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Regulation of *Brassica rapa* chloroplast proliferation in vivo and in cultured leaf disks

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Summary. To understand the regulatory mechanisms of chloroplast proliferation, chloroplast replication was studied in cultured leaf disks cut from plants of 25 species. In leaf disks from *Brassica rapa* var. *perviridis*, the number of chloroplasts per cell increased remarkably in culture. We examined chloroplast replication in this plant in vivo and in culture media with and without benzyladenine, a cytokinin. In whole plants, leaf cells undergo two phases from leaf emergence to full expansion: an early proliferative stage, in which mitosis occurs, and a differentiational stage after mitosis has diminished. During the proliferative stage, chloroplast replication keeps pace with cell division. In the differentiational phase, cell division ceases but chloroplast replication continues for two or three more cycles, with the number of chloroplasts per cell reaching about 60. In the leaf disks, the number of chloroplasts per cell increased from about 18 to 300 without benzyladenine, and to over 600 with benzyladenine, indicating that this cytokinin enhances chloroplast replication in cultured tissue. We also studied changes in ploidy and cell volume between in vivo cells and cells grown in culture with and without benzyladenine. Ploidy and cell volume increased in a manner very similar to that of the number of chloroplasts, suggesting a relationship between these phenomena.

Keywords: *Brassica rapa* var. *perviridis*; Leaf disk; Chloroplast replication; Ploidy; Cell size; Benzyladenine.

Abbreviation: BA N^6-benzyladenine.

Introduction

It is generally accepted that chloroplasts arose from a bacterial endosymbiont, and that they proliferate by division (Gray 1989). However, plastids can divide only in cells that are under the control of cell nuclei, whereas bacteria can divide on their own. This control by nuclei must have been necessary to transform bacteria into organelles in ancient times and is likely to be still important to the maintenance of cell functions. In recent years, the machinery of chloroplast division has begun to be elucidated at a rapid rate. It was demonstrated by ultrastructural observation that electron-dense plastid-dividing rings (PD rings) form at the plastid division site (Mita and Kuroiwa 1988, T. Kuroiwa et al. 1998). PD rings appear at the initiation of chloroplast division and continue to constrict the membrane until the final division stage (Miyagishima et al. 1998). PD rings appear to be the main plastid-dividing machinery. In a recent report, it was shown that the bacterial division gene *ftsZ* is conserved in plants (Osteryoung and Vierling 1995) and is involved in plastid division (Strepp et al. 1998, Osteryoung et al. 1998). FtsZ localizes as a ring structure at the plastid division site (Mori et al. 2001a, b; Vitha et al. 2001). Plastid division is thought to occur with the cooperation of factors from both cyanobacteria (FtsZ) and host cells (PD rings, etc.) (Miyagishima et al. 2001).

In spite of remarkable progress in the study of plastid replication, the mechanisms that regulate the number of chloroplasts per cell remain totally unclear. There are a number of studies that suggest relationships between nuclear DNA content and organelle numbers (Butterfass 1973, Tymms et al. 1982) and between cell size and organelle numbers (Ellis and Leech 1985, Pyke and Leech 1987). However, these studies do not go beyond the level of circumstantial evidence, and there are exceptions to their suppositions that indicate other possibilities. Whether these factors control chloroplast replication positively, and how they regulate it, remains unclear and needs to be verified.

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The experimental systems in which the chloroplast number per cell can be changed will be useful to study the regulatory mechanisms of chloroplast replication. It was reported that chloroplasts proliferate in leaf disks cut from spinach and tobacco cultured on nutrient medium (Possingham and Smith 1972, Boasson et al. 1972). In this system, the number of chloroplasts per cell increases remarkably in the absence of cell division. This system does not use toxic chemicals and is expected to reflect the state in vivo. Moreover, in recent years, the machinery of chloroplast division has begun to be elucidated as described above and various biological methods have also become available. Now, new information can be expected using the leaf-disk system. For these reasons, we searched for a superior experimental system by culturing leaf disks from various plants and have established a chloroplast multi-proliferation system using *Brassica rapa.*

We examined chloroplast replication in vivo and in cultures with and without N^6-benzyladenine (BA), a cytokinin. Chloroplast replication was enhanced by culturing and further enhanced by added BA. Ploidy and cell volume increased in a manner very similar to that of the chloroplast number, both in vivo and in the culture containing BA.

