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Behavior of organelles and their nucleoids in the shoot apical meristem during leaf development in *Arabidopsis thaliana* L.

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Abstract. The behavior of organelle nucleoids and cell nuclei was studied in the shoot apical meristem and developing first foliage leaves of *Arabidopsis thaliana*. Samples were embedded in Technovit 7100 resin, cut into thin sections and stained with 4′-6-diamidino-2-phenylindole to observe DNA. Fluorimetry was performed using a video-intensified microscope photon-counting system. The DNA content of individual mitochondria was more than 1 Mbp in the shoot apical meristem and the young leaf primordium, and decreased to approximately 170 kbp in the mature foliage leaf. In contrast, the DNA content of individual plastids was low in the shoot apical meristem and increased until day 7 after sowing. Application of 5-bromo-2′-deoxyuridine, an analogue of thymidine, was usesd to investigate DNA synthesis in situ. The activities of DNA synthesis in the mitochondria and plastids changed according to the stage of development. Mitochondrial DNA was actively synthesized in the shoot apical meristem and young leaf primordia. This strongly suggests that the amount of mitochondrial DNA per mitochondrion, which has been synthesized in the shoot apical meristem and young leaf primordium, is gradually reduced due to continual divisions of the mitochondria during low levels of mitochondrial DNA synthesis. Synthesis of DNA in the plastid became active in the leaf primordia following DNA synthesis in the mitochondria, and the small plastids were filled with large plastid nucleotids. This enlargement of the plastid nucleoids occurred before the synthesis of ribulose-1,5-bisphosphate carboxylase/oxygenase and the development of thylakoids.

Key words: *Arabidopsis* – Leaf development – Mitochdonrion – Organelle – Plastid – Shoot apical meristem

Introduction

Higher plants have two apical meristems, a root apical meristem and a shoot apical meristem, both of which supply cells to tissues. These cells in the meristem maintain embryonic characteristics and continue to divide throughout the lifespan of the plant. The structure and function of plant cells change during their development from embryonic cells in the meristem to differentiated cells, such as mesophyll, in foliage leaves. In particular, the structures of two energy-converting organelles, mitochondria and plastids, which contain their own genomes, change drastically. Plastids change their configurations, including their volume and thylakoid system, as a result of differentiation (Kirk and Tilney-Bassett 1978). Mitochondria change their configurations as well (Bendich and Gauriloff 1984).

In addition to the changes in the volume and internal membranes of the mitochondria and plastids, the amount of DNA of individual organelles also changes drastically. The numbers of nucleoids in each organelle and the amount of DNA in each nucleoid change during development (reviewed in Possingham and Lawrence 1983; Kuroiwa 1982; Kuroiwa 1991). In the root apical meristem, mitochondrial DNA (mtDNA) has been shown to be actively synthesized in the lower part of the meristem just above the quiescent center before the activation of DNA synthesis in cell nuclei (Kuroiwa et al. 1992; Suzuki et al. 1992; Fujie et al. 1993a). Organelle DNA has even been shown to be synthesized in the quiescent center of *Arabidopsis* seedlings (Fujie et al. 1993b). Therefore, mtDNA is amplified and forms large mitochondrial nucleoids (mt-nucleoids) in the lower part of the root apical meristem. Synthesis of DNA in the mitochondria becomes inactive before the cessation of cell proliferation, and the large amount of

Abbreviations: BrdU = 5-bromo-2′-deoxyuridine; DAPI = 4′-6-diamidino-2-phenylindole; $DiOC_6$ = 3,3′-dihexyloxacarbocyanine; mtDNA = mitochondrial DNA; mt-nucleoid = mitochondrial nucleoid; ptDNA = plastid DNA; pt-nucleoid = plastid nucleoid; Rubisco = ribulose-1,5-bisphosphate carboxylase/oxygenase

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mtDNA in the large mitochondria is divided among daughter mitochondria by repeated divisions to form the smaller mt-nucleoids which are observed in mature root cells (Kuroiwa et al. 1992; Fujie et al. 1993a). Such specific amplification and distribution of the mtDNA have also been observed in egg cells of *Pelargonium zonale* (Kuroiwa and Kuroiwa 1992). It is possible that a similar amplification of mtDNA occurs in the shoot apical meristem. The abundance of specific mitochondrial genes per cell of wheat leaf decreases during cellular differentiation (Topping and Leaver 1990). Moreover, the level of mtDNA in each type of tissue changes during development (Lamppa and Bendich 1984). However, as far as we know, there has been no report of the DNA content in each mitochondrion in the shoot apical meristem, and the DNA content of individual mitochondria during leaf development has not been studied thoroughly. It is difficult to observe and quantify mtDNA in tissue because the volume of mitochondria and the amount of DNA in individual mitochondria in foliage leaves are quite small compared to those of plastids. However, it has recently become possible to observe a very small amount of DNA in tissue by using Technovit 7100 resin (Kuroiwa et al. 1990).

In contrast to mtDNA, the dynamics of plastid DNA have been studied by many investigators. The DNA content in each plastid changes according to its developmental stage (Scott and Possingham 1980; Kuroiwa et al. 1981; Boffey and Leech 1982; Lawrence and Possingham 1986 and reviewed by Possingham and Lawrence 1983; Kuroiwa 1991). It has also been observed in wheat leaves that pt-nucleoids (plastid nucleoids) change shape as they develop (Sellden and Leech 1981; Miyamura et al. 1990). In etioplasts of monocotyledons, the pt-nucleoids become ring-shaped structures (Hashimoto 1985; Miyamura et al. 1986). However, the behavior of pt-nucleoids and the changes in the shape of the nucleoids in dicotyledons have not been completely elucidated in the shoot apical meristem and young leaf primordia. Moreover, the role of the amplified ptDNA in individual plastids has not yet been clarified.

In this study, the development of mitochondria and plastids was studied in the shoot apical meristem and the first foliage leaves of *Arabidopsis* seedlings. We observed the relation between the configurations of these organelles and the features of their nucleoids by staining sections with 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and 4′-6-diamidino-2-phenylindole (DAPI) simultaneously. Changes in the DNA contents of the organelles were measured directly using microphotometry, and DNA synthesis in the organelles was studied by applying 5-bromo-2′-deoxyuridine (BrdU) during leaf development. The relationship between pt-nucleoids and thylakoids was also investigated to determine the role of thylakoids in pt-nucleoid dynamism. The expression of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) was investigated by immunostaining.

