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Amyloplast Nucleoids in Sycamore Cells and Presence in Amyloplast DNA of Homologous Sequences to Chloroplast Genes

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Employing specific dye-stain followed by fluorescence microscopy, evidence was obtained for the presence of amyloplast nucleoids in protoplasts as well as in the amyloplasts isolated from the suspension- cultured cells of sycamore (Acer pseudoplatanus L.), which are devoid of chlorophylls and grow heterotrophically. We have isolated and partially characterized amyloplast DNA. The presence of six homologous sequences to chloroplast genes encoding (i) apoprotein of P700, (ii) 32kDa protein (photogene 32), (iii) α subunit of CF_1, (iv) large subunit of RuBisCO, (v) ß and ε subunits of CF_1 and (vi) 16S ribosomal RNA, has been revealed by hybridization experiments, using gene probes from maize chloroplast DNA.

Among the plastid genomes of plant cells, the chloroplast genome has been most extensively studied (1). Amyloplasts present in the non-photosynthetic plant tissues are known to accumulate starch as a storage form of carbohydrate (2). In spite of the fact that amyloplasts and chloroplasts are frequently postulated to be ontogenically related, neither the functional nor the structural nature of the former organelle is substantively characterized (3). The isolation of amyloplasts from different plant sources has been reported (4-8), and the nucleic acid content has been estimated (7). Identity of chloroplast and amyloplast DNA has been claimed in the case of potato (9,10). It is well recognized that upon illumination, proplastids or etioplasts in some plant tissues such as potato tuber and etiolated wheat seedlings are transformed to the

Abbreviations: DAPI, 4'-6-diamidino-2-phenylindole; MEM, mannitol-EDTA-MOPS; MOPS, morpholinopropane sulfonic acid; RuBisCO, ribulose 1,5-bisphosphate carboxylase/oxygenase.

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chlorophyll-bearing chloroplasts (11). One can thus surmise that there exist identical genetic messages in these two different types of organelle. On the other hand, cultured cells of sycamore derived from the cambium tissues are non-photosynthetic in situ, and the amyloplasts never transform to chloroplasts. As it is likely that mechanism(s) operating in either formation or breakdown of reserve starch in amyloplasts is distinguishable from those for the assimilation starch in chloroplasts, the characterization of genes in amyloplasts is thought to be crucially important to obtain insight into the biochemical function of this unique organelle.

Materials and Methods

Suspension culture of sycamore cells and amyloplast preparation

Sycamore cells (Acer pseudoplatanus L.) were grown in the liquid medium as previously described (11). Cells (200g fresh weight) were collected at the exponential phase of growth after sucrose starvation for 15 hr, rinsed with the culture medium supplemented with 0.5 M mannitol and incubated for 90 min in the same medium containing 1% (w/v) Cellulase Onozuka RS (Yakult Co. Ltd., Japan) and 0.1% Pectolyase Y23 (Seishin Co. Ltd., Japan). The resulting protoplasts were filtered through Miracloth and washed with 0.5 M mannitol/2% (w/v) sucrose solution, resuspended in MEM buffer (0.5 M mannitol/1 mM $EDTANa_2$/10 mM MOPS, pH 7.5) containing 7 mM ß-mercaptoethanol, 0.4 mM phenylmethylsulfonylfluoride, 0.4 mM spermidine and 0.1 mM sodium tetrathionate. The final preparation of protoplasts was disrupted by forcibly passing the suspension twice through a 20 µm Nylon mesh fitted to a syringe (12). The broken protoplast preparation was centrifuged (100 g, 5 min) and the pellet suspended in MEM buffer containing 40% (v/v) Percoll. The suspension was then filtered through Miracloth and the filtrate included as the 40% layer in discontinuous layers of Percoll i.e., 50,40,20 and 10% (v/v) in MEM buffer. After centrifugation (100g, 10 min), yellow pelletable amyloplast preparation obtained was gently rinsed with 1 ml of MEM, and subjected to the experiments written below.

Fluorescence microscopy

The staining of DNA with DAPI was performed according to the procedure of Nishibayashi and Kuroiwa as previously reported (14), using protoplasts and amyloplasts. In each case, the specimens were subjected to the high resolution epifluorescence microscopy.

Characterization of DNA

DNA was extracted from amyloplasts with Sarkosyl and purified by a first ethidium bromide-CsCl gradient centrifugation (60,000 rpm, 12 hr, rotor Beckman Vti 65) followed by a second centrifugation (60,000 rpm, 12 hr) in a CsCl gradient containing bisbenzimide Hoechst 33258 (Sigma) as an intercalating agent for the double strand DNA (15). After removal of Hoechst 33258, the DNA was precipitated with ethanol. The procedure yielded 20-25 µg DNA. The digestion of DNA by EcoRI and 0.7% agarose gel electrophoresis were performed using conventional techniques. The DNA fragments obtained were transferred to Genescreen (New England Nuclear) using the Southern blotting technique (16). DNA probes containing genes from maize chloroplast DNA came from Dr. L. Bogorad, Harvard University. They were labeled with [^{32}P]deoxy CTP by nick translation. After hybridization according to the instruction manual of New England Nuclear, the labeled bands on Genescreen were detected by radioautography.

Results & Discussion

As presented in Fig. 1 a-c, the DAPI staining of intact sycamore protoplasts shows a strong fluorescence from nuclei, but fluorescence was also detectable as small spots corresponding to mitochondrial nucleoids (dark arrowheads) and as larger and irregular areas corresponding to amyloplast nucleoids (white arrowheads). Phase contrast (a), fluorescence (b), and superposition of fluorescence and phase contrast images (c) distinctly show the localization of mitochondrial nucleoids within small spherical particles as well as amyloplast nucleoids within the 1-8 starch grain containing organelles. The dye-stained images of isolated amyloplasts are shown in Fig. 1 d-f. Although the amyloplast preparations were slightly squashed (d), they represent the nearly

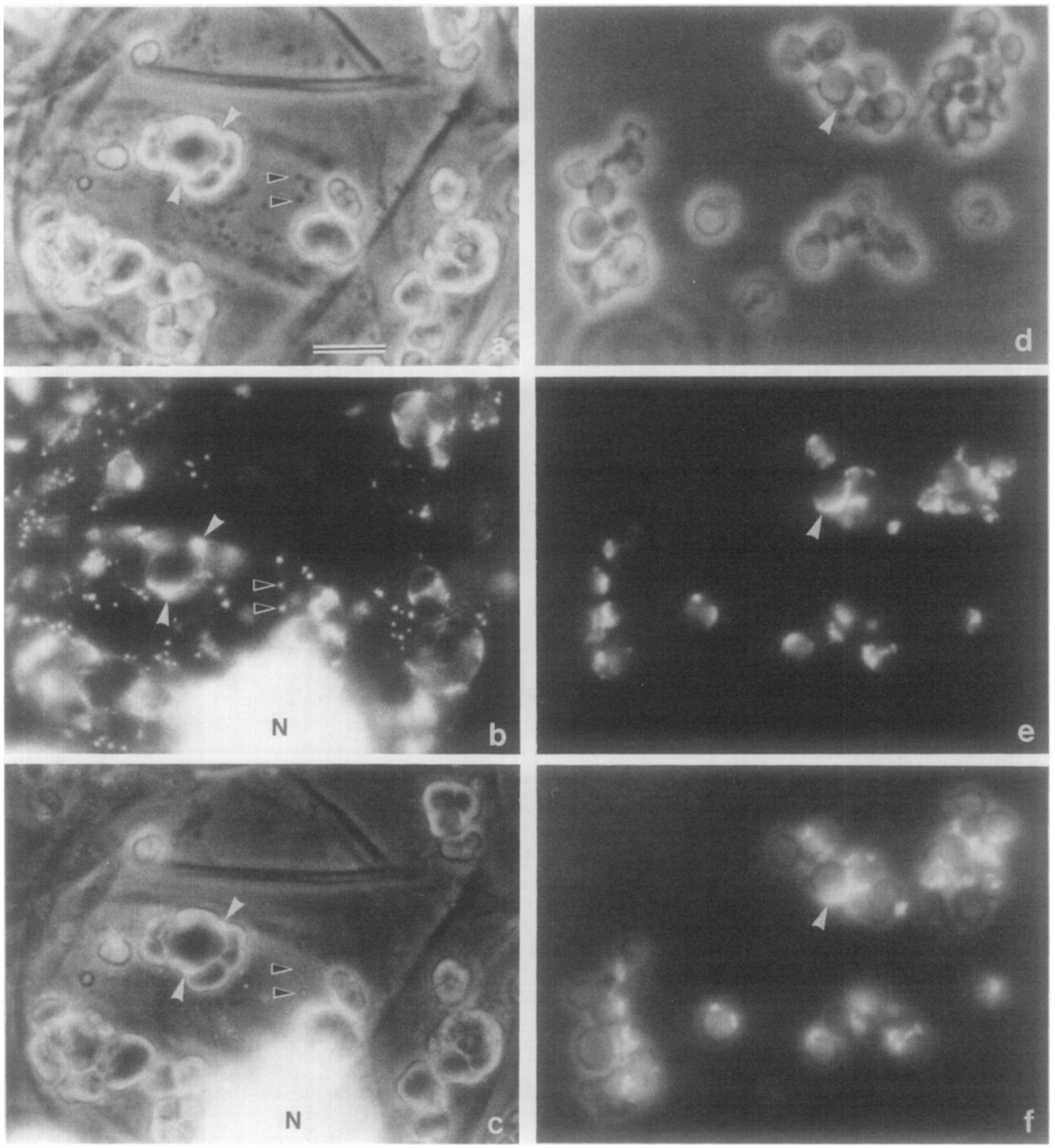

Fig. 1. DAPI staining of protoplasts and amyloplasts.

Phase-contrast (a,d), fluorescence (b,e) and phase-contrast/fluorescence (c,f) photomicrographs showing cell nuclei (N), amyloplast (white arrowheads) and mitochondria (black arrowheads) in protoplasts (a-c) and isolated amyloplasts (d-f) of sycamore cells. White and black arrowheads are markers showing the same field and feature amyloplast nucleoids and mitochondrial nucleoids, respectively (b,c,e,f). In the protoplast, the amyloplasts contained 1-8 starch grains and irregularly shaped nucleoids, which emit larger blue-white fluorescence comparing to the mitochondrial nucleoids. The isolated amyloplasts also contained several starch grains, and irregularly shaped nucleoids can be seen among these starch grains (scale bar-5 μm).

identical type of fluorescence images (e,f), which reinforces the existence of amyloplast nucleoids. Results also indicate that the envelope of the organelles is retained during the isolation steps involved. This was in fact confirmed by an electron microscopy which shows the presence of a double envelope (results not shown).

Upon centrifugation of the DNA extracted from amyloplasts in ethidium bromide-CsCl only one band was discernible under the UV light. The second CsCl gradient centrifugation with Hoechst 33258 yielded one major band in addition to two very faint bands which we could not analyze and which were presumably derived from either contaminating or damaged DNA components. The DNA from the major band was precipitated, cleaved with EcoRI and the fragments, separated by 0.7% agarose gel, were subjected to the characterization of gene components (Fig. 2 A). The calculated size of the DNA after digestion by endonuclease Pst I was approximately 110 kbp, which is among the shortest lengths so far reported from plastids of different species (17).

The hybridization of labeled probes of maize chloroplast genes with EcoRI digested fragments of amyloplast DNA indicated the presence of seemingly homologous sequences in the amyloplast genome. A homologous sequence for the apoprotein of P700 was detectable in the second band (B). A sequence equivalent to photogene 32 coding for the 32kDa protein was detected in the fifth fragment (C). A similar sequence coding for the α subunit of CF_1 was located in the third fragment (D). A homologous sequence to the gene for the large (A) subunit of RuBisCO and a similar sequence for β and ε subunits of CF_1 were both located in the eighth fragment (E,F). It is worthy of noting that in the maize chloroplast DNA, these three genes are shown to be located close to each other (18), and our observation indicates that probably a similar situation exists in the amyloplast DNA. The sequence for 16S ribosomal RNA can be seen on the fourth and the seventh bands (G). These fragments are duplicated with the expected similarity to the inverted repeat regions in many chloroplast DNAs (19,20).

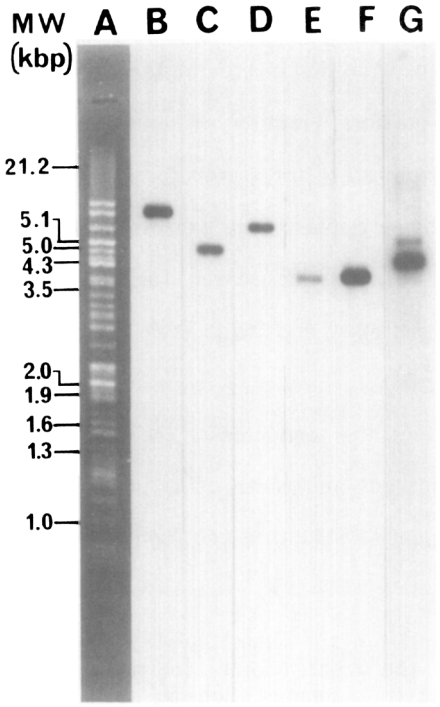

Fig. 2. Hybridization of amyloplast DNA .h chloroplast gene probes.
Electrophoretic pattern of amyloplast DNA digested by EcoRI (A) and radioautographs of Southern hybridizations with probes for maize chloroplast genes coding the apoprotein of P700 (B), 32 kDa protein (C), α subunit of CF_1 (D), large subunit of RuBisCO (E), ß and ε subunits of CF_1 (F), and 16S ribosomal RNA (G). Molecular weights (MW) are indicated at the left in kilobase pairs (kbp).

Overall, our experimental results strongly suggest an analogy between the DNAs from amyloplasts and chloroplasts. Since the structure and the function of these organelles are clearly distinguishable, the expression of the amyloplast genome and its regulation are evidently of great interest for future investigations. Among the products directed by the gene sequences which we can now localize on the amyloplast DNA, five of them are directly involved in photosynthesis [apoprotein of P700, 32kDa protein, α , ß and ε subunits of CF_1, large (A) subunit of RuBisCO], but none of them are necessary elements of amyloplasts which lack the photosynthetic apparatus. The product of the 16S ribosomal RNA gene should be involved in protein synthesis. It is thus an intriguing question to elucidate whether or not amyloplasts are able to synthesize proteins, especially in relation to the source of energy involved. Our attempts to identify in organello translation activity have been so far unsuccessful; a possible reason for this is the structural fragility of the

amyloplasts, but the absence of protein-synthesizing machinery in the organelle can also not be excluded. Another interesting area of research which should be pursued concerns the possible location of genetic message(s) in amyloplasts relating to the biochemical steps for starch formation. We are now attempting to clarify the problem of amyloplast gene expression by (i) the construction of a physical map of the DNA, (ii) the search for the gene products using the immuno-detection of proteins and (iii) the hybridization of mRNA with amyloplast DNA.

In view of the fact that amyloplasts are the site of starch synthesis in storage organs such as seeds and roots, it is imperative that every effort be given to elucidate the genome structure and function of the amyloplast genome.

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