**Introduction**

Eukaryotic cells replicate by cell division preceded by DNA synthesis. To preserve the genetic composition of self-replicating organelles in a cell, these organelles and their DNAs must replicate in step with cell division, indicating the presence of some control mechanism between the cell nucleus and the organelles.

Organelle DNAs of both mitochondria and plastids are associated with specific proteins to form compact structures in situ. These compactly organized DNA-protein complexes are called mitochondrial nuclei (nucleoids) and plastid nuclei (nucleoids). The number, structure, distribution, and protein composition of plastid nuclei vary considerably during plastid differentiation and development (Kuroiwa et al., 1981; Hansmann et al., 1985; Miyamura et al., 1986; Reiss and Link, 1985; Nemoto et al., 1988, 1990). Microautoradiographs of the root meristem of *Pelargonium zonale* and *Arabidopsis thaliana* have revealed that mitochondrial DNA synthesis is not correlated with cell division, and morphological changes of mitochondrial nuclei are accompanied by changes in the rate of mitochondrial DNA synthesis (Kuroiwa et al., 1992; Fujie et al., unpublished data). However, it is difficult to analyze the molecular mechanisms of multiplication and distribution of these organelles in higher plants in vivo. Using cultured cells growing under controlled conditions, rather than the tissue or organs of higher plants in vivo, should permit such analysis at the molecular level. In tobacco cultured cells (BY-2), preferential synthesis of plastid DNA (ptDNA) and multiplication of plastids were observed following the renewal of medium (Yasuda et al., 1988). However, no direct information has been available on organelle DNA synthesis in mitochondria and plastids, and especially proliferation of mitochondria in higher plants in vitro, because it has been difficult to distinguish between the synthesis of mitochondrial DNA...
(mtDNA) and ptDNA by microautoradiography. In the present study, we were able to visualize DNA synthesis in the nuclei of both organelles individually by indirect immunofluorescence microscopy of Technovit sections stained with anti-BrdU antibody.

The mechanisms of multiplication and distribution of mitochondria and plastids are generally explained in terms of the preservation of the genetic composition in step with cell division. In this study, therefore, we first examined whether the behavior of both organelles in the tobacco cultured cell line reflected that in the in vivo meristematic cells of root apical meristem. The cytological and molecular biological analyses showed preferential organelle DNA synthesis unconnected to the DNA synthesis of the cell nucleus, both in vivo and in vitro.

**Materials and methods**

**Plant materials**

To prepare the root meristem, seeds of tobacco cv. Bright Yellow 2 were soaked with water on two layers of filter paper at 25°C in the light for 3 days. Tobacco cultured cells, line BY-2 derived from *Nicotiana tabacum* L. cv. Bright Yellow 2, were propagated on a gyratory shaker at 130 revs/min at 26°C in the dark as previously described (Yasuda et al., 1988; Nemoto et al., 1988). To maintain the cultured cell line, 2 ml of stationary phase cells were transferred weekly into 95 ml of fresh Linsmaier and Skoog (1965) medium, as modified by Nagata et al. (1981). For sampling or observation of the cultured cells, 2 ml of completely stationary phase cells (9-10 days old) were transferred into the fresh medium. The growth of the BY-2 cells was monitored by counting the numbers of cells per ml under a microscope.

**Labeling cells with 5-bromodeoxyuridine (BrdU) and indirect immunofluorescence microscopy with anti-BrdU antibody**

Three-day-old seedlings with primary roots approximately 3 mm long were pulse-labeled for 1 h with 100 µM BrdU, a halogenated pyrimidine analogue of thymidine, and with additional 10 µM 5-fluorodeoxyuridine to enhance the incorporation of BrdU. Roots of seedlings were fixed with 4% paraformaldehyde and embedded in Technovit 7100 resin (Kulzer and Co., GmbH, Wehrheim) as previously described (Kuroiwa et al., 1990). Thin serial sections were cut with a glass knife on an MT-6000 XL Ultramicrotome (RMC-Eiko Co., Kawasaki), and stuck to a coverslip by 70% ethanol. Cultured cells were pulse-labeled with BrdU for 1 h as above at 0, 3, 6, 12, 24, 72, 120, 168 and 216 h after the subculture. The labeled cultured cells were treated with Pectolyase Y23 and Cellulase YC (both from Seishin Pharmaceutical Co., Tokyo) to remove cell walls (Okada et al., 1986), fixed with Carnoy’s solution (25% glacial acetic acid in ethanol) for 30 min, and stuck to a coverslip. The samples on coverslips were double-stained with 4’,6-diamidino-2-phenylindole (DAPI) and indirect immunofluorescence stained with anti-BrdU monoclonal antibody (Becton Dickinson Immunocytometry Systems Co., California) and fluorescein isothiocyanate (FITC)-conjugated second antibody (goat anti-mouse Igs, Tago Inc., California) as described previously (Munaut et al., 1990). Labeling patterns of organelles were observed under an epi-fluorescence microscope (Olympus BHS-RFK, Olympus Optical Co., Tokyo). Photographs were taken at magnifications of ×50 and ×330 on 35 mm Kodak Ektachrome 400 film (Eastman Kodak Co., New York).

**Morphological observation of organelles and organelle nucleus**

The cell walls of cultured cells were removed enzymatically as described above. Released protoplasts were fixed and stained in a drop of 1% glutaraldehyde and 1 µg/ml of DAPI in TAN buffer (17% (w/v) sucrose, 20 mM Tris-HCl (pH 7.65), 0.5 mM EDTA, 1.2 mM spermidine, 7 mM 2-mercaptoethanol and 0.4 mM PM5F). The fixed protoplasts were burst by applying slight pressure to the coverslips placed on top of them. They were then observed under an epi-fluorescence microscope equipped with a phase-contrast objective. Photographs were taken at a magnification of ×330 on 35 mm Fuji Neopan 400 film (Fuji Photo Film Co., Tokyo).

**Quantitative analysis of organelle DNA by Southern blot hybridization**

Total DNA from tobacco cultured cells was isolated by a method described previously (Corriveau et al., 1990). After digestion of total DNA by HindIII, the restriction fragments were separated in a 1% agarose gel by electrophoresis for 1.5 h at 50 V. Blotting to nylon membrane filters and hybridization were performed by the method of Southern (1975). For quantitative loading, preliminary Southern blot hybridization experiments were performed with a probe of cloned nuclear-DNA fragment, containing rice nuclear genes for both 26 S and 18 S rRNA, donated by Dr. Y. Sano (National Institute of Genetics, Mishima). To define the invariability of the copy number of rRNA genes during the culture, another hybridization experiment was conducted with a probe of the psaH gene, supplied by Dr. Obokata (Hokkaido University, Sapporo). About 5 µg of HindIII-digested total DNAs, which were standardized to contain equal volumes of total nuclear DNA by preliminary hybridization experiments, were loaded and then hybridized with two probes of plastid and mitochondria. Labeling of probes with [α-32P]dCTP was performed with a DNA labeling kit under the conditions recommended by the manufacturer (Amersham International PLC, Amersham). For the detection of ptDNA, a 2.5 kb cloned fragment containing the psaA and psaB genes of tobacco ptDNA was used as a probe. For mtDNA, a 2.0 kb cloned fragment containing the entire coding region of rice coxl gene (Kadowaki et al., 1989) was used. The intensity of hybridization signals was estimated by a densitometer (Densitograph AE-6900, Atto Co., Tokyo).

**Results**

**Organelle DNA synthesis in root apical meristem**

DNA synthesis in the root apex of tobacco was examined by staining with DAPI and indirect immunofluorescence staining with anti-BrdU antibody after pulse-labeling with BrdU for 12 h (Fig. 1a-f). There were four types of cells in the root apex, based on the labeling patterns of organelles and cell nuclei. The first type, in which only organelles were labeled with BrdU and identified by green-yellow fluorescence of FITC of second antibody to anti-BrdU antibody, was observed primarily in the neighborhood of the quiescent center (Fig. 1c and d). The second type (both organelles and the cell nucleus were labeled) was observed above the quiescent center (Fig. 1c and d). The third type, in which only the cell nucleus was labeled, was observed in the root apical meristem above the quiescent center (Fig. 1e and f). The last type, in which neither of the organelles nor the cell nucleus was labeled, was observed in root cap cells (Fig. 1a and b). In the root apex of tobacco, cell
nucleus DNA synthesis and cell division occurred actively throughout the entire root meristem from about 130 µm to 400 µm from the root tip, as is typical in root meristems of dicotyledons. In contrast, organelle DNA synthesis occurred mainly in a specific region neighboring the quiescent center, about 130 µm from the root tip. Furthermore, morphological changes of mitochondrial nuclei, from long ellipsoids to granules, were observed as the distance of cells from the quiescent center increased.

*Morphological changes of mitochondrial nuclei and plastid nuclei in cultured cell line*

The above results suggest that organelle DNA synthesis is not related to cell division. However, it is difficult to analyze further the behavior of organelles during cell division at the molecular level by such observations in vivo. Cultured cells are more suitable for analysis biochemically or molecular biologically than the tissue or organs of higher plants in vivo. The tobacco cultured cells of BY-2 multiplied about 50-fold in a week when 2 ml of suspension of 9-day-old cell clusters was transferred to fresh medium (Fig. 2). The mitotic indices from 48 to 120 h after the transfer were 4-5%. The 4-day period from about 24 h to 120 h after the transfer was determined to be the logarithmic growth phase and the mean duplication time was estimated to be about 18.8 h. The period beyond 120 h was determined to be the stationary phase. Both organelles and their nuclei were examined morphologically by phase-contrast microscopy and DAPI-fluorescence microscopy at 0, 24, 72, 120, 168 and 216 h after the transfer (Fig. 3). These morphological changes are illustrated in Fig. 7, below. At the time of transfer, spherical plastids and elongated mitochondria were observed but their fluorescence was quite weak (Fig. 3a, b, c and d). Within 24 h after the transfer, plastids were extremely elongated and string-like. The number of pt-nuclei apparently increased to the maximum and then fluorescence became stronger (Fig. 3e, f, g and h). Small granular mitochondria with relatively intensely fluorescent nuclei were also observed during this time. After the third day, the number and fluorescence intensity of pt-nuclei decreased gradually, and elongated plastids were divided into small pieces (Fig. 3i-x). However, the number and fluorescence intensity of mt-nuclei increased until the third day and then decreased slowly. After 120 h, mitochondria were growing longer gradually (Fig. 3m-r).

Changes of DNA content of organelles during cell culture

The above results indicate that morphological changes of organelles occur during the proliferation of cultured cells and in the in vivo root meristem. In the root meristem, the synthesis of mtDNA occurred only in mitochondria with long ellipsoidal mt-nuclei just above the quiescent center. Since morphological changes of both organelles and organelle nuclei occurred along with the proliferation of cells in the culture, the DNA content of organelles must also change during these processes. To confirm the above hypothesis, total DNAs were isolated from cultured cells at 0, 12, 24, 48, 72, 120, 168, 216, 264 and 312 h after the transfer, and analyzed by quantitative Southern hybridization (Figs 4 and 5). *HindIII* digestions of total DNAs, which contained equal amounts of nuclear DNA determined by hybridization signals from a nuclear probe of rRNA genes (7.0 kb bands in Fig. 4a), were loaded and then hybridized with two probes specific for ptDNA and mtDNA. The possibility that the copy number of rRNA genes varied during the culture was eliminated by an additional hybridization experiment with a probe of nuclear single-copy gene *psaH* (data not shown). The probe specific for ptDNA hybridized into two DNA fragments consisting of a major 3.6 kb band and a minor 2.3 kb band (middle and lower bands in Fig. 4a). The probe specific for mtDNA hybridized into a DNA fragment of 4.1 kb (Fig. 4b). The hybridization signal intensities of both organelles changed over the time course of the culture as compared to the standardized hybridization signals of cell nuclear DNA (upper bands in Fig. 4a). Hybridization signal intensities, which indicated the relative amount of organelle DNAs per cell, increased suddenly at 12 h after the transfer, reached a peak between 24 and 72 h, and then gradually subsided (Fig. 5). These results suggest that the amount of organelle DNA per cell increased suddenly, 3.2- and 4.4-fold in plastids and mitochondria, respectively, due to preferential organelle DNA synthesis without cell division within 24 h after the transfer, and then decreased gradually according to the distribution of organelles by cell division.

Organelle DNA synthesis in a cultured cell line

Although the above results indicate that organelle DNA is synthesized immediately following the renewal of medium, they do not directly show that preferential organelle DNA synthesis occurs prior to cell nucleus DNA synthesis during culture. To confirm the synthesis of organelle DNA prior to that of cell nuclei, cultured cells pulse-labeled with BrdU were examined by immunofluorescence microscopy with antibody against BrdU. Preferential synthesis of organelle DNA following medium renewal was observed in individual cells of BY-2 by indirect immunofluorescence staining with anti-BrdU antibody as well as in the root meristem. The cells were classified into four types according to the labeling patterns of organelles and cell nuclei (Fig. 6). In
Fig. 3. Epi-fluorescence (a, c, e, g, i, k, m, o, q, s, u and w) and phase-contrast (b, d, f, h, j, l, n, p, r, t, v and x) images of mitochondria, plastids and cell nuclei in BY-2. Cells harvested at 0 (a, b, c and d), 24 (e, f, g and h), 72 (i, j, k and l), 120 (m, n, o and p), 168 (q, r, s and t) and 216 (u, v, w and x) h after transfer into fresh medium were treated with cellulolytic enzymes to remove cell walls, and observed under an epi-fluorescence microscope after staining with DAPI. Small pictures (right two files: bar, 10 µm) show parts of the cytoplasm of the large pictures (left two files: bar, 10 µm) at higher magnification. Small arrowheads and large arrowheads show mitochondria and plastids, respectively.
Preferential DNA synthesis in *N. tabacum*

the cells of the first type, neither organelles nor the cell nucleus was labeled (N−O−; Fig. 6a and b). In the second type of cells, only organelles were labeled (N−O+; Fig. 6c and d). Both organelles and the cell nucleus were labeled in the third type of cells (N+O+; Fig. 6e and f). Only the cell nucleus was labeled in the last type of cells (N+O−; Fig. 6g and h). The proportions of these four different types of cultured cells were followed during culture (Table 1), and the changes in frequencies of labeled organelles and cell nuclei are shown in Fig. 7. At the time of the transfer, 99% of the cells were type of N−O−; and only 1% of the cells were N−O+. The frequency of N−O+ type cells increased immediately, reached peaks of 56-63% after 6 to 72 h, and decreased suddenly at 120 h after the transfer. N+O− cells were observed after 6 h and reached a maximum frequency of 40% at 72 h after the transfer. Only a few N+O− cells were observed after 24 h, and their frequency reached a peak of 24% at 120 h after the transfer. Table 1 and Fig. 7 clearly show that the DNA synthesis in organelles preferentially occurred ahead of that in cell nuclei. The frequency of labeled organelles suddenly increased from 1 to 93% within only 24 h and immediately decreased after 72 h. Although the frequency of labeled organelles stayed within the range of 93-96%, the intensity of that labeling slightly decreased after 24 h. On the other hand, the frequency of labeled cell nuclei increased 5-33% from 6 to 24 h after that of organelles and gradually increased to 48% at 120 h after the transfer, when the frequencies of labeled organelles and cell nuclei gradually decreased. These results clearly show that organelle DNAs are synthesized preferentially ahead of cell nuclear DNA just after the renewal of medium.

**Discussion**

In the present study, mtDNAs of root meristem cells were found to be synthesized in a specific region neighboring the quiescent center. In this region, DNA synthesis of cell nuclei is observed at low frequency by fluorescence staining with DAPI and indirect immunofluorescence staining with anti-BrdU antibody and FITC-conjugated second antibody in a thin section of BrdU-labeled root meristem of tobacco embedded in Technovit 7100 resin. Essentially the same phenomenon of preferential synthesis of organelle DNA was observed in cultured cells.

**Table 1. Change in the proportion of four different cell types classified by their labeling patterns**

<table>
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<tr>
<th>Labeling pattern</th>
<th>0</th>
<th>3</th>
<th>6</th>
<th>12</th>
<th>24</th>
<th>72</th>
<th>120</th>
<th>168</th>
<th>216</th>
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<tr>
<td>N−O−</td>
<td>99</td>
<td>68</td>
<td>36</td>
<td>12</td>
<td>5</td>
<td>0</td>
<td>35</td>
<td>76</td>
<td>98</td>
</tr>
<tr>
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<td>32</td>
<td>60</td>
<td>59</td>
<td>63</td>
<td>56</td>
<td>17</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>N+O+</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>29</td>
<td>30</td>
<td>40</td>
<td>24</td>
<td>10</td>
<td>0</td>
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<td>11</td>
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</tbody>
</table>

**Fig. 4.** Quantitative Southern blot hybridization patterns of tobacco total DNAs extracted according to the time course after transfer. *Hind*III-digested total DNAs were measured in contrast with the hybridization signals of cell nuclear DNA (7.0 kb bands in a). The probe specific for plastid DNA hybridized to two DNA fragments of a major 3.6 kb band and a minor 2.3 kb band (a), and the probe specific for mitochondrial DNA hybridized to a DNA fragment with 4.1 kb (b).

**Fig. 5.** Relative changes in the DNA levels of both mitochondria (●) and plastids (○) per cell nucleus in tobacco BY-2 cells during the culture. Relative values for organelle DNA amounts were determined by measuring the intensities of hybridization signals with a densitometer.

**Fig. 6.** DNA synthesis and morphological changes of organelles and cell nucleus during cell culture. Upper drawings indicate morphological changes of plastid (pt) and mitochondrion (mt). Black dots show organelle nuclei and open circles show starch grains. Filled areas under the drawings indicate the activity of DNA synthesis of organelles (pt & mt) and cell nucleus (N). Thickness of areas reflects percentage of cells labeled with BrdU.

**Fig. 7.** DNA synthesis and morphological changes of organelles and cell nucleus during cell culture. Upper drawings indicate morphological changes of plastid (pt) and mitochondrion (mt). Black dots show organelle nuclei and open circles show starch grains. Filled areas under the drawings indicate the activity of DNA synthesis of organelles (pt & mt) and cell nucleus (N). Thickness of areas reflects percentage of cells labeled with BrdU.
DNA is found in the root meristem of *Pelargonium zonale* and *Arabidopsis thaliana* (Kuroiwa et al., 1992; Fujie et al., unpublished data). These results emphasize that DNA synthesis of organelles is unconnected to cell division. Moreover, in tobacco cultured cells, quantitative Southern blot analysis and immunofluorescence microscopy with anti-BrdU antibody show that large amounts of preferential mitochondrial and plastid DNA synthesis occur prior to cell nuclear DNA synthesis just after stationary phase cells are transferred to fresh medium (Fig. 7). Organelle DNA synthesis of in vitro cultured cells resembles that of in vivo root meristematic cells. However, the patterns of morphological changes of both mitochondria and plastids seem to depend upon the different growth conditions. In tobacco root meristem, the mitochondrial nuclei changed from long ellipsoids with a high frequency of DNA synthesis to granules with a low frequency of DNA synthesis as the distance from the quiescent center increased (Fig. 1). The same pattern was also observed in the root meristem of *P. zonale* (Kuroiwa et al., 1992). However, in the cultured cell line BY-2, elongated, ellipsoidal mitochondria were observed only in stationary phase cells 120 h following transfer, instead of during the logarithmic growth phase of mtDNA (Figs 3 and 7). mtDNA synthesis was not observed in these long ellipsoidal mitochondria. On the other hand, the granular mitochondria present in the logarithmic phase, vigorously synthesized mtDNA. The frequency of mtDNA synthesis increased gradually until 72 h after the transfer and then decreased slowly. The mitochondria in the cultured cells do not completely resemble those in the root meristem and are more similar to those in more differentiated meristematic cells. The morphological difference between the mitochondria in the cultured cells and those in the root meristem might aid in understanding the relationship between cell differentiation and morphological changes of mitochondria.

The morphological changes of plastids in the cultured cells were more obvious than those of mitochondria (Figs 3 and 7). These changes were not observed in the root meristem. The spherical plastids became extremely elongated and string-like during the first 24 h after the renewal of medium. The elongated plastids broke into small pieces after the third day (Fig. 7). A similar observation was reported in BY-2 cells subcultured weekly (Yasuda et al., 1988). In this case, the plastid division occurred between 24 and 48 h after the subculture. This is also supported by the observation that the surface area of a dumbbell-shaped plastid is reduced to half of its original value during this time. However, the two culture systems differ in that the extremely elongated string-shaped plastids were not observed in BY-2 subcultured weekly. The string-shaped plastid seems to be observed only when completely stationary phase cells are transferred to fresh medium as a 9-day subculture. Miyamura et al. (1990) observed ellipsoid and elongated sausage-shaped plastids, in which several spherical and cup-shaped pt-nuclei were located side by side, in the leaf base of *Triticum aetivum*. They suggested that the division of plastids occurs in this area. From the results presented here, plastid elongation seems to occur along with the preferential synthesis of ptDNA. This preferential synthesis may be in preparation for several plastid divisions following multiple cell divisions without organelle DNA synthesis.

The preferential synthesis of organelle DNA prior to that of cell nuclear DNA, both in vivo and in vitro, suggests that organelle DNA is synthesized and stored in large amounts to prepare for the following multiple cell divisions without organelle DNA synthesis. This behavior of organelles was observed in both in vivo and in vitro systems. In the in vitro culture system, the preferential synthesis of organelle DNA is apparently induced by the stimulation of medium renewal. Moreover, the multiplication and differentiation of organelles can also be controlled artificially by modifying the medium. For example, when BY-2 cultured stationary phase cells were transferred into medium with additional benzyladenine, the proplastids were converted to amyloplasts and this was accompanied by changes in the transcriptional activities of plastid genes (Sakai et al., 1992). A culture system like BY-2 offers a good in vitro experimental system in which to study the molecular mechanism of replication, multiplication and distribution of organelles.

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