Material and methods

Plant material

In initial experiments, *Spinacea oleracea*, *Brassica rapa* var. *perviridis*, *B. rapa* var. *laciniifolia*, *B. rapa* var. *chinensis*, *Nasturtium officinale*, and *Angelica keiskei* were purchased from local supermarkets. Seedlings of *Pisum sativum*, *Glycine max*, *Phaseolus vulgaris*, *Ipomoea batatas*, *Cucumis sativus*, *Cichorium endivia*, *Solanum tuberosum*, *Capsicum annuum*, and *Oryza sativa* were purchased from local markets and grown in a greenhouse. Seeds of *Canavalia gladiata* and *Zea mays* were purchased from Takii Shubyo (Kyoto, Japan) and grown in a greenhouse. *Trifolium repens*, *Calystegia japonica*, *Taraxacum officinale*, *Persicaria longisetum*, and *Fagopyrum dibotrys* were gathered from the campus of the University of Tokyo (Tokyo, Japan). *Nicotiana tabacum*, *Arabidopsis thaliana*, and *Cucurbita moschata* were obtained from a neighboring laboratory. In later experiments, seeds of *B. rapa* var. *perviridis* cv. Gokurakuten were purchased from Takii Shubyo and grown on nutrient soil at 22 °C under a cycle of 12 h light and 12 h darkness.

Culture of leaf disks

In the first experiment, leaf disks were excised initially from medial parts of leaves that were as small as possible and in later experiments from 3 cm long leaves of *B. rapa* var. *perviridis* and were cultured on sterile nutrient agar. Leaves were sterilized by a 4 min treatment in 0.1% (v/v) sodium hypochlorite and washed three times with sterile distilled water. Disks with a diameter of 2 mm were cut from the leaves with a sterile stainless steel punch and were cultured on Murashige and Skoog medium (Dainippon Pharmaceutical Co., Osaka, Japan) containing 1% agar and 5% sucrose at 25 °C under a cycle of 16 h of light and 8 h of darkness. Occasionally, 0.5 μg of BA per ml was added to the medium.

Chloroplast number determination

Protoplasts were generated to count the number of chloroplasts per cell. Leaf disks or leaves were digested for 30 to 80 min in an enzyme solution: 1–2% (w/v) cellulase YC (Seishin Pharmaceutical Co., Tokyo, Japan), 0.1% (w/v) pectolyase Y-23 (Seishin Pharmaceutical Co.), 0.03% (w/v) macerozyme R-10 (Seishin Pharmaceutical Co.), pH 5.5. When protoplasts were fragile, 1 mM $CaCl_2$ and 5% potassium dextran sulfate were added to the solution. The protoplasts obtained were fixed with 1% (w/v) glutaraldehyde in TAN buffer (0.25 M sucrose, 20 mM Tris-HCl [pH 7.6], 0.5 mM EDTA, 1.2 mM spermidine, 7 mM 2-mercaptoethanol, and 1.4 mM phenylmethylsulfonyl fluoride) and were stained with 1 mg of 4′,6-diamidino-2-phenylindole per ml in TAN buffer. After staining, a cover slip was placed over the sample to squash it. Chloroplasts of mesophyll cells were counted with an epifluorescence microscope (BHS-RFC; Olympus, Tokyo, Japan) under either ultraviolet (for DNA) or blue (for chloroplasts) excitation.

Estimation of mitotic indices

Basal, medial, and apical parts of leaves 1, 3, and 5 were dissected into small pieces (1 by 1 mm) and were fixed in 75% ethanol–25% acetic acid at room temperature for 1 h. The fixed samples were incubated in 1 N HCl at 60 °C for 5 min. The samples were washed in distilled water and stained with standard Schiff's reagent (Feulgen and Rossenbeck 1924) for 2 h. Stained samples were washed in 10% potassium metabisulfite solution for 2 h. Following these procedures, a cover slip was placed over the sample, which was then squashed. Mesophyll cells displaying evidence of mitosis such as condensed DNA or chromosomes were counted under an epifluorescence microscope (BHS-RFK; Olympus). For each sample, 1000 cells were examined.

Estimation of nuclear DNA content by fluorimetry

Dissected medial parts of *B. rapa* leaves or leaf disks and roots from seedlings germinated on wet filter paper were fixed together and prepared as described above. Nuclei in telophase in root meristem cells were used as controls indicating 2N. Fluorescence intensities from root meristem cells and mesophyll cells were quantified with a video-intensified microscope photon-counting system (VIMPICS; Hamamatsu Photonics Ltd., Hamamatsu, Japan) connected to an epifluorescence microscope, by accumulating photos with illumination for 2 s and subtracting the background without illumination for 2 s.

