Subcellular reorganization of mitochondria producing heavy DNA in aging wheat coleoptiles

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Abstract Unusual closed membrane vesicles containing one or more mitochondria were isolated from homogenates of aging wheat coleoptiles. Very similar (or the same) bodies were shown to exist in situ in vacuoles of undividing cells in the apical part of intact senescent coleoptiles. Vesicles isolated from coleoptile homogenate free of nuclei by 10 min centrifugation at $1700 \times g$ and traditional mitochondria (sedimented at between $4300 \times g$ and $17\,400 \times g$) are similar in respiration rate, composition and content of cytochromes and sensitivity to respiration inhibitors. However, vesicles contain about 2-fold more Ca^{2+} ions than free mitochondria do. The specific feature of vesicles containing mitochondria in aging coleoptiles is an intensive synthesis of heavy ($\rho = 1.718$ g/cm^3) mitochondrial DNA (H-mtDNA). Thus, aging in plants is accompanied by an increased selective H-mtDNA production and change in subcellular organization of mitochondria.

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Key words: Aging; Apoptosis; DNA synthesis; Heavy DNA; Mitochondria; Mitochondrial DNA; Plant; Wheat

1. Introduction

The periodic synthesis of an unusual fraction of heavy ($\rho = 1.716$–1.718 g/cm^3) DNA localized in mitochondria (H-mtDNA) was detected [1–3] in aging zones of the wheat seedling coleoptile and leaf in cells with strong apoptotic internucleosomal DNA fragmentation [4].

In contrast to wheat nuclear DNA (nDNA, $\rho = 1.700$ g/cm^3), H-mtDNA is enriched with GC base pairs, it seems to contain N^6-methyladenine but not 5-methylcytosine [5–8] and is represented by a population of open circle molecules with a contour length between 0.1 and 0.6 μm [3]. Synthesis of this DNA seems to be common to all higher plants investigated: it was detected in aging but not young organs of various gymnosperms and angiosperms (monocots and dicots) [8]. Similarly to aging animal cells [9,10] the amount of mtDNA in coleoptiles increases with age and H-mtDNA accumulates in the form of discrete fragments that are similar in size to products of apoptotic fragmentation of wheat nuclear DNA [4,11–13]. Unfortunately, the precise localization of H-mtDNA synthesis in the cell was unknown.

The main goal of this work was to identify and investigate the structural and functional features of the wheat coleoptile mitochondria that possess the unique property of heavy DNA synthesis programmed in the plant ontogenesis.

2. Materials and methods

2.1. Growing of seedlings

Seedlings of the winter wheat variety Mironovskaya 808 were grown in darkness at 26°C in the presence of 10 U/ml benzylpenicillin [1,4]. Control for passage of the cycles of nDNA and mtDNA synthesis was done as described earlier [4,13]. Seedlings (about 1000) were cut off above the seed, washed with sterile water and incubated for a
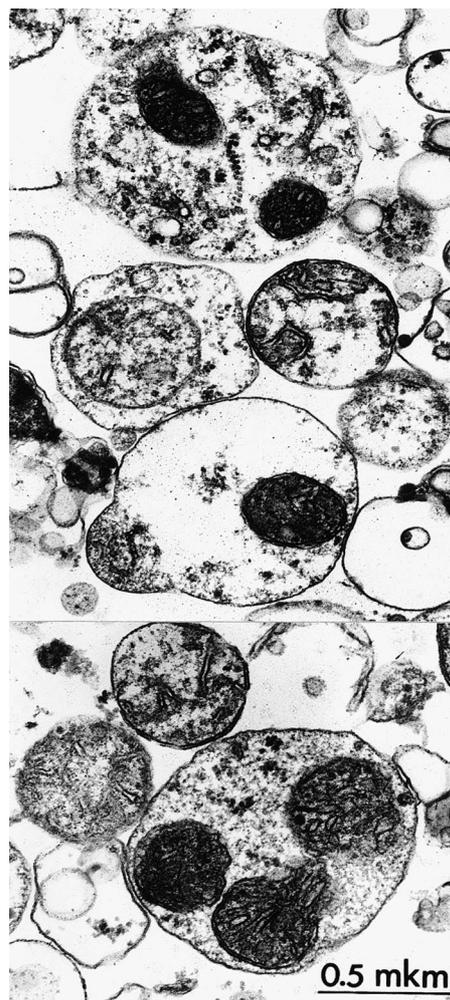
Fig. 1. Fraction of fast sedimenting ($1700 \times g$) subcellular particles (vesicles) isolated from coleoptiles of 8-day-old wheat seedlings.

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period of the DNA cycle synthesis in the presence of the DNA precursor [methyl-^{3}H]thymidine (50 µCi/ml, Institute of Molecular Genetics, Russian Academy of Sciences, Moscow).

2.2. Isolation and fractionation of subcellular particles

Seedlings were washed with sterile distilled water after incubation with label, coleoptiles were isolated and ground with broken glass in a mortar with pestle at 0°C in a medium for isolation of mitochondria (0.4 M sucrose, 5 mM EGTA, 1 mM DTE, 20 mM HEPES-Tris, pH 7.5 and 1 mg/ml BSA) [14] at a plant mass to medium ratio of 1:2 (g/ml). Cell debris was removed by filtration of homogenate through four gauze and one Miracloth (Calbiochem, USA) layers, and filtrate was centrifuged at $17\,400 \times g$ (10 min, 2°C, rotor JA-20, Beckman, USA). The sediment was suspended in 20 ml isolation medium, and the fraction of subcellular particles (vesicles) was sedimented by 10 min centrifugation at $4350 \times g$. The fraction of mitochondria was then isolated by 10 min centrifugation at $17\,400 \times g$ of the supernatant obtained after separation of vesicles. Sediment of mitochondria was suspended in a medium without BSA (0.4 M sucrose, 20 mM HEPES-Tris, pH 7.5), and mitochondria were isolated by centrifugation under the same conditions. Sediment of particles obtained by centrifugation at $4350 \times g$ was suspended in the isolation medium without BSA and fractionated by 10 min sequential stepwise centrifugation at $300 \times g$, $600 \times g$, $1090 \times g$, $1700 \times g$, $2600 \times g$, and $4350 \times g$. Besides, the particle fractions sedimented for 10 min by sequential stepwise centrifugation at $300 \times g$, $600 \times g$, $1090 \times g$, $1700 \times g$, $2600 \times g$, $4350 \times g$, $5930 \times g$, $7740 \times g$, $9800 \times g$, $12\,100 \times g$, and $17\,400 \times g$ were isolated from total cell homogenate. Each fraction was suspended in the isolation medium (without BSA), divided into aliquots, and the protein and DNA contents and (or) radioactivity incorporated in DNA and the DNA buoyant density in respective aliquots were determined.

2.3. Determination of protein and DNA

Protein was measured by the method of Lowry et al. [15] using the standard kit (Sigma, USA). The DNA amount in fractions was determined spectrophotometrically [16], and the DNA buoyant density was measured as described earlier [1–4].

2.4. Analysis of respiration and cytochromes of subcellular particles

The oxygen consumption by the isolated particles was measured using the stationary Clark's oxygen electrode in polarograph LP-7E (Czech Republic). The measurements were carried out at room temperature in a medium containing 0.3 M sucrose, 10 mM KCl, 2 mM MgSO$_4$, 5 mM KH$_2$PO$_4$, 10 mM HEPES-Tris, pH 7.5 and 5×10^{-7} M carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; respiration was initiated by the respiration substrate 5 mM succinate (+2 µM rotenone). Cytochrome spectra in a wavelength interval from 530 nm to 650 nm were obtained at room temperature using an Aminco DW-2000 spectrophotometer. Reduction of cytochromes with dithionite was performed in the presence of 2 mM KCN and 2 mM butylhydroxamic acid. The Ca^{2+} content in subcellular fractions isolated and washed off Na$^+$ was measured by the method of flame photometry.

2.5. Electron microscopy

The electron microscopy investigation of the isolated subcellular particle fractions and serial sections of coleoptile was carried out by standard procedures described earlier [2].

3. Results and discussion

After removal of nuclei (10 min, $600 \times g$) the fraction of unusual closed membrane vesicles containing mitochondria was isolated from homogenates of wheat senescent coleoptiles by centrifugation at $1700 \times g$ for 10 min. The electron microscopy investigation of the fraction showed that it is a suspension of specific subcellular particles with a quite unusual organization. These particles with a single membrane are filled up with cytoplasmic matrix and contain one or more mitochondria (Fig. 1). Sometimes ribosomes and microtubules may also be seen in these vesicles. Analyzing the ultra-thin sections of intact coleoptiles from 8-day-old wheat seedlings, we observed in situ polymorphous suspended cytoplasmic bodies (Fig. 2) that are very similar or even identical to vesicles isolated in vitro. In an intact plant these bodies are localized in the cellular vacuoles and similarly to isolated vesicles they are covered with a single membrane and filled
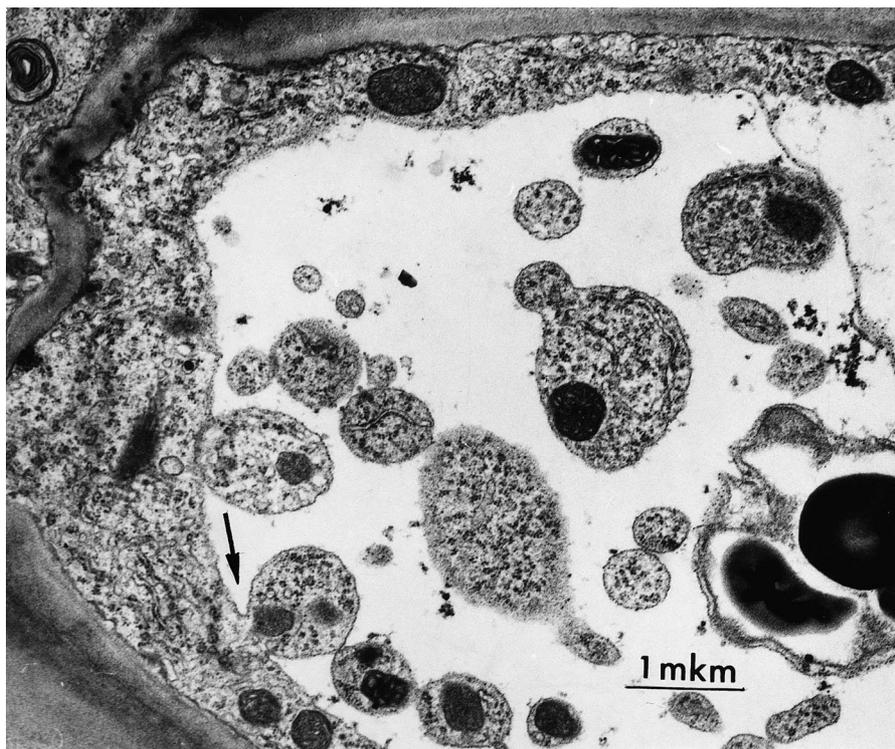
Fig. 2. Structure of parenchyma cells in the apical part of coleoptiles. Vesicles formed are seen in the cellular vacuole. Vesicles that are forming are shown by arrows.
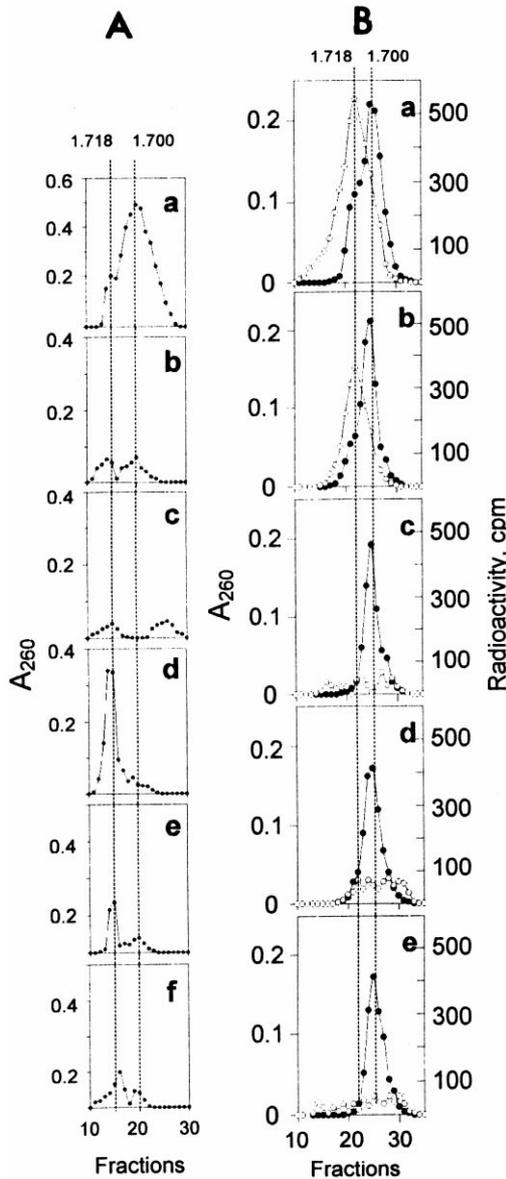
Fig. 3. Distribution in the CsCl density gradient of radioactivity and UV light absorption of DNA isolated from different subcellular fractions of homogenate of coleoptiles of 8-day-old wheat seedlings. Abscissa: fraction numbers (from tube bottom). Ordinates: absorption at 260 nm (left), radioactivity, cpm (right). Numbers at the top give the buoyant density values (g/cm^3). Open circles: radioactivity; filled circles: UV light absorption. Subcellular fractions were obtained by consecutive stepwise 10 min centrifugation at certain rotor speeds of homogenate of coleoptiles unlabeled (A) or labeled (B) with [^{3}H-methyl]thymidine. A: Fractions represent particles sedimented under condition: (a) $300 \times g$; (b) $600 \times g - a$; (c) $1090 \times g - a + b$; (d) $1700 \times g - a + b + c$; (f) $17400 \times g - a + b + c + d + e$. B: Fractions isolated under condition: (a) $1090 \times g$; (b) $3070 \times g - a$; (c) $4350 \times g - a + b$; (d) $7740 \times g - a + b + c$; (e) $14600 \times g - a + b + c + d$.

up with cytoplasm containing mitochondria. We observed how these bodies may be formed in intact cells: tonoplast forms the protrusions filled with cytoplasm and certain mitochondria translocate into them (Fig. 2). The outgrowths formed are then separated and, as a result, the suspended polymorphous cytoplasmic bodies bound up with a membrane and containing mitochondria appear in vacuoles. Thus, the electron microscopy study demonstrated that the in vitro iso-

lated unusual fast sedimenting particles (Fig. 1) are not artifacts but they do exist in an intact plant. They are most common for cells of old coleoptiles. In young coleoptiles (3-day-old seedlings) these bodies were observed very rarely only in the cells of the extreme apical coleoptile part that is the most senile part of the organ. Up to now such cytoplasmic bodies were not isolated.

H-mtDNA ($\rho = 1.718$ g/cm^3) practically free of nDNA ($\rho = 1.700$ g/cm^3) is present mainly in the particle fraction sedimented at $1700 \times g$ with removed nuclei (Fig. 3A, d). After incubation of seedlings in the presence of [^{3}H]thymidine the peak of radioactivity incorporated into DNA with buoyant density corresponding to that of newly formed H-mtDNA was observed in the fast sedimenting (about $1090 \times g$ and $3070 \times g$) particles (Fig. 3B, a,b). There is no such peak in the traditional mitochondrial fraction sedimented at more than $3070 \times g$ (Fig. 3B, c,d,e) and no significant radioactivity in the CsCl density gradient zone with $\rho = 1.718$ g/cm^3 was observed. Therefore, synthesis of H-mtDNA is located in the fraction of vesicles or vesicular but not free mitochondria. By the most detailed fractionation of subcellular particles and accurate determination of the DNA content and radioactivity in the fractions isolated it was found that H-mtDNA is most actively synthesized in particles (vesicles) sedimented at $1700 \times g$. The yield of the $1700 \times g$ fraction (by protein) from coleoptile is strongly increased with age (data not shown).

Similarly to free mitochondria the vesicles consume oxygen intensively. The respiration rate of vesicular and free mitochondria is rather similar; it corresponds to 145 ± 15 and 160 ± 20 µM O$_2$/min/mg protein, respectively.

Inhibitors of the respiration chain effectively decreased the respiration rate of mitochondria of both kinds. For example, myxothiazol (Q-cycle inhibitor) in a concentration of 2×10^{-6} M suppressed the respiration of vesicles and free mitochondria by 77% and 67%, respectively. The system of the CN-
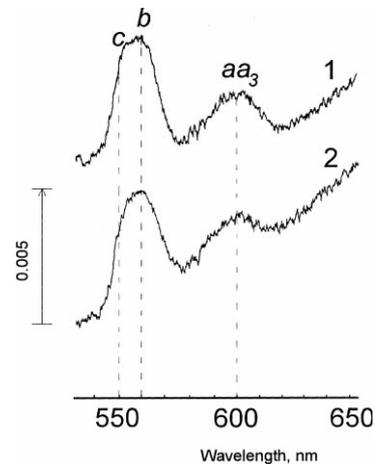
Fig. 4. Spectra of cytochromes isolated from vesicles and mitochondria of 8-day-old coleoptiles of wheat seedlings. Vesicles were obtained by 10 min centrifugation of coleoptile homogenate at $2600 \times g$ after removal of the $600 \times g$ fraction. Free mitochondria were sedimented at $17400 \times g$ for 10 min after removal of particles by centrifugation at $4350 \times g$. Cytochromes were reduced with respiration substrate (succinate) and dithionite in the presence of 2 mM potassium cyanide and 2 mM butylhydroxamate. Curve 1: cytochromes from vesicles; 2: cytochromes from mitochondria (2). Absorption is represented in optical units. Abscissa: wavelength, nm.

insensitive (alternative) oxidation way was inhibited by 2 mM butylhydroxamic acid in vesicles and free mitochondria by 18% and 11% respectively. Cyanide (2 mM) added after myxothiazol additionally decreases the residual respiration rate of both fractions by 15–20%. The content and composition of cytochromes in vesicles and mitochondrial fraction isolated from coleoptiles of 8-day-old seedlings are similar (Fig. 4). Fraction of vesicles contains about 2-fold more Ca^{2+} (195 nmol/mg protein) compared with free mitochondria (83 nmol/mg protein). It may be due to the fact that vesicles are localized inside the vacuole, which could be a sort of depot of excessive Ca^{2+}. It also cannot be ruled out that products of pronounced apoptotic degradation of nuclear DNA in these aging coleoptiles [4,13] may accumulate in such vacuoles and from there the pool of precursors for superproduction of H-mtDNA in vesicles.

Thus, in addition to free mitochondria some population of mitochondria in plants may exist and function in special suspended vesicles formed by vacuolar membranes of undividing cells. This population of mitochondria has never before been observed in plant cells. The principal specific feature of mitochondria of these vesicles in senescent cells only is a strong synthesis of heavy GC-enriched mitochondrial DNA (H-mtDNA, GC = 56–58%). Synthesis of H-mtDNA starts at a certain moment in plant ontogenesis during aging of the individual organ and results in superproduction of H-mtDNA [13]. The biological meaning of intensive H-mtDNA synthesis in aging plant organs is still unclear.

Anyhow, aging associated with apoptosis in wheat coleoptile cells seems to be accompanied by some maturation or probably formation of such mitochondria that intensively synthesize H-mtDNA in special vesicles located in vacuoles. Unfortunately, the true functional role of H-mtDNA and vesicles described is still unknown. It may be assumed that the new form of cytoplasm organization (vesicles) observed may to some extent discriminate between mitochondria and preserve their structure, activity and, in particular, ability to synthesize H-mtDNA in the process of cell aging and death.

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