Materials and methods

Culture of A. thaliana, and fixation and embedding of samples. Seeds of *Arabidopsis thaliana* (L.) ecotype Colombia were sown on rockwool and incubated with a solution that contained 0.1% Hyponex (Murakami bussan Co., Tokyo, Japan) under continuous light at 25° C. The seedlings were harvested from 3 to 10 d after sowing, and the shoot apical meristems and the first foliage leaves were used for each experiment. Cells from the distal portion of the palisade tissue were used for microscopic observations of the first foliage leaves. The samples were fixed for 12 h at 4° C in 4% paraformaldehyde which was buffered with sodium cacodylate at pH 7.0, dehydrated through an ethanol series and then embedded in Technovit 7100 resin (Kulzer and Co., Wehrheim, Germany; Kuroiwa et al. 1990). Thin serial sections (0.5–1.0 μm thick) were cut with a glass knife on an MT-7 Ultramicrotome (RMC-Eiko Corp., Kawasaki, Japan). Sections were placed on a drop of distilled water on a cover slip and air-dried (Kuroiwa et al. 1990).

Fluorescence-microscopic observation of DNA. For observation of DNA, sections were stained with 1 $\mu g \cdot ml^{-1}$ DAPI in TAN buffer (0.5 mM EDTA, 1.2 mM spermidine, 0.05% 2-mercaptoethanol, 10 mM Tris-HCl at pH 7.6; Nemoto et al. 1988) and/or 100 $\mu g \cdot ml^{-1}$ $DiOC_6$ in ethanol. Stained samples were then observed under an Olympus BHS-RFC epifluorescence microscope (Kuroiwa et al. 1986, 1990). Photographs were taken at magnifications of ×66, ×330 and ×500 on 35 mm Fuji Neopan 400 PRESTO film (Fuji photo film Ltd., Tokyo, Japan).

Estimation of organelle DNA content by fluorimetry. The amount of DNA in each mt-nucleoid was examined directly using a video-intensified microscope photon-counting system (VIMPCS; Hamamatsu Photonics Ltd., Hamamatsu, Japan) which was connected to an Olympus epifluorescence microscope BHS-RFC, as described previously (Kuroiwa et al. 1986). The fluorescence intensities of the samples were expressed as approximate multiples of that of T4 phage embedded in Technovit 7100 resin, which contains approximately 170 kbp DNA (Freifelder 1970). The arbitrary unit "T" was used to express the amount of DNA (Miyamura et al. 1986; Fujie et al. 1993a).

Mitochondria were small enough in the shoot apical meristems and the first foliage leaves for the amount of DNA in each of mitochondrion in a section (0.5–1.0 μm thick) to be considered to represent the entire DNA content of the mitochondrion. Since the axes of the plastids in the foliage leaves were much longer than the thickness of the section, the entire amount of DNA contained in each plastid was estimated by the following formulae:

$$Cp = Vp^*Cv, \quad VP = 4/3^*\pi^*(a/2)^*(b/2)^2$$

where Cp = content of DNA per plastid, Vp = volume of each plastid (assuming that the plastids are ellipsoid), Cv = DNA content per volume, a = major axis and b = minor axis.

The average content of DNA per volume (Cv) was determined for each plastid, by the following formulae

$$Cv = N^*Ac/Vc, \quad Vc = \pi^*(a/2)^*(b/2)^*d,$$

where N = number of spots of DAPI in each section of plastid, Ac = average DNA content per spot, Vc = volume of each plastid section, and d = thickness of the section.

The pt-nucleoids of the plastids in the first foliage leaves were so complex and condensed from 3.5 to 5 d after sowing that the pt-nucleoids within one plastid could not be distinguished from each other. Thus, the DNA content of the pt-nucleoid was measured by assuming that each plastid contained only one nucleoid from 3 to 5 d after sowing (based on this assumption, N = 1 during this period).

Investigation of DNA synthesis using BrdU. For the study of DNA synthesis, BrdU was applied as an analogue of thymidine. Seedlings that were cultured for 3.5–7.5 d were incubated in an aqueous solution which contained 10 μM BrdU, 1 μM 5-fluorodeoxyuridine

(FldU) and 0.1% hypone for 12 h, and then embedded in Technovit 7100 resin as mentioned above. Sections (0.5–1.0 μm thick) were cut and attached to coverslips. Attached samples were treated with anti-BrdU mouse monoclonal antibody (Becton Dickinson immunochemistry Systems, San Jose, Calif., USA) and anti mouse-immunoglobulin goat antibody conjugated with fluorescein isothiocyanate (FITC; TAGO, Inc., Burlingame, Calif., USA) as described previously (Suzuki et al. 1992). Treated samples were observed under an epifluorescence microscope equipped with appropriate filters. No appreciable signals were observed on sections of seedlings which were cultured without BrdU as negative control (data not shown).

Localization of Rubisco. The expression of Rubisco during leaf development was investigated by immunostaining. The sections attached to the cover slips were incubated in 20% bovine serum albumin (BSA) for 30 min at 37° C, and then incubated with rabbit antiserum to pea Rubisco large subunit for 2 h at a 1:500 dilution in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) at 37° C. Coverslips were washed in PBS several times, incubated in 5% BSA for 5 min and incubated with FITC-conjugated goat anti-rabbit IgG (Cappel, Durham, N.C. USA) at a dilution 1:60 in PBS at 37° C. After two washing in distilled water, the coverslips were stained with 1 $\mu g \cdot ml^{-1}$ DAPI in TAN buffer. The treated samples were mounted in 50% glycerol which contained 1 $mg \cdot ml^{-1}$ *n*-propyl gallate, and were observed with an epifluorescence microscope equipped with appropriate filters. The specificity of the rabbit antiserum to the pea Rubisco large subunit was examined by immunoblotting and detecting the band corresponding to the Rubisco large subunit (data not shown).

Results

Growth of seedlings from 3–10 d after sowing. The first foliage leaves were visible 5 d after sowing and continued growing. The leaves expanded on day 10 (Fig. 1). Therefore, the development of the first foliage leaves in this study was observed from 3 to 10 d.

Organelles and organelle nucleoids in the shoot apical meristem. The features of the mitochondria and plastids were investigated in the shoot apical meristem and the DNA contents of these organelles were investigated in each layer of the meristem by microphotometry. Three superimposed cell layers, L1 (outermost layer), L2 (second layer) and L3 (innermost layer), could be recognized in the shoot apical meristem of *Arabidopsis* seedlings (Fig. 2a, b). In L1 and L2, the mt-nucleoids were thin and rope-shaped (Fig. 2b, c); they contained a large amount of DNA. The DNA content of individual mitochondria was 7.1±10.6 T (1.2 Mbp) and 5.4±3.9 T (0.9 Mbp) in L1 and L2, respectively (Table 1). The plastids were oval-shaped or thick rope-shaped and contained a small aggregation of pt-nucleoids. The DNA content of individual plastids was 25.3±19.2 T (4.3 Mbp) and 17.1±7.2 T (2.9 Mbp) in L1 and L2, respectively (Table 1). In L3, the mitochondria and the plastids were smaller than those in L1 and L2 (Fig. 2d). Each mitochondrion in L3 contained less DNA (3.0±3.1 T; 0.5 Mbp) than those in L1 and L2. Some of the plastids in L3 resembled those in L1 and L2, while others were more dilated. The DNA content of each plastid was 6.7±2.2 T (1.1 Mbp), which was less than that in L1 and L2. The DNA contents of the organelles in the shoot apical meristem were closer to those in the lower part of the root apical meristem than to those of mature foliage leaves (Table 1; Figs. 2, 3).