Cell volume determination

Palisade cells were used to measure cell volumes, as these cells have regular shapes and can be modeled as simple bodies. Leaf disks or leaves were digested for 60 min at room temperature in an enzyme solution: 0.1% (w/v) pectolyase Y-23, 0.05% (w/v) macerozyme R-10, pH 5.5. After digestion, samples were vortexed briefly and centrifuged for several seconds. Long and short axes of cells were measured with an Image Processor ARUGAS-20 (Hamamatsu Photonics K.K., Hamamatsu, Japan) connected to an inverted microscope (IX71; Olympus). Cells were approximated to cylinders and cell volumes were calculated from their axis lengths.

Immunofluorescence microscopy

Protoplasts were obtained from leaf disks as described above and fixed in 3% (w/v) paraformaldehyde dissolved in 50 mM piperazine-N,N′-bis(2-ethanesulfonic acid) [pH 6.8], 10 mM EGTA, 5 mM $MgSO_4$, and 0.5 M sucrose for 1 h at room temperature, according to a fixation method for microtubules (Tanaka 1991). After fixation, samples were washed twice with phosphate-buffered saline (PBS), affixed to cover

slips coated with poly-D-lysine (M_r, 15000 to 30000; Sigma), and squashed beneath cover slips. After treatment with 0.05% Triton X-100 in PBS for 15 min, the samples were blocked with 5% bovine serum albumin (BSA) in PBS for 30 min at 37 °C. The cover slips were then exposed to rabbit anti-LlFtsZ antibody (1:100 dilution) for 1 h at 37 °C. After washing with 1% BSA in PBS for 30 min and blocking with 5% BSA in PBS for 30 min, the samples were labeled with goat anti-rabbit immunoglobulin G conjugated with fluorescein isothiocyanate (Biosource, Camarillo, Calif., U.S.A.) diluted 1:100 in 5% BSA in PBS. Samples were viewed with an epifluorescence microscope (BHS-RFK; Olympus).

Results

Cultures of leaf disks from various plants

To identify a system that lends itself well to chloroplast replication studies, we cultured leaf disks cut from medial parts of leaves of 25 plants for seven days, with *Spinacea oleracea* as a control, and counted the number of chloroplasts per cell (Fig. 1). Leaf disks from 19 plants survived culturing. The culture of six plants, *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Trifolium repens*, *Solanum tuberosum*, and *Capsicum annuum*, was unsuccessful because each withered within seven days. In *Nicotiana tabacum*, chloroplasts increased about fourfold, and in *Spinacea oleracea*, chloroplasts increased fivefold. In the members of the family Brassicaceae, as a whole, chloroplasts proliferated well. In *B. rapa* var. *chinensis* and *Nasturtium officinale*, chloroplast numbers increased more than twofold, and in *B. rapa* var. *laciniifolia*, more than threefold. The greatest increase, more than sixfold, was seen in *B. rapa* var. *perviridis*. Among the other families, chloroplasts slightly increased in ten plants: *Pisum sativum*, *Phaseolus vulgaris*, *Canavalia gladiata*, *Ipomoea batatas*, *Calystegia japonica*, *Cucurbita moschata*, *Taraxacum officinale*, *Persicaria longisetum*, *Fagopyrum dibotrys*, and *Angelica keiskei*. Chloroplasts increased more than twofold in three plants: *Glycine max*, *Cichorium endivia*, and *Cucumis sativus*. These results led us to use *B. rapa* var. *perviridis* for further experiments.

Measurements of the basic growth features of B. rapa

We measured the overall leaf growth for use in experimental design. Figure 2 shows the growth of leaves 1 to 8 over the period from days 10 to 40 after sowing. From leaf 1 to leaf 5, the later-emerging leaves had larger final sizes, whereas leaves emerging after leaf 5 all attained nearly the same size. We decided to use leaves 1, 3, and 5 in later experiments. Leaf 1 fully expanded soon after emergence, and its final size was small (4 cm). Leaf 5 continued to grow over a longer period, and its final size was significantly larger than that of leaf 1 (12 cm). Leaf 3 had features between those of leaf 1 and leaf 5, with a final size of 7 cm.

To examine the relationship between mitosis and chloroplast replication in vivo, we first counted mitotic figures in

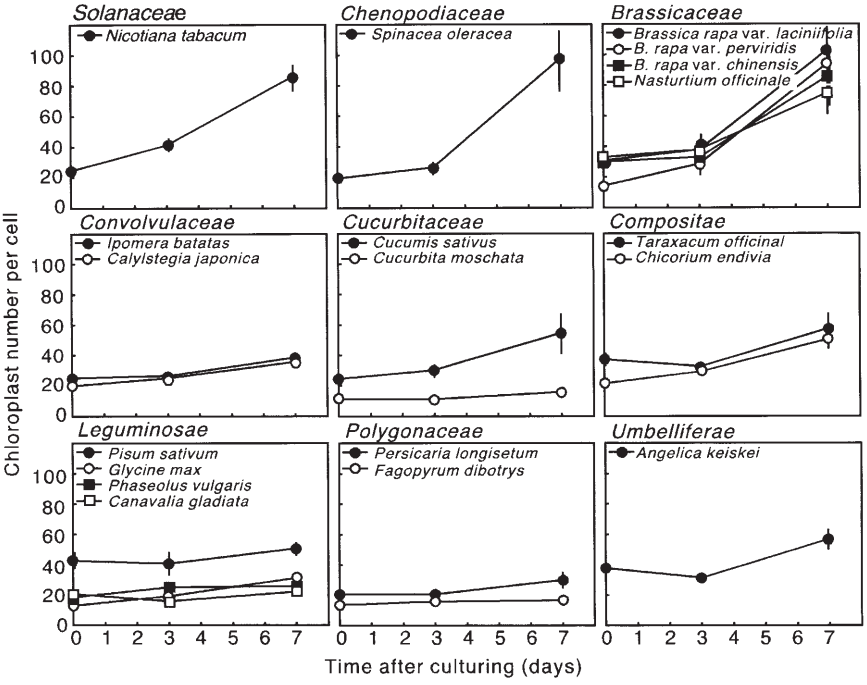

Fig. 1. Changes in chloroplast numbers per cell in cultures of 19 different plant species. Leaf disks from plants were cultured for 7 days (16 h light and 8 h dark) at 25 °C on nutrient medium. The number of chloroplasts per cell increased more in members of the Brassicaceae than in those of other families

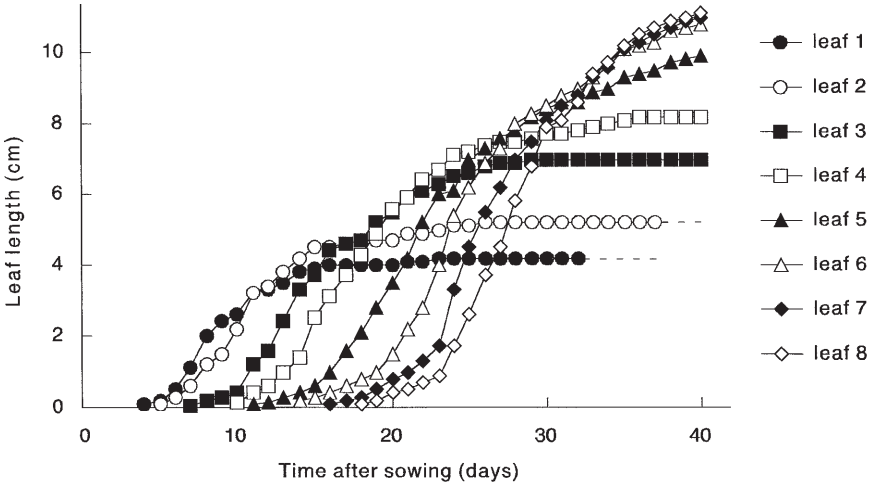

Fig. 2. Growth of leaves 1–8 of *B. rapa* from days 10 to 40 after sowing. In leaves 1 to 5, the later the leaf emerged the larger the final size, whereas subsequent leaves attained almost the same final size

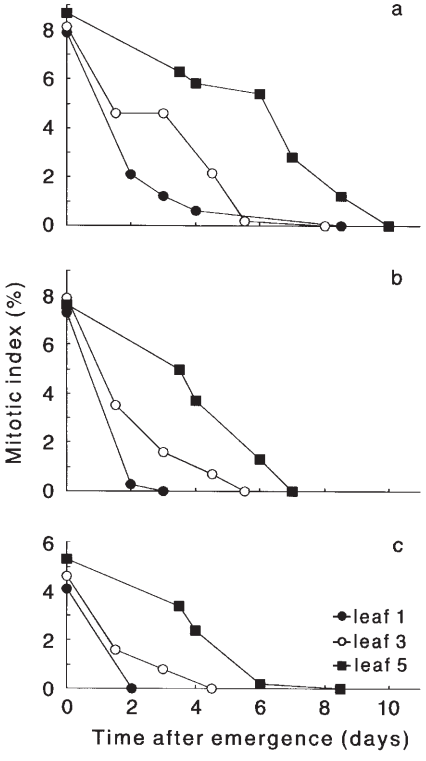

Fig. 3a–c. Changes in the mitotic index along with leaf expansion in *B. rapa* leaves 1, 3, and 5, from leaf emergence (1 mm in length) to full expansion. **a** Basal parts. **b** Medial parts. **c** Apical parts. In all parts, the mitotic index began to drop soon after leaf emergence, but the decrease was more rapid in the more apical parts

the basal, medial, and apical parts of the leaves (Fig. 3). The mitotic index of leaf 1 at day zero after leaf emergence (1 mm in length) was 8% in the basal part, 8% in the medial part, and 5% in the apical part. In all parts, the mitotic index soon began to drop, but the decrease was more rapid in the more apical parts: it reached zero percent after 8 days in the basal part, after 3 days in the medial part, and after 2 days in the apical part. The tendencies of the mitotic indices of day zero in leaves 3 and 5 were almost the same as that of leaf 1, but the decrease became slower in later leaves. We defined the developmental stage in which mitosis occurs as the proliferative stage and the stage following the diminishing of mitosis as the differentiational stage.

Using medial regions of leaves, we investigated the increase in the number of chloroplasts in vivo from leaf emergence (2 mm in length) to full expansion (Fig. 4a). In leaf 5, chloroplasts numbered about 13 per cell at day zero and remained constant during the proliferative stage. In the differentiational stage, the number gradually increased until about 60. This indicates that the chloroplasts divided about two more times after nuclear division and cytokinesis had ceased. The same tendency was seen in leaves 1 and 3, and their initial and final numbers were almost the same as those of leaf 5.

We also studied the changes in cell volume from leaf emergence (2 mm in length) to full expansion and the ploidy level from the end of the proliferative stage to full leaf expansion in vivo, as these factors are thought to affect the regulation of chloroplast numbers (Fig. 4b, c). Cell volume was determined using only palisade cells because spongy parenchyma cells have irregular shapes and their volume is difficult to determine. In all leaves, the cell volume did not change drastically during the proliferative stage but greatly increased in the differentiational stage. In leaf 5, cell volumes of 210 μm^3 at day zero, and 690 μm^3 at the end of the proliferative stage, rapidly increased at the beginning of the differentiational stage to 1.2×10^4 μm^3, about 60 times the volume at day zero. We observed cross sections of leaves. Spongy parenchyma cells became larger in the same manner and in the same degree as palisade cells (data not shown). Nuclear DNA content also began to increase soon after cells had ceased mitosis. In leaf 5, a rapid increase in ploidy was seen at the initiation of the

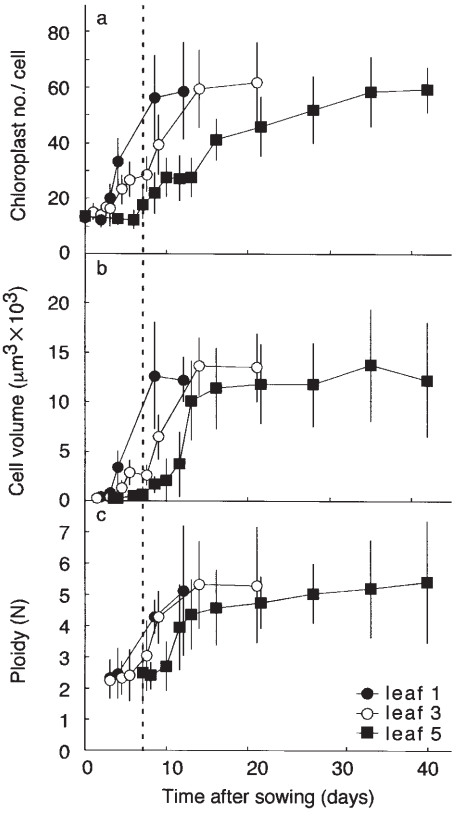

Fig. 4 a–c. Changes in chloroplast numbers per cell, cell volume, and ploidy in medial parts of leaves 1, 3, and 5, from leaf emergence (2 mm in length) to full expansion. Dotted line expresses the boundary between the proliferative stage and the differentiational stage of leaf 5. **a** Number of chloroplasts per cell. **b** Cell volume. **c** Ploidy. The number of chloroplasts per cell was about 13 at day zero and increased to about 60. In expanded leaves, the cell volume increased to about 60 times that of day zero, and ploidy reached about 5.4N

differentiational stage, which reached an average of 5.4N. This tendency was the same in leaves 1 and 3. The pattern of cell growth and the increase in ploidy mirrored the increase in chloroplast numbers.

Cultures of leaf disks and effects of benzyladenine

While the chloroplast number per cell was constant in the proliferative stage, it increased in the differentiational stage. We took leaf disks from leaves of the differentiational stage. Also required were leaves large enough to sample several leaf disks and younger leaves with high viability. Since mitosis continues longer in the basal parts of leaves, and cells are likely to be older in the apical parts, we decided to use the middle leaf parts. In leaves 3 and 5, we decided to use the leaves at days 6 and 7 after emergence, since on those days mitosis had ceased and the leaves had reached 3 cm in length, the minimum size needed for culturing. To test leaf 1, leaves from day 8 after emergence, 3 cm in length, were used, since the cells ceased mitosis before the leaf grew adequately large.

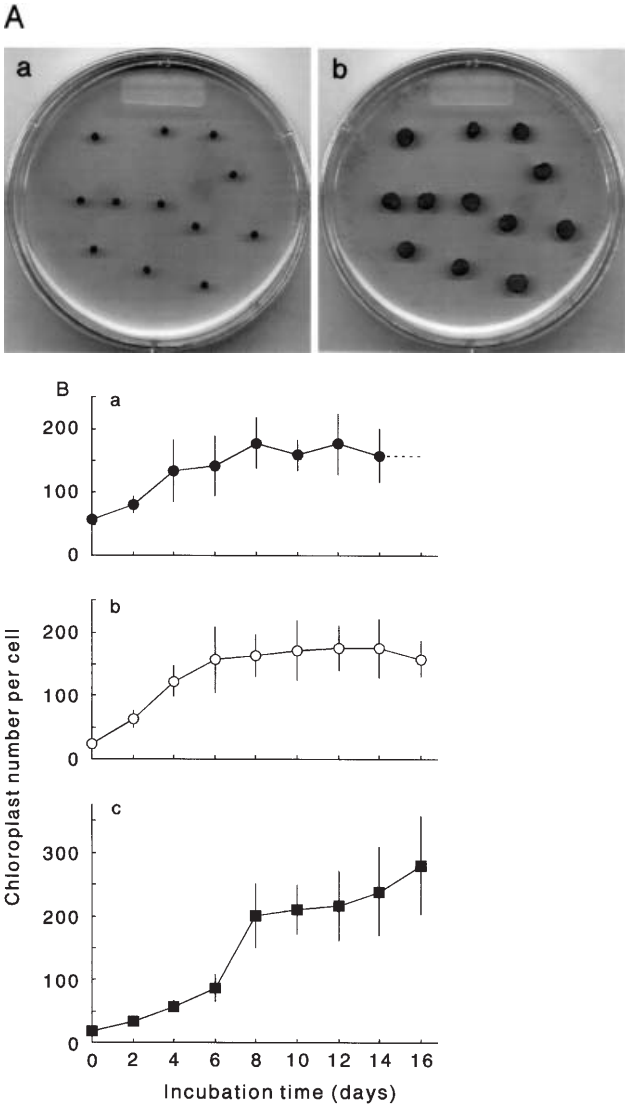

Fig. 5 A, B. Changes in chloroplast numbers per cell during 16 days of culture of leaf disks from leaves 1, 3, and 5. **A** Disks from leaf 5 at day 0 (**a**), and at day 16 (**b**). Leaf disks increased their volume. **B** Changes in the number of chloroplasts per cell in leaves 1 (**a**), 3 (**b**), and 5 (**c**): the number of chloroplasts per cell increased from 56 to about 170, 24 to about 180, and 18 to about 280, respectively

On the basis of the above results, the middle parts of leaves 1, 3, and 5 that had reached 3 cm in length were cultured for 16 days, and the numbers of chloroplasts per cell were counted (Fig. 5). Leaf disks grew from initial 2 mm to about 6 mm in diameter and their thickness increased from about 0.2 mm to about 0.6 mm. The leaf disks increased in size in all three dimensions (Fig. 5A). In all leaves, chloroplast division occurred more times in leaf disks than in whole plants (Fig. 5B). In leaves 1 and 3,

the chloroplasts stopped dividing a few days after culturing, but the number of chloroplasts per cell increased from 56 to about 170 and from 24 to 180, respectively. In leaf 5, the number of chloroplasts increased from 18 to about 280, which indicates that each chloroplast replicated four times during culture. Since disks from leaf 5 were also in better condition than those from other leaves, we decided to use leaf 5 to examine the effects of BA.

We examined whether chloroplast replication was accelerated by the addition of cytokinin. We investigated chloroplast proliferation in disks from leaf 5 cultured with 0.5 μg of BA per ml for a 16-day period (Fig. 6a). In our system, chloroplast replication was greatly enhanced by BA. The number of chloroplasts increased logarithmically for 10 days and then slowed to a linear rate of increase until day 16. During culture, the chloroplasts replicated about five times, resulting in about 630 chloroplasts in a single cell on day 16, twice as many as in cells from medium lacking BA. To examine whether BA has effects on factors thought to affect chloroplast numbers, we also investigated the cell volume and ploidy in leaf disks cultured for 16 days with and without 0.5 μg of BA per ml; the addition of BA drastically increased the cell volume and ploidy (Fig. 6b, c). The cell volume averaged $1.2 \times 10^5\ \mu m^3$ without BA and $3.8 \times 10^5\ \mu m^3$ with BA on day 16, an increase of more than threefold with BA. Nuclear ploidy was about 12N without BA and 33N with BA on day 16, again about a threefold increase. The patterns of cell growth and ploidy increase were very similar to the changes in chloroplast numbers: a logarithmic increase for ten days and a linear increase after day 10. On day 16, giant cells containing more than 600 chloroplasts and large nuclei were observed (Fig. 7). The size of these chloroplasts was similar to those at day zero. Many constricted chloroplasts were observed, suggesting that chloroplasts continued to divide. Using immunofluorescence, we also visualized FtsZ with a ringlike structure appearing at the division site in each chloroplast (Fig. 8). Chloroplast DNA was distributed equally to each daughter chloroplast, and chloroplast proliferation in this system was normal.

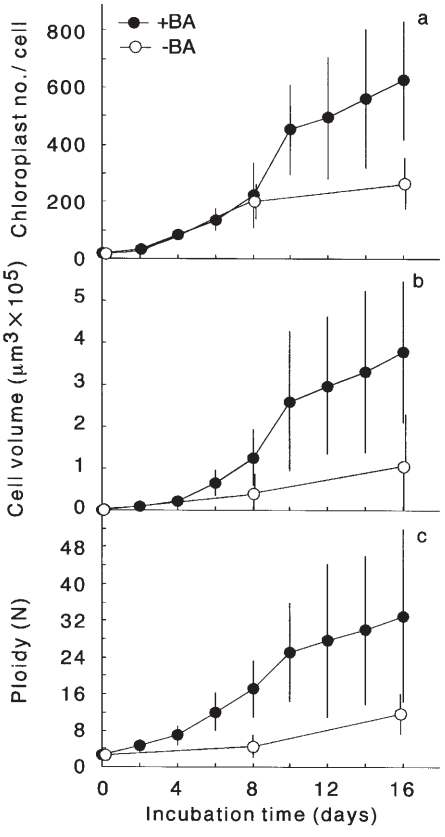

Fig. 6a–c. Changes in chloroplast numbers per cell, cell volume, and ploidy during 16 days of culture with or without BA. Leaf disks were cultured on nutrient medium containing or lacking 0.5 μg of BA per ml. **a** Number of chloroplasts per cell. **b** Cell volume. **c** Ploidy. Chloroplast number, cell volume, and ploidy were all increased by BA; values on day 16 increased from 280 to 630 chloroplasts, $1.2 \times 10^5\ \mu m^3$ to $3.8 \times 10^5\ \mu m^3$, and 12N to 33N, respectively

Discussion

Culture of leaf disks from various plants

In the culture of leaf disks from 25 plant species, we found that the number of chloroplasts per cell increased remarkably in members of the family Brassicaceae, especially in *B. rapa*, comparable to spinach. Like spinach, *B. rapa* is also useful for biochemical and physiological experiments because of its large size. In addition to these, *B. rapa* is a superior material in other aspects which spinach does not have. The germination and the growth of *B. rapa* are well synchronized. Genome information from *Arabidopsis thaliana* can be exploited because *B. rapa* is a close relative. Transformation methods are available for species in the family Brassicaceae, and it is informative to study chloroplast proliferation in these species because there are over forty vegetables that belong to this family.

Measurement of the basic growth features of B. rapa

The growth of *B. rapa* leaves showed the pattern typical of rosette leaves, gradually expanding in size from small to relatively large. In leaf development, the stage in which

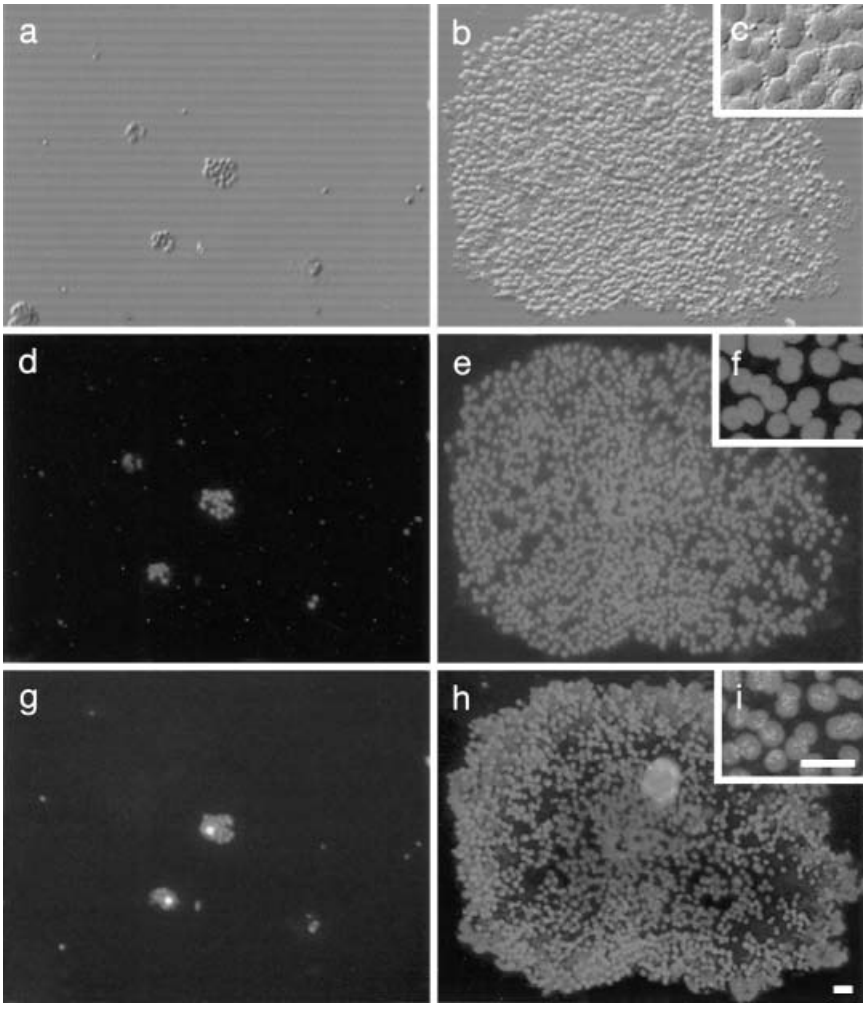

Fig. 7 a–i. Day zero and 16-day-old cells from leaf disks cultured with BA. Protoplasts were derived from samples from each day and squashed under cover slips. **a**, **d**, and **g** Day zero. **b**, **e**, and **h** Day 16. The insets show magnified images. **a–c** Differential interference contrast. **d–f** Chloroplast fluorescence. **g–i** 4′,6-diamidino-2-phenylindole staining. Large nuclei and over 600 chloroplasts are seen in the cell from day 16. The size of the chloroplasts was almost the same as those of day zero cells. Bars: 10 μm

mitosis occurs is defined as the proliferative stage, and the stage after mitosis has ceased is the differentiational stage. During the proliferative stage, chloroplast replication kept pace with mitosis, as the number of chloroplasts per cell was constant (Fig. 4). We postulate that the mechanisms that loosely connect chloroplast replication with mitosis include nuclear DNA replication, nuclear division, cell growth, and cytokinesis (Fig. 9a, c). It is possible that this loose linkage is an alteration of the tight linkage of monoplastidic cells. All monoplastidic cells must have mechanisms that ensure that the chloroplast divides before or along with the cell. For example, in *Cyanidioschyzon merolae*, whose cells contain one nucleus, one chloroplast, and one mitochondrion, the organelles divide before the nucleus and the cell divide (Suzuki et al. 1994). The division machinery of chloroplasts is well conserved in photosynthetic eