotein of P700 and 16 S rRNA [(1985) Biochem. Biophys. Res. Commun. 133, 140–146]. However, employing either enzyme assays or immunological techniques, RuBisCO and CF_1 cannot be detected. Northern blot analysis of total cell RNA showed only the presence of transcripts for the 16 S rRNA gene. Thus, amyloplast DNA appeared to be little transcribed in the sycamore cells. The digestion pattern of the specific endonuclease shows that some methylation occurs in the amyloplast DNA, but several regions of DNA are shown to be potentially active as templates for in vitro transcription. Transcriptional control of gene expression in amyloplasts of sycamore cells is postulated.

(Sycamore cell) *amyloplast DNA* *Gene expression* *Transcription* *Methylation*

1. INTRODUCTION

In contrast to accumulation of extensive knowledge of chloroplast DNA, relatively little is known about the mechanism(s) which regulate gene expression in different types of plastids and at different developmental stages [1]. From analytical studies on the light-mediated transition of etioplasts to chloroplasts during the greening process, transcriptional regulation of gene expression has been proposed [2]. Results from recent investigations dealing with the maize plastid photogenes [3], as well as those of transcriptionally active complexes isolated from mustard (*Sinapis alba*) [4], support this hypothetical view and accordingly, the regulatory role of phytochrome in the overall process has been implicated [5].

The suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.) are heterotrophic organisms which contain mainly one type of differentiated plastid (amyloplasts) and, previously, the presence of several homologous sequences in the amyloplast DNA to genes for photosynthesis and 16 S rRNA was reported [6]. Since amyloplasts, uniquely differentiated organelles which accumulate starch, are depleted of the photosynthetic machinery, we can readily surmise that they may not contain any gene products relating to photosynthesis. Consequently, we have attempted to investigate the mechanism(s) governing gene expression in these organelles, and evidence showing that the amyloplast genome is little expressed has been obtained. We propose that the regulatory mechanism presumably takes place at the level of transcription.

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Abbreviations: CF_1, coupling factor of chloroplasts; ELISA, enzyme-linked immunosorbent assay; RuBisCO, ribulosebisphosphate carboxylase/oxygenase

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2. MATERIALS AND METHODS

Both protoplasts and amyloplasts of sycamore cells were prepared according to the methods described in [6]. Mitochondria free from other organelles were prepared after fractionation of the 10000 × *g* pellets of the disrupted protoplasts as described [7], except for the use of 10 mM Mops-NaOH (pH 7.5). DNA was extracted from each organellar fraction as reported [6]. The extraction of RNA from sycamore cells was performed following the procedure reported by Schmidt et al. [8], except for the repeated precipitation by LiCl. DNA was radioactively (^{32}P) labeled in vitro by nick translation [9]. Partially hydrolyzed RNA was labeled in vitro by T_4 polynucleotide kinase in the presence of RNase inhibitor (RNasin, 1 unit/μl) as described [10]. The digestion of DNA by restriction endonucleases and subsequent agarose gel electrophoresis of the digested fragments were performed using conventional techniques. The transfer of nucleic acids to GeneScreen (New England Nuclear) membranes and the hybridization conditions for Northern and Southern blot analyses were carried out according to the instruction manual of New England Nuclear. The in vitro transcription of circular amyloplast DNA by *E. coli* RNA polymerase in the presence of RNase inhibitors (RNasin, 1 unit/μl) was carried out according to Bogorad et al. [10]. The use of [α-^{32}P]UTP resulted in the radiolabeling of the transcripts. For the isolation of transcripts, the template DNA (0.4 μg) was digested with 2 units of RNase-free DNase (Worthington) for 15 min at 37°C. The labeled RNA was then collected by ethanol precipitation after phenol extraction, using sonicated salmon sperm DNA (20 μg) as a carrier.

Plasmids containing the maize chloroplast genes *rbcL* (large subunit of RuBisCO), *psbA* (32 kDa protein), *atpA* (α-subunit of CF_1), *atpB* (β-subunit of CF_1), *atpE* (ϵ-subunit of CF_1), and *psaA* (apoprotein of P700) and 16 S rDNA came from Dr Bogorad's laboratory (Harvard University).

3. RESULTS AND DISCUSSION

Using the total extracts of either sycamore cells or isolated amyloplasts, we have been unable to detect the presence of RuBisCO or CF_1 as assayed by the following procedures: (i) enzyme activity measurements (for RuBisCO); (ii) Western blotting; (iii) ELISA. Although we cannot totally exclude the possibility that the immunochemical cross-reactivity of the antibodies used (maize origin) were not sensitive enough, this appears unlikely because the pea chloroplast extracts have given positive results under identical assay conditions. These findings, together with the absence of the photosynthetic apparatus in the amyloplast, appear to indicate that the products of the genes *rbcL, psbA, atpA, atpB, atpE* and *psaA* are not present in sycamore cells.

As the next step in attempting to determine whether these genes are transcribed, we performed the Northern blot analysis of the RNA for detecting transcripts. As presented in fig.1, the hybridization experiments using probes of the 7 different genes mentioned above gave a positive signal only with 16 S rDNA. The band of higher M_r likely represents the precursor of 16 S rRNA. The negative signals for the other genes may indicate that they are not transcribed or that the transcripts formed are rapidly degraded.

We next carried out the Southern blot hybridization of the ^{32}P-labeled cell RNA with the *Eco*RI-digested fragments of amyloplast DNA. As shown in fig.2, only two fragments gave a positive signal even after prolonged exposure to the X-ray film (lanes 3 and 5). It has been previously demonstrated that these fragments contain the 16 S rDNA [6], which is consistent with the results obtained by Northern analysis (fig.1). In comparison, when analogous experiments were performed with mitochondrial DNA, potentially high signals were obtained (lanes 2 and 4). These results indicate that there are abundant transcripts of mitochondrial DNA. From these findings, it is reasonable to conclude that the amyloplast DNA in the sycamore cells is not actively transcribed. Hansmann et al. [11] have reported that DNA of the chromoplast, another type of differentiated plastid, is also not active. It merits description also that throughout our investigation, we have been unable to detect RNA pellets in the CsCl gradient centrifugation during the step of repeated amyloplast DNA extraction; no amyloplast RNA can be isolated even after the use of RNase inhibitors.

In spite of the fact that our results indicate the presence of transcripts of 16 S rDNA in the

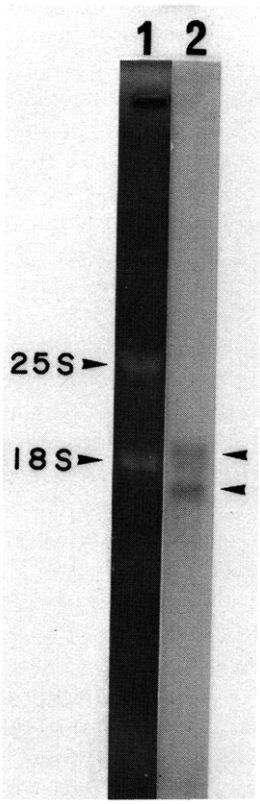

Fig.1. Northern blot analysis of transcripts for amyloplast 16 S rDNA. Purified cell RNA (12 μg) was denatured by glyoxal and electrophoresed (30 V, 12 h) on 1.2% agarose gel run in 10 mM Na phosphate (pH 7.0). Fluorescence of RNA after ethidium bromide staining of the agarose gel. 25 S and 18 S RNA are indicated on the left (lane 1). Autoradiograph of the GeneScreen membrane after Northern blotting of RNA followed by hybridization with nick-translated plasmid containing the 16 S rDNA gene from maize chloroplast DNA (4×10^6 cpm) is shown on the right (lane 2, arrows indicate 16 S RNA and its possible precursor).

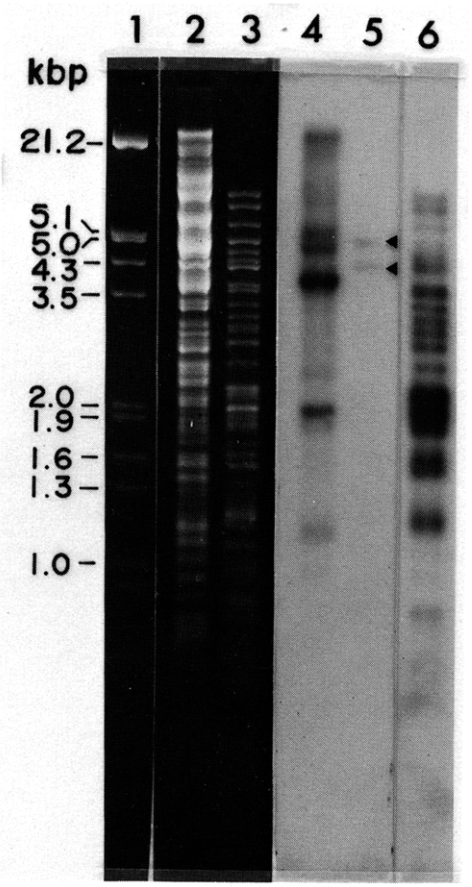

Fig.2. Southern blot analysis of transcription products of amyloplast and mitochondrial DNA. *Eco*RI digests of mitochondrial DNA (1 μg) and amyloplast DNA (0.8 μg) were electrophoresed on 0.7% agarose gel, visualized by ethidium bromide staining and transferred to GeneScreen membranes. Ethidium bromide fluorescence of 0.5 μg of *Hin*dIII/*Eco*RI fragments of λDNA (lane 1), mitochondrial DNA (lane 2), and amyloplast DNA (lane 3). Autoradiographs of the DNA from lanes 2 and 3 after Southern transfer to GeneScreen and hybridization with ^{32}P-labeled cell RNA (5×10^6 cpm) (lane 4 and 5, arrows indicate 16 S rDNA). Autoradiograph of amyloplast DNA from lane 3 after Southern transfer to GeneScreen and hybridization with ^{32}P-labeled in vitro transcription products of amyloplast DNA (1.5×10^6 cpm, lane 6; see text for further details).

amyloplast DNA (figs 1 and 2), we must rigorously determine whether it comes from cross-hybridization with either cytosolic or mitochondrial RNA species. Furthermore, it should be mentioned that this gene sequence is reported to be present in the maize mitochondrial DNA [12], and indeed our preliminary experiments have shown that certain homologous sequences exist between the amyloplast and mitochondrial genomes (not shown). However, 16 S transcripts would be amyloplast rRNA, because mitochondria contain 18 S rRNA instead of 16 S rRNA in maize [13].

It is tempting to speculate that the low magnitude of the expression of the amyloplast

genome is ascribable to transcriptional control. Among several mechanisms, DNA methylation is known to play a key role in the transcriptional activity of eukaryotic genomes [14]. Methylated DNA is not usually found in chloroplasts [15], except for the case of *Chlamydomonas reinhardi* [16], in which maternal inheritance is implicated (review [17]). The digestion pattern of the amyloplast DNA using the isoschizomers *Bst*NI and *Eco*RII showed that methylation of the internal cytosine residues occurs in some 5′-CC$^{A}_{T}$GG-3′ sequences, because *Eco*RII did not completely cleave the amyloplast DNA (fig.3, lanes 3 and 4; different cleaving products are marked by white squares). The different patterns observed are not likely to be derived from an incomplete digestion with *Eco*RII, because a prolonged incubation with a large excess of enzyme did not alter the pattern. Such a methylation of the internal cytosine residues did not occur in 5′-CCGG-3′ sequences as evidenced by the identical patterns obtained by the isoschizomers *Msp*I and *Hpa*II (lanes 1 and 2). The presence of a similar type of methylation has been reported in the mitochondrial DNA of *Physarum polycephalum* [18].

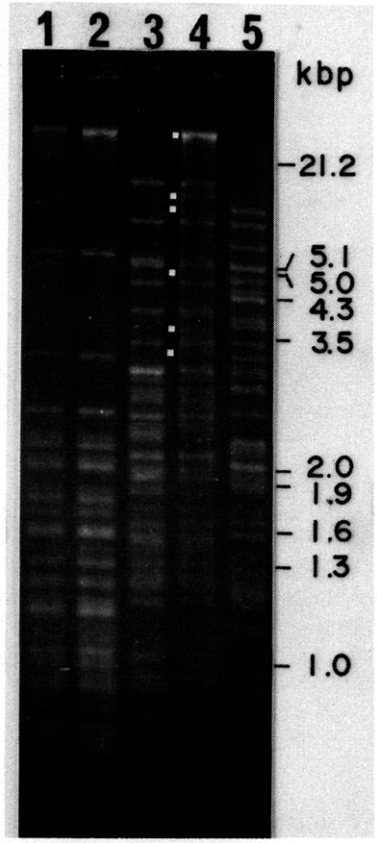

Fig.3. Methylation of amyloplast DNA at *Eco*RII/*Bst*NI sites. Amyloplast DNA was digested by restriction endonucleases (10 units each, 2 h) according to the instructions of the manufacturer and electrophoresed on 0.7% agarose gels in the presence of ethidium bromide: *Msp*I (lane 1); *Hpa*II (lane 2); *Bst*NI (lane 3); *Eco*RII (lane 4); *Eco*RI (lane 5). Each lane contained about 1 μg of DNA. Different cleavage sites at *Eco*RII/*Bst*NI are indicated by white squares between lanes 3 and 4.

Assuming that the methylation of such type and/or other structural modification of the amyloplast DNA might affect transcription, we have examined the in vitro transcription of circular amyloplast DNA with *E. coli* RNA polymerase. The radiolabeled transcripts were purified and subjected to hybridization with *Eco*RI fragments of the amyloplast DNA. As clearly shown in fig.2 (lane 6), the transcripts are able to hybridize with most of the fragments. These results indicate that many regions of the DNA molecule are potentially active as templates for in vitro transcription.

Based on the results obtained in the present investigation, we propose that the amyloplast genome is mostly inactive except for rDNA and it is presumably regulated at the transcriptional level. In contrast mitochondrial genome is actively transcribed.

Future rigorous experimentation will be needed to clarify the detailed mechanism(s) of regulation, e.g., function of methylation, in the hope that the general mechanism of the regulation of the chloroplast-type genome in non-green plastids such as amyloplasts can be elucidated. Another interesting area of investigation is the mechanism for transmembrane transport of the nuclear genome-encoded enzymes engaged in the starch metabolism (both biosynthesis and degradation such as α-amylase) (D. Macherel et al., submitted). Fig.4 is a schematic illustration of the expression of amyloplast DNA.

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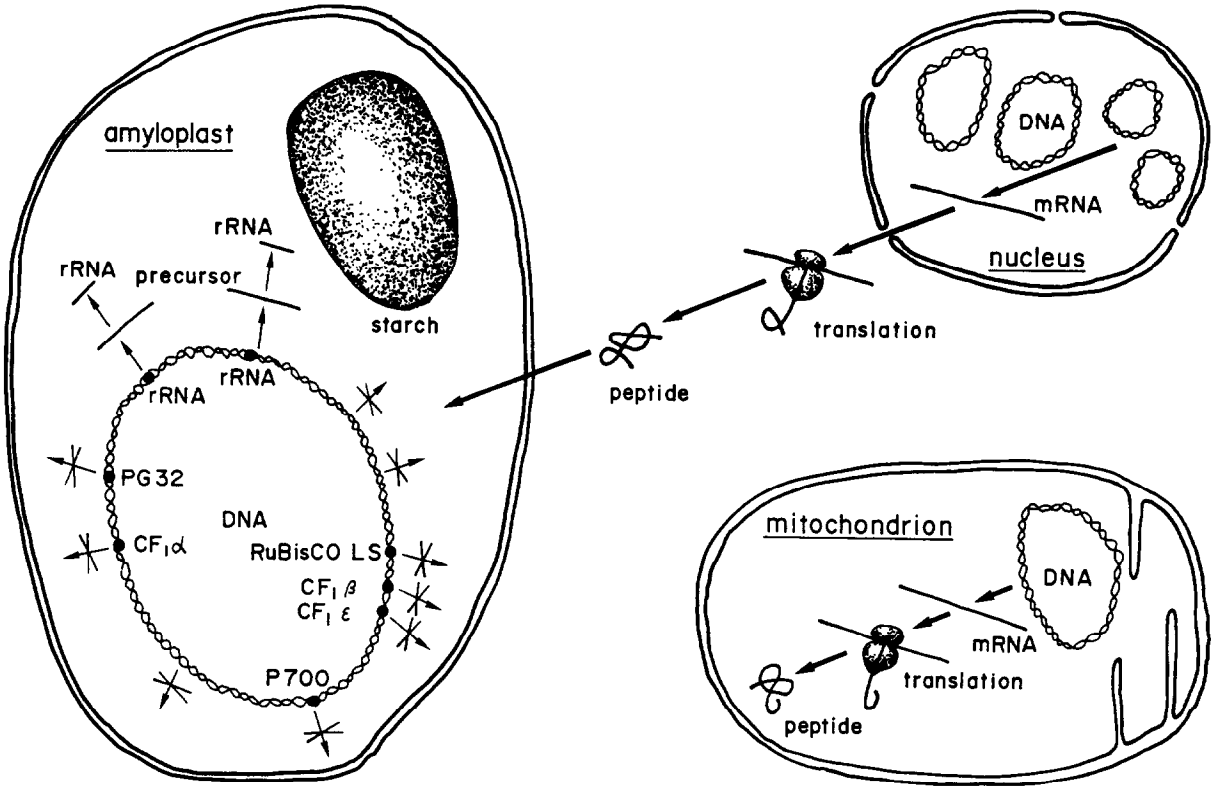

Fig.4. Schematic representation of expression of amyloplast and mitochondrial DNAs and transport of nuclear DNA-encoded proteins into amyloplast.

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