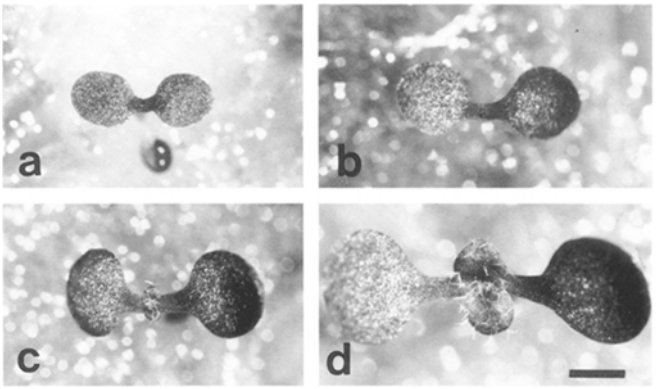


Fig. 1a–d. *Arabidopsis* seedlings cultured on rockwool. **a** Three days after sowing; **b** 5 d after sowing; **c** 7 d after sowing; **d** 10 d after sowing. At 3 d after sowing, cotyledons have elongated and leaf primordia are evident. Bar = 1 mm

Table 1. DNA contents of mitochondria and plastids in each of the cell layers (L1–L3) within the shoot apical meristem and in the root apical meristem of 3-d-old seedlings of *Arabidopsis*. Values are means ± SD; $n = 100$

DNA content of apical meristem (T)[a]				
	Shoot[b]			Root
	L1	L2	L3	(0–100 μm)[c]
Mitochondria (T)	7.1±10.6	5.4±3.9	3.0±3.1	4.5±3.1
Plastids (T)	25.3±19.2	17.1±7.2	6.7±2.2	13.1±7.5

[a] 1 T is approximately 170 kbp
[b] L1 is the outermost layer
[c] Distance from central cells

Behavior of mitochondria, plastids and their nucleoids in the first foliage leaves. Both the mitochondria and plastids changed their shapes and DNA contents during the development of the first foliage leaves. At day 3, large mitochondria were observed in the primordia of the first foliage leaves (Fig. 3A, B). Some of these large mitochondria contained rod-shaped mt-nucleoids, which resembled those in L1 and L2 of the shoot apical meristem (Fig. 2b). Plastids in this stage were small, ovoid or rod-shaped, and contained several pt-nucleoids.

At day 4, large rope-shaped mitochondria were still observed in the first foliage leaves. Plastids were ovoid and a few layers of thylakoids were observed by staining with $DiOC_6$ (Fig. 3C, D). In this stage, dumbbell-shaped plastids were also observed. This existence of dumbbell-shaped plastids suggests that the plastids were increasing rapidly during this stage. The DNA content of the plastids was larger than that in the shoot apical meristem. The pt-nucleoids were thick and rod-shaped, and occupied almost all of the stroma.

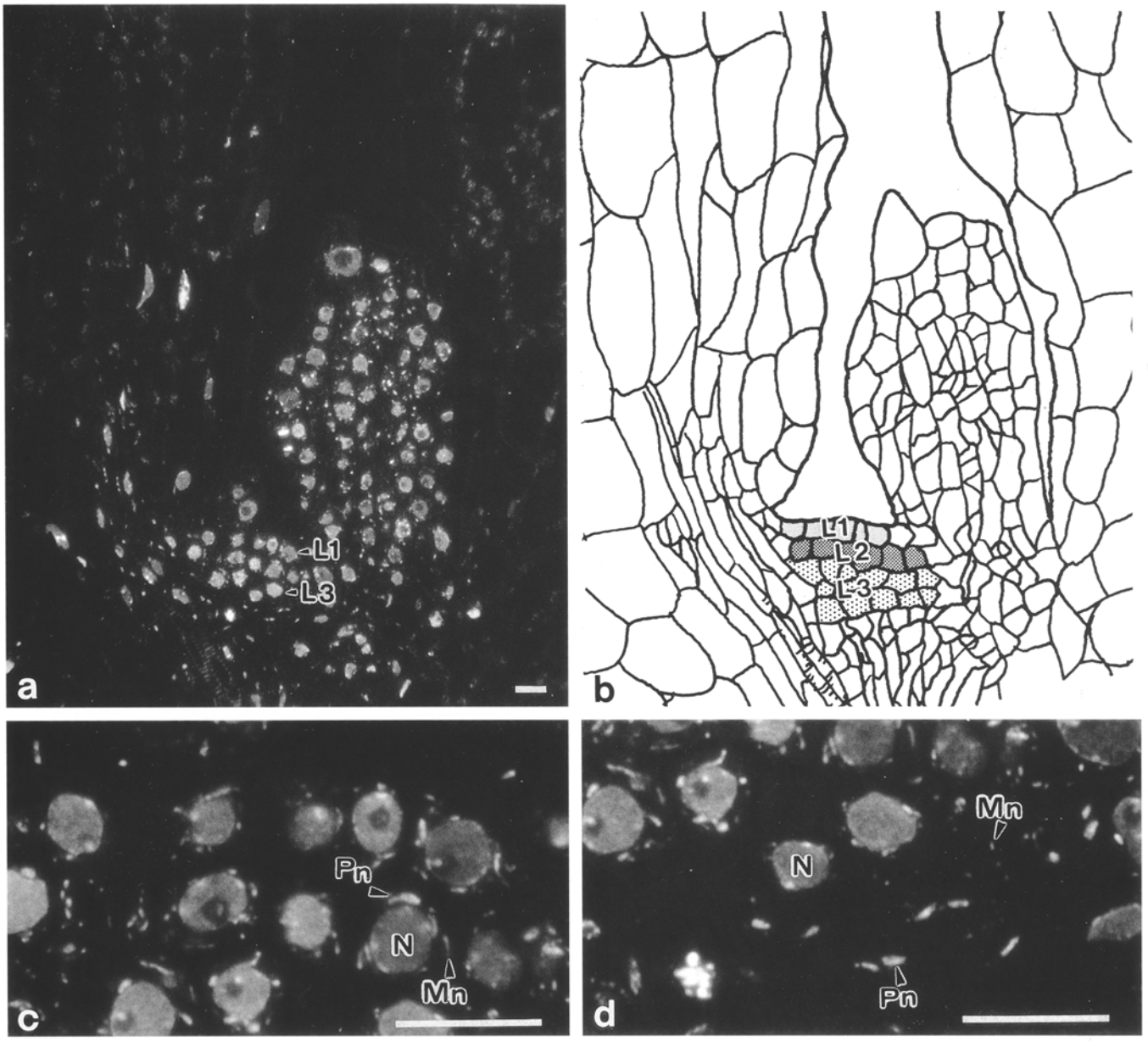


Fig. 2a–d. Details of the shoot apical meristem of 3-d-old *Arabidopsis* seedlings. The sections were stained with DAPI. Large bright spots indicate cell nuclei and small spots indicate organelle nucleoids. **a** Low magnification of a shoot apex. **b** Diagram of a shoot apex of *Arabidopsis*. **c** Higher magnification of L1 and L2. **d** Higher magnification of L2 and L3. *N*, cell nucleus; *Mn*, mt-nucleotid; *Pn*, pt-nucleotid; *L1*, one of the cells in layer L1; *L3*, one of the cells in layer L3. Bars = 10 μm

At day 5, cells that comprised the palisade were observed (Fig. 3E, F). The volume of the mitochondria had decreased rapidly and most of the mt-nucleoids had become small and spherical. The plastids had increased in volume and developed thylakoids could be observed by staining with $DiOC_6$. The pt-nucleoids were segregated from each other, and had become smaller. The DNA content of each plastid appeared to have increased remarkably, since the volume of each plastid had increased. These pt-nucleoids were rod-shaped or disk-shaped and were distributed throughout the thylakoids.

At day 7, the mitochondria had become very small and the DNA content of each mitochondrion was further decreased. The volume of plastids had further increased and thylakoids were better developed. In these mature chloroplasts, the pt-nucleoids were smaller and were observed between the thylakoids (Fig. 3G, H). Starch grains were observable as large dark areas within the chloroplasts.

Change in organelle DNA contents during development of the first foliage leaf. To determine how the DNA in individual organelles was amplified and distributed with development, the amounts of DNA within individual mitochondria and plastids in the developing first foliage leaves were measured directly using VIMPCS (Fig. 4). We used cells from the distal portion of the palisade to measure these DNA contents.

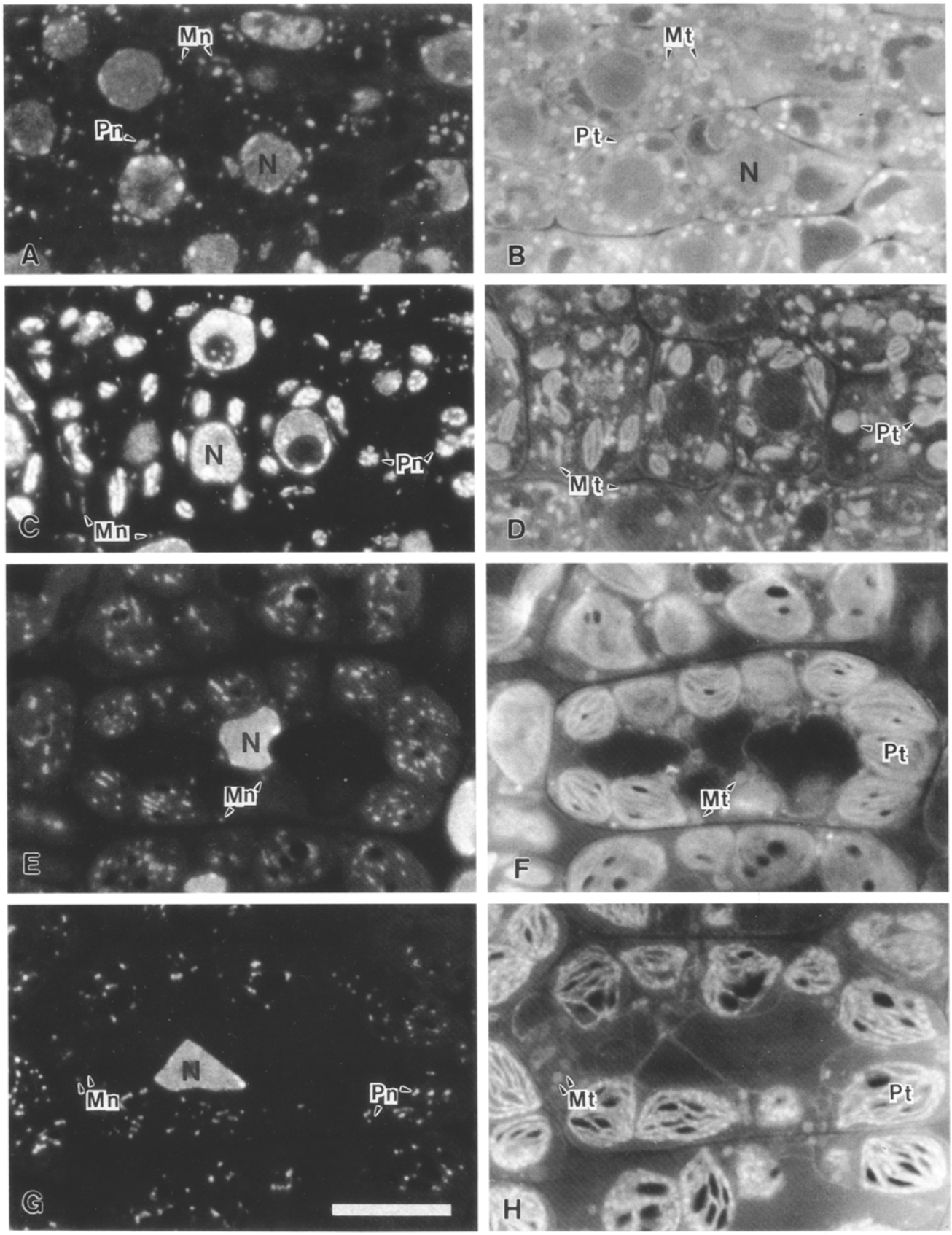


Fig. 3A–H. Epifluorescence micrographs of thin sections of the first foliage leaves of *Arabidopsis* stained with DAPI (**A**, **C**, **E**, **G**) and $DiOC_6$ (**B**, **D**, **F**, **H**). The mt-nucleoids are long, slim bright spots or small bright spots. Cells from 3- (**A**, **B**), 4- (**C**, **D**), 5- (**E**, **F**), and 7-d-old (**G**, **H**) seedlings are shown. The cells in the palisade are shown in **E–H**. *N*, cell nucleus; *Mt*, mitochondrion; *Mn*, mt-nucleoid; *Pt*, plastid; *Pn*, pt-nucleoid. Bar = 10 μm

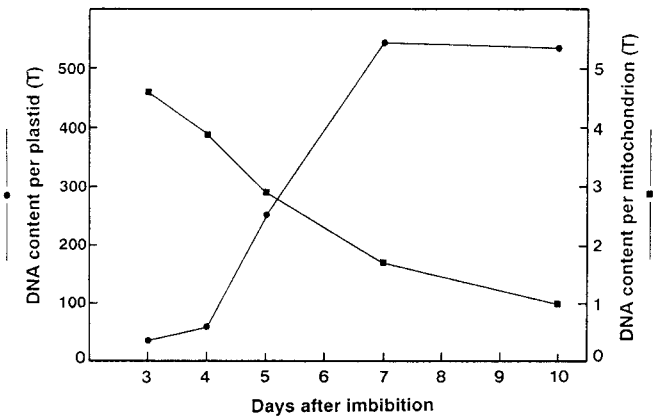


Fig. 4. Changes in the DNA content of individual mitochondria and plastids from the first foliage leaves of *Arabidopsis*. "T" is an arbitrary unit used to express the DNA contents as a multiple of the DNA content of T4 phage (1 T is approximately 170 kbp of DNA). The DNA content of individual mitochondria (■–■) is large in the young leaf primordia and decreases as development progresses. The DNA content of individual plastids (●–●) is small within the small young leaf primordia, but increases rapidly

In the leaf primordia of 3-d-old seedlings, the DNA content of each mitochondrion was approximately 4.6 T (780 kbp) and that of each plastid was 34.7 T (5.9 Mbp). These values were closer to those in the shoot apical meristem than to those in photosynthetically active mesophyll cells in the mature leaf (e.g. foliage-leaves of 10-d-old seedlings). During leaf development, the DNA content in each mitochondrion decreased, while that in each plastid increased rapidly, especially from 3 to 5 d after sowing. By day 10, the DNA content of individual mitochondria had fallen to about 1 T (170 kbp). On the other hand, the DNA content of individual plastids had increased to 535 T (91 Mbp). This means that the DNA content of individual plastids was amplified by more than a factor of ten from 3 to 10 d after sowing.

These results suggest that mtDNA and ptDNA are synthesized in a specific stage during leaf development. Synthesis of DNA in mitochondria seemed to be active in young leaf primordia, and became inactive during the early stage of leaf development. On the other hand, DNA synthesis in plastids seemed to be activated following mtDNA synthesis before the development of thylakoids at day 4, and to continue for a longer time than that in mitochondria. This hypothesis could be verified directly by investigating DNA synthesis in the mitochondria and plastids in the first foliage leaves.

Expression of Rubisco protein in developing first foliage leaves. We studied the relationship between the amounts of organelle DNA and the expression of Rubisco protein, one of the major protein in chloroplasts. If the amplification of ptDNA plays an important role in the rapid protein synthesis in developing plastids, then this amplification should occur before the massive expression of Rubisco protein. Thus, the expression of Rubisco protein was examined by immunostaining using anti-Rubisco-large-subunit antiserum in the first foliage leaves.

In 3-d-old seedlings, some plastids contained large pt-nucleoids in the leaf primordia (Fig. 5A). Appreciable Rubisco signals could not be detected on such plastids (Fig. 5B). Thus, amplification of ptDNA seemed to occur before the expression of Rubisco protein and the development of thylakoids. At day 4, the plastids contained amplified ptDNA and Rubisco was weakly expressed in the young chloroplasts (Fig. 5C, D). At day 5, strong Rubisco signals were detected in the stroma of the chloroplasts. At this stage, the pt-nucleoids were scattered among the developed thylakoids (Fig. 5E, F).

Synthesis of DNA in cell nuclei and organelle nucleoids in the shoot. During leaf development, the time and location of DNA synthesis in the cell nuclei and organelle nucleoids were studied by applying BrdU, an analogue of thymidine. Figure 6 shows longitudinal sections of *Arabidopsis* shoots incubated with BrdU. The seedlings were incubated with BrdU for 12 h at various times after sowing. The cell nuclei and the organelle nucleoids which incorporated BrdU could be clearly identified by immunostaining using anti-BrdU antibodies.

Some cell nuclei were labeled with BrdU in the shoot apical meristem from 3.5 to 4 d after sowing (Fig. 6a, b). In the case of *Arabidopsis* seedlings, we could not identify particular regions where DNA synthesis in cell nuclei was especially inactive, such as occurs in the quiescent center in the root apical meristem. Under higher magnification, some cell nuclei and organelle nucleoids labeled with BrdU could be observed in the meristem (Fig. 6c, d). The labeling frequencies indicate that the activities of DNA synthesis in the cell nuclei, mt-nucleoids and pt-nucleoids had changed in their respective manners during development of the first foliage leaf.

The distal halves of the first foliage leaves were used to study DNA synthesis. Mitochondrial DNA was labeled with BrdU in young leaves from 3.5 to 4 d after sowing (Fig. 6e, f). Synthesis of DNA then decreased gradually during leaf development. However, DNA synthesis in plastids seemed to be more active from 4.5 to 5 d (Fig. 6g, h) than from 3.5 for 4 d after sowing. The activity of BrdU incorporation into mitochondria was very low from 5.5–6 d after sowing (Fig. 6i, j). Active ptDNA synthesis continued for a rather long time and some pt-nucleoids were labeled with BrdU as late as 5.5 to 6 d after sowing.

These activities of DNA synthesis were evaluated by counting the cell nuclei, mitochondria and plastids that incorporated BrdU over a 12-h period at various times after sowing (Table 2). The labeling frequency in cell nuclei was high from 3.5 to 5.5 d after sowing and decreased by day 7.5. Synthesis of DNA was active in mitochondria only during the early stage of leaf development. The labeling frequency in mitochondria decreased gradually as development progressed, so that by day 7.5, when the leaves seemed to have matured, this value was only $6.7 \pm 8\%$. The labeling frequency in cell nuclei was higher than that in mitochondria throughout development, and some labeled cell nuclei were observed as late as 7.5–8 d after sowing (18%). However, the labeling frequency in plastids was higher than that in cell nuclei

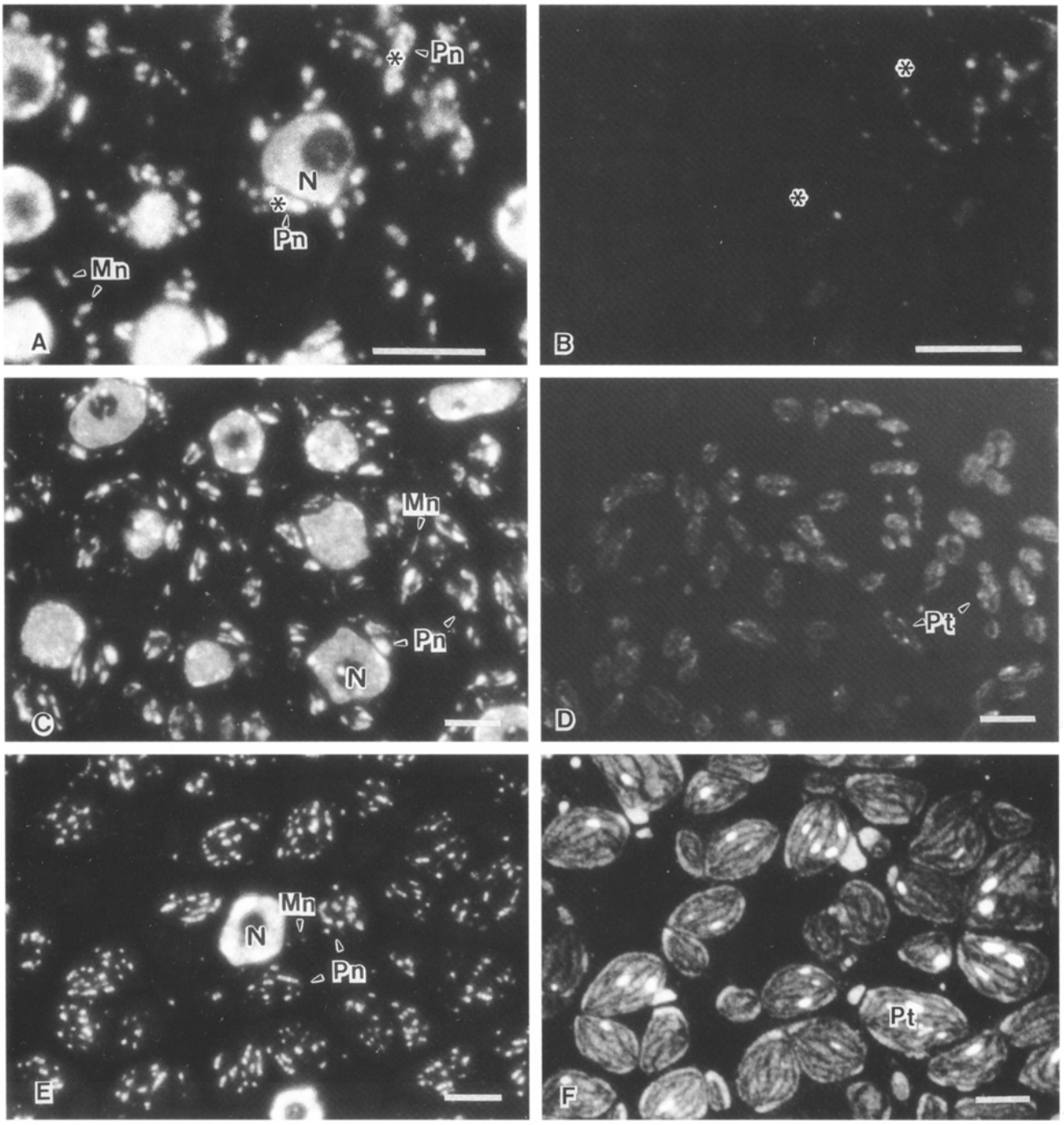
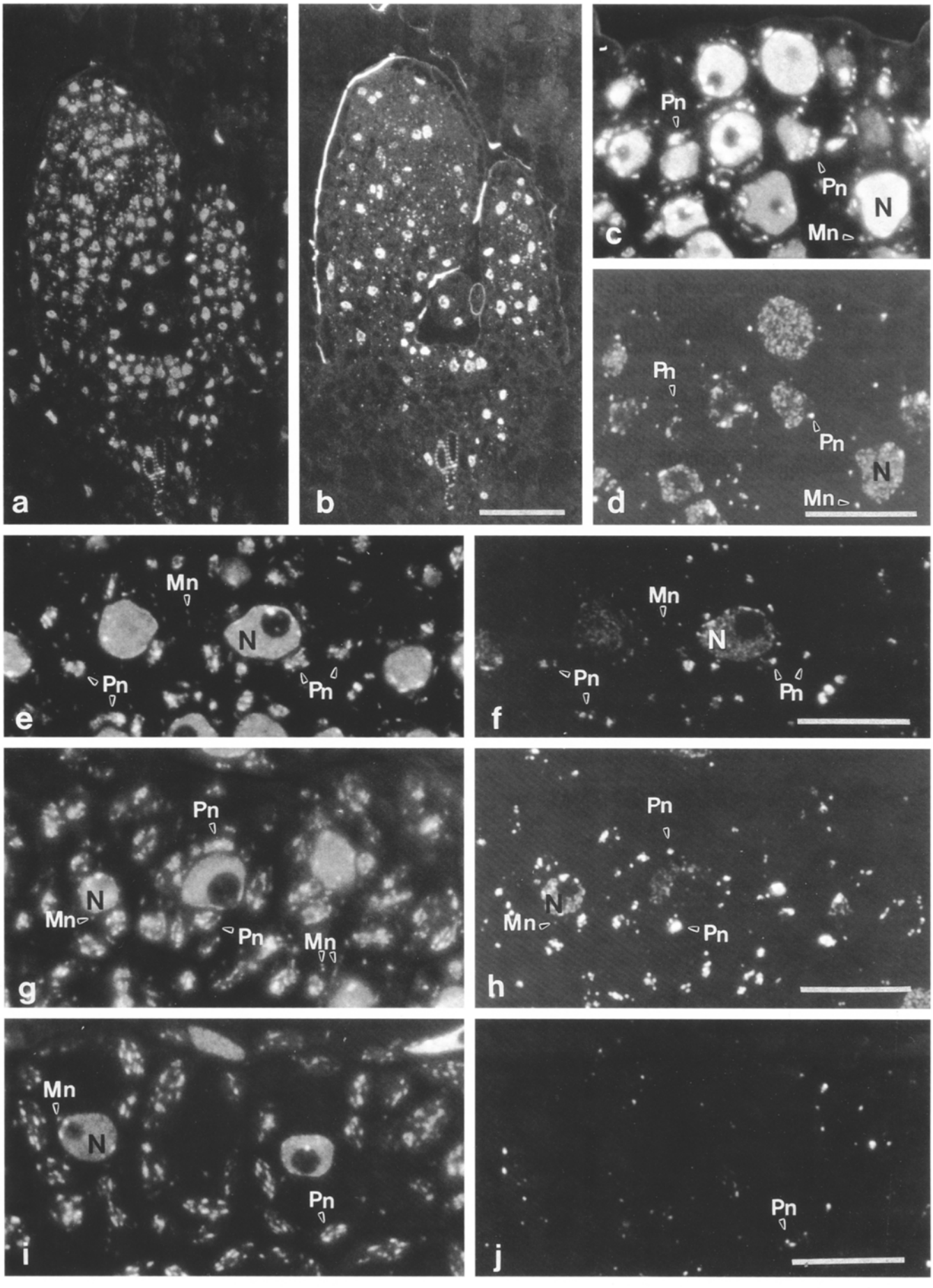


Fig. 5A–F. The localization of Rubisco by immunostaining in the developing first foliage leaves of *Arabidopsis*. **A**, **C**, **E** sections were stained with DAPI and cell nuclei (*N*), mt-nucleoids (*Mn*), and pt-nucleoids (*Pn*) are shown. **B**, **D**, **F** sections were stained with antisera against Rubisco large subunit and FITC-conjugated anti-rabbit IgG. **A**, **B** Cells in 3-d-old seedlings. *Asterisks* indicate the location of the same plastids. Appreciable signals were not detected in 3-d-old seedlings. **C**, **D** Sections of 4-d-old seedlings. Plastids (*Pt*) were labeled by antibodies, but no signals were observed on mitochondria. **E**, **F** Sections of 5-d-old seedlings. Plastids were developed and pt-nucleoids were observed between the developed thylakoids. Bar = 5 μm

throughout development, and plastids showed active DNA synthesis even after that in cell nuclei was inactive.

Discussion

The shoot apical meristem of *Arabidopsis* seedlings consists of superimposed cell layers, L1, L2 and L3, as reported by Satina et al. (1940). The development of the vegetative shoot apex, including the direction of the cell-division plane, has been studied and compared in the apices of wild and mutant plants of *Arabidopsis* (Medford et al. 1992). In our present study, we observed changes in the fine structures of mitochondria and plastids during leaf development. The fine structures of the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mitochondria and plastids in the shoot apical meristem were similar to those in the root apical meristem. In both of the apical meristem, the large rope-shaped mitochondria had large mt-nucleoids that contained more than 1 Mbp of DNA. These large mt-nucleoids become small nucleoids which contained about 170 kbp (1 T) of DNA during leaf development, as has been observed in the root apical meristem of *Arabidopsis* (Fujie et al. 1993a). We have already reported that the amount of DNA in each mitochondrion increases during the early stage of cell proliferation in the root apical meristem of several species such as *Pelargonium zonale* (Kuroiwa et al. 1992), *Nicotiana tabacum* (Suzuki et al. 1992), and *Arabidopsis thaliana* (Fujie et al. 1993a). We have also examined very large mitochondria which contain several mega-base pairs of DNA in the ovules of *Pelargonium zonale* (Kuroiwa and Kuroiwa 1992). Therefore, the appearance of large mitochondria which contain large amounts of DNA seems to be a characteristic feature of the active meristem of angiosperms.

The plastid is a typical organelle in plant cells and has been studied by varous methods. Kowallik and Herrmann (1972) studied the distribution of pt-nucleoids in individual chloroplasts by reconstructing them from serial electron-microscopy sections and showed that the DNA in chloroplasts is distributed within several regions separated by thylakoids. Previous studies of plastid development and of the dynamism of pt-nucleoids have been reported (reviewed by Possingham and Lawrence 1983; Kuroiwa 1991). Using microphotometry, the amount of DNA in each plastid has been studied in plastids that have been removed from their surrounding tissue (Kuroiwa et al. 1981; Lawrence and Possingham 1986; Miyamura et al. 1986). Removing the organelles from tissue is suitable for measuring the DNA contents of the plastids, but this process necessarily makes it difficult to determine where the plastids were located before removal. Therefore, the DNA contents of individual mitochondria and plastids have not been precisely measured in each part of the shoot apex despite the importance of this information in understanding leaf development. In the present study, the DNA contents of these organelles were measured by distinguishing each cell in the tissue using Technovit 7100 resin and DAPI staining (Kuroiwa et al. 1990). This method has enabled us to measure the DNA contents of individual organelles in

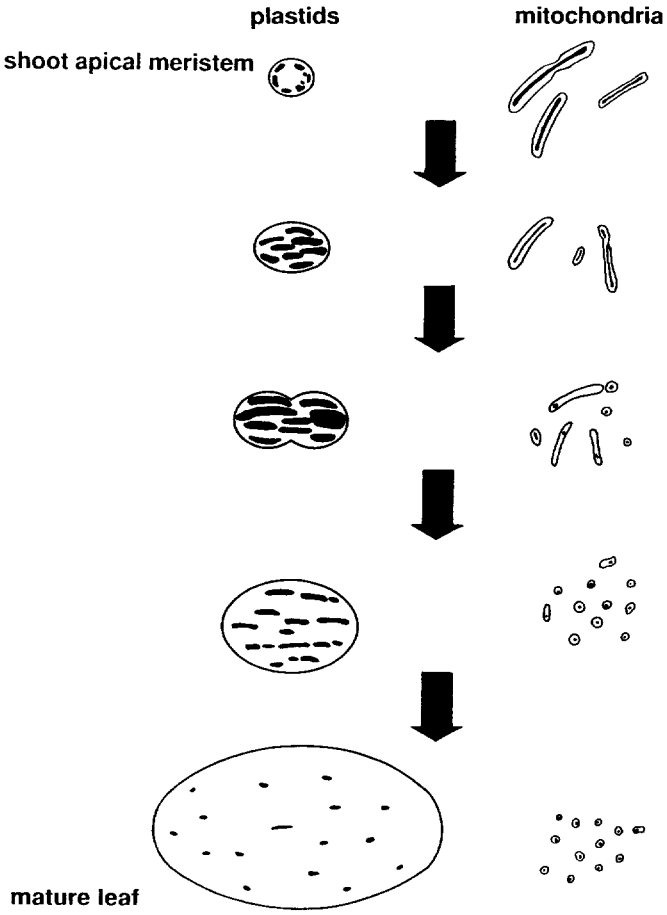

Fig. 7. A diagram of organelle development in the first foliage leaves of *Arabidopsis*. In the shoot apical meristem, the mitochondria were rather large and contained a large amount of DNA. The plastids were small and contained less DNA than developed plastids. These characteristics are similar to those in the root apical meristem. In these young plastids, thylakoids were not conspicuous. In the young leaf primordium, mitochondria were still large and plastids were still small. During leaf development, the mitochondria and the mt-nucleoids became progressively smaller while the plastids rapidly increased in volume and continued to divide. These growing plastids contained a large amount of DNA, and their stroma was occupied by large pt-nucleoids. This rapid ptDNA amplification occurred before development of the thylakoid systems. In the mature mesophyll, the mitochondria became very small granules which contained very small amounts of DNA, while plastids enlarged and the thylakoid system developed. The pt-nucleoids become small and were scattered throughout the thylakoid system

Fig. 6a–j. Epifluorescence micrographs of sections showing where DNA synthesis occurred in the shoot apical meristem (**a–d**) and in the first foliage leaves (**e–j**) of 3.5- to 4-d-old (**a–d**, **e**, **f**) 4.5- to 5-d-old (**g**, **h**) and 5.5- to 6-d-old (**i**, **j**) *Arabidopsis* seedlings. To detect DNA synthesis, BrdU was applied to the seedlings for 12 h. The BrdU that had been incorporated into DNA was detected by immunostaining using anti-BrdU antibodies and FITC-conjugated secondary antibodies. The cell nuclei (*N*), mt-nucleoids (*Mn*) and pt-nucleoids (*Pn*) were stained with DAPI and emitted bluish-white light (**a**, **c**, **e**, **g**, **i**). Some of the cell nuclei and organelle nucleoids incorporated BrdU and were stained with antibodies (**b**, **d**, **f**, **h**, **j**). Bars = 50 μm (**a**, **b**) and 10 μm (**c–j**)

Table 2. Percentages of cell nuclei, mitochondria and plastids in the distal halves of the first foliage leaves that were labeled by BrdU in 12 h. Values are means ± SD of five independent experiments

	Beginning of BrdU treatment (d)			
	3.5	4.5	5.5	7.5
Cell nuclei	55 ± 14	74 ± 8	52 ± 12	18 ± 7
Mitochondria	32 ± 26	11 ± 16	11 ± 20	6.7 ± 8
Plastids	75 ± 18	90 ± 13	79 ± 24	64 ± 22

each portion of the shoot apical meristem. Synthesis of DNA in individual plastids has been studied using conventional microautoradiography by many investigators (reviewed by Possingham and Lawrence 1983; Kuroiwa 1991). However, it has been difficult to simultaneously examine DNA contents and DNA synthesis activities of plastids. In the present study, we examined the relationship between the DNA content of individual plastids and ptDNA synthesis during leaf development. Synthesis of DNA in plastids occurred before the appearance of characteristic chloroplast features, such as developed thylakoids or expression of Rubisco. Thus, DNA synthesis and amplification of DNA in each plastid appear to be some of the initial events in plastid development.

The present study is the first to reveal the behavior of individual mt-nucleoids in shoot apical meristems and in developing foliage leaves. As observed in the root apical meristem (Fujie et al. 1993a), DNA synthesis in mitochondria was active during the early stage of cell proliferation, but decreased during the latter stage. With regard to leaf development, once the plastids became chloroplasts, BrdU incorporation by mitochondria fell to a very low level. Then DNA synthesis in the cell nuclei concluded. Finally, the activity of DNA synthesis in plastids decreased by 7.5 d after sowing. This difference in DNA synthesis activities among the cell nuclei, mitochondria and plastids suggests that each has a different system to control DNA synthesis. This control mechanism seems to be very important since amplification of organelle DNA occurs during the very early stage of leaf development and precedes many other events.

The observed increase in the DNA content of individual organelles is not peculiar to mitochondria and plastids. With regard to cell nuclei, it is well known that cell nuclear DNA is synthesized without mitosis and the DNA content of each cell nucleus is amplified during development (reviewed by Nagl 1982). This amplification of the DNA content in the cell nucleus, known as endoreduplication, is important for the expression of some enzyme activities in some species. The inhibition of nuclear DNA synthesis by hydroxyurea suppresses the expression of hydrolytic enzyme activities in germinating cotyledons of *Vaccaria pyramidata* (Bernhardt et al. 1993). The amplification of the DNA content in individual mitochondria may be related to the complex genome of plant mitochondria (Kuroiwa et al. 1992; Fujie et al. 1993a). On the other hand, ordinary plastids have a simple genome and it would appear that there is no necessity for ptDNA amplification during the early stage of development to maintain the genome of individual plastids. In this study, we have shown that ptDNA amplification started before the expression of Rubisco protein and the development of thylakoids. Therefore, it is possible that the amplification of DNA content in individual plastids may be important in supplying many transcripts, including rRNA, to the plastids which require many ribosomes to convert from proplastids to chloroplasts. During wheat leaf development, relative levels per cell of some mitochondrial genes (*coxII, cob, atpA*) decrease five- to tenfold and their transcript levels per cell also decrease following the gene changes (Topping and Leaver 1990). The levels of the genes and transcripts were determined by quantitative hybridization in this latter study. It is possible that mitochondria which contain a large amount of DNA exist in the basal meristematic region of a wheat leaf.

The DNA content of individual plastids is a very effective indicator of plastid development, since it increases during the very early stage of development, and few other characteristics that can be detected by light microscopy are known in these young plastids. The dynamic changes in the DNA contents in the mitochondria and plastids of *Arabidopsis* were accompanied by changes in the activities of DNA synthesis in the cell nuclei, mitochondria and plastids. Three phases of chloroplast division and chloroplast DNA synthesis were proposed from observations of expanding spinach leaves (Scott and Possingham 1983; Lawrence and Possingham 1986). The first phase, when the DNA amounts per plastids remain relatively low, corresponds to 3–4 d after sowing, and the second phase, when the DNA amounts per plastid increase, corresponds to 4–7 d. Investigating mtDNA synthesis is important in the early phase of leaf development (such as phase 1) to study the cell-differentiation interaction of the mitochondria, plastids and cell nuclei. The third phase, when DNA amounts per plastids fall, may correspond to the stage after 7 d. In addition to the change of ptDNA, the amounts of DNA of individual mitochondria also changed in the *Arabidopsis* shoot. The mtDNA level of individual mitochondria was high at 3 d (phase 1) and decreased four- to fivefold at the end of phase 2.

Figure 7 depicts organelle differentiation in *Arabidopsis,* including changes in nucleoids. The changes in the DNA contents of organelles, and their DNA synthesis activities, seemed to correspond to the reduction of mitochondria and the development of plastids, i.e. the change from sink-type cells to source-type cells. It has also been reported that mitochondrial functions are regulated developmentally in some plant species (Azcón-bieto et al. 1983; Hill et al. 1992). Further study of the behavior of organelle nucleoids will help in understanding respiration in mitochondria and the expression of the photosynthesis mechanism in plastids of developing leaves.

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