A Rapid, Simple Method for Nuclei Isolation from Plant Protoplasts

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KANJI OHYAMA, LAWRENCE E. PELCHER, AND DAVE HORN
National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan S7N 0W9

ABSTRACT
A rapid, simple method for nuclei isolation and purification from soybean (Glycine max L. Merr.) protoplasts is described. The isolated nuclei exhibited active amino acid incorporation and RNA synthesis, but DNA synthesis was not detectable. Analysis by CsCl density gradient centrifugation showed that DNA isolated from nuclei had a single band, while DNA isolated from protoplasts consisted of three bands comprised of nuclear DNA, mitochondrial DNA, and chloroplast DNA.

Isolated nuclei have been used recently to investigate biochemical aspects in higher plants (2, 3). However, it is difficult to obtain adequate yields of intact nuclei in a short period of time from plant materials. This paper describes a rapid, simple procedure for isolation of intact nuclei from plant protoplasts. As criteria of the integrity and purity of nuclei, DNA analysis in CsCl density gradient centrifugation was done and the biological activity of the nuclei was ascertained by measuring amino acid incorporation, RNA and DNA synthesis.

MATERIALS AND METHODS

Cell Culture and Preparation of Protoplasts. Cell material used in this work was soybean (Glycine max L. Merr.) (SB-1). The cells were maintained in suspension culture with 1-B5 medium (5). Protoplasts were prepared as described previously (7).

Preparation of Nuclei from Protoplasts. Protoplasts prepared from 300 ml of SB-1 cell culture (approximately 13.5 ml packed volume after centrifugation at 100g for 5 min) were washed with 0-B5 (sucrose-free) medium containing 0.275 M sorbitol and suspended to make a total volume of 20 ml of 0-B5 (sucrose-free) medium containing 10 mM MgCl₂, 1 mM 2-mercaptoethanol, and 0.5% of Triton X-100 (medium A) plus 0.275 M sorbitol. The protoplasts were disrupted by a Dounce homogenizer with 10 gentle strokes. The homogenate was passed once through a layer of Miracloth and twice through triple layers of Miracloth. Filtrate was layered on 4 ml of medium A containing 0.4 M sorbitol and centrifuged at 400g for 5 min in an International model HN centrifuge or a Sorvall model GLC-2 centrifuge. The nuclei pellet was suspended in medium A containing 0.275 M sorbitol to make a total volume of 2 ml. This step was repeated twice to remove debris, cytoplasmic organelles, and starch granules. Nuclei suspension was layered on gradients of 5 ml of 0.5 M sorbitol in medium A and 2 ml of 1 M sorbitol in medium A, and centrifuged at 100g for 3 min. The top layer, containing the nuclei, was withdrawn and spun down at 400g for 5 min and the pellet suspended in 2 ml of 0.275 M sorbitol in medium A. This process was repeated at least twice to ensure complete removal of larger particles such as small unbroken protoplasts (checked under a light microscope). The nuclei were suspended in 1 to 2 ml of 0.01 M tris-HCl buffer (pH 7.5) containing 0.275 M sorbitol, 10 mM MgCl₂, 1 mM 2-mercaptoethanol, and 0.1% of Triton X-100 (Fig. 1), and used for assaying amino acid incorporation, RNA and DNA synthesis and for DNA isolation and analysis. Nuclei number was determined by counting on a hemacytometer.

Amino Acid Incorporation. Amino acid incorporation was assayed by incubating nuclei (approximately 10⁸) in a total volume of 0.5 ml reaction mixture containing 0.01 M tris-HCl (pH 7.5), 0.275 M sorbitol, and 0.1 μCi of [¹⁴C]-amino acid mixture (260 mCi/mmol). The reaction mixture was incubated at 30°C for an appropriate period of time. After incubation, the nuclei were washed three times with medium A containing 0.275 M sorbitol by centrifugation (800g for 3 min), suspended in 0.5 ml of 2% SDS, and lysed. For total uptake, 0.1 ml of the lysate was transferred to a scintillation vial and counted with toluene-Triton X-100 (2:1) scintillation fluid containing 2-(4-tert-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxidiazole (Butyl-PBD, 5 g/l). To the rest of the lysate, 0.5 ml of 10% trichloroacetic acid was added. The precipitate was collected on a glass fiber filter and washed with 5% trichloroacetic acid, ethanol, and ether, successively. The filter was then dried and counted in liquid scintillation counter to provide data on the incorporation of radioactivity into the acid-precipitable fraction.

RNA Synthesis. RNA synthesis was measured by incubating nuclei (approximately 10⁸) in 0.5 ml of standard reaction mixture containing 0.01 M tris-HCl (pH 7.5), 0.275 M sorbitol, 0.6 M of each of ATP, GTP, UTP, and 2 μCi of [³H]-CTP (20.3 Ci/mmol), 6 mM of P-enolpyruvate, 20 μg of P-enolpyruvate kinase, 100 mM KCl, 4 mM MgCl₂, and 1 mM 2-mercaptoethanol. The reaction mixture was incubated at 30°C for an appropriate period of time. Total uptake and incorporation of [³H]-CTP into nuclei were determined by procedures described for amino acid incorporation assay.

DNA Synthesis. DNA synthesis was determined by incubating nuclei (approximately 10⁸) in a total volume of 0.5 ml reaction mixture containing 0.01 M tris-HCl (pH 7.5), 0.275 M sorbitol, 1 mM 2-mercaptoethanol, and dATP, dTTP, and dCTP each at 0.5 mM, 2 μCi of [³H]-dGTP (9.4 Ci/mmol), 4 mM MgCl₂, and 5 mM ATP. The reaction mixture was incubated at 30°C for an appropriate period of time. Total uptake by nuclei and incorporation of [³H]-dGTP into the acid-precipitable fraction were determined by procedures described in amino acid incorporation assay.

DNA Isolation from Protoplasts and Nuclei, and Properties in CsCl Density Gradient Centrifugation. DNA isolation for protoplasts was described previously (8). For DNA isolation from nuclei, nuclei were suspended to 1 ml of 0.15 M NaCl-0.015 M trisodium citrate (1 x SSC) and lysed by addition of 1 ml of a 10% SDS solution. The DNA was spooled immediately by

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addition of cold ethanol. Crude DNA was dissolved into 2.7 ml of 0.1 × SSC containing 0.01% Sarkosyl and the solution adjusted to 1 × SSC with 10 × SSC. This solution was then treated once with RNase (100 μg/ml) for 30 min and pronase (100 μg/ml) for 30 min, successively. The purified DNA was subjected to CsCl density gradient analysis performed in a Spinco E analytical ultracentrifuge (6).

RESULTS AND DISCUSSION

Previous methods of nuclei isolation utilized mechanical breakage of the cell wall of plant cells (3, 9), and sucrose density high speed centrifugation (2, 3). The method described here employs a gentle disruption of protoplasts with 0.5% Triton X-100, together with a low speed centrifugation. As shown in Figure 1, the round nuclei were intact and with distinct nucleoli indicating complete removal of the cytoplasm from the nuclear membrane. Purity of the isolated nuclei was determined by subjecting DNA isolated from nuclei to CsCl density gradient ultracentrifugation. Total DNA isolated from protoplasts showed three components; nuclear DNA (1.693 g/ml), chloroplast DNA (1.695 g/ml) from leukoplasts (4), and mitochondrial DNA (1.706 g/ml). On the other hand, DNA isolated from nuclei appeared as a very sharp single band indicating nuclear DNA (1.693 g/ml) (Fig. 2). Assuming that 0.1 μg of DNA produces a detectable absorbance peak we could have detected cytoplasmic DNA contamination at less than 2% level. Apparently the treatment with Triton X-100 at low speed centrifugation repeated a few times is sufficient to remove organelles such as chloroplasts (leukoplasts) and mitochondria. Yield of nuclei was 10⁷ to 10⁸ from 300 ml of SB-1 cell suspension culture. A 300-ml suspension culture of SB-1 cells (3.0 × 10⁶ cells/ml) yielded approximately 5.5 × 10⁷ purified nuclei were recovered. This represents a yield of approximately 2.3%. This yield would provide adequate quan-

![Photo micrographs of purified nuclei from soybean protoplasts. A: stained with carbol fuchsin; × 2,400. B: nonstain; × 2,400. N: nucleus; Nu: nucleolus.](image)

![CsCl density gradient centrifugations of DNA isolated from protoplasts (A) and from nuclei (B). Micrococcus lysodeikticus DNA (1.731 g/ml) as density marker; mit-DNA: mitochondrial DNA (1.706 g/ml); c-DNA: chloroplast DNA (1.695 g/ml); n-DNA: nuclear DNA (1.693 g/ml).](image)

![Kinetics of amino acid incorporation (A); RNA synthesis (B); DNA synthesis (C) by isolated nuclei. (–––): total uptake; (○–○): acid-precipitable incorporation of ¹⁴C-amino-acids (A), ³H-CTP (B), and ³H-dGTP (C), respectively. Values are pmol of radioactive incorporated compounds under assay conditions.](image)
ties of nuclei for biochemical investigations such as amino acid incorporation, RNA synthesis and DNA synthesis.

The isolated nuclei were assayed for amino acid incorporation, RNA and DNA synthesis. The nuclei took up 14C-amino-acids and incorporated them into an acid-precipitable fraction (Fig. 3A). The isolated nuclei were also active in 3H-CTP uptake thus demonstrating RNA synthesis (Fig. 3B). The incorporation of 3H-CTP into acid-precipitable fraction continued for at least 2 hr. The product (acid-precipitable fraction) was sensitive to pancreatic RNase (100 µg/ml, 30 min, 30 C). DNA synthesis could not be detected in the isolated nuclei (Fig. 3C). This is not unexpected because DNA synthesis by nuclei requires cytoplasmic factors (1).

The described procedures permit isolation of nuclei, of high purity, from plant protoplasts. The preparations were free from contamination of cytoplasmic organelles as demonstrated by CsCl buoyant density gradient analysis. The isolated nuclei exhibited active amino acid incorporation and RNA synthesis, indicating that they would be suitable for investigations on genetic expression in eukariotic cells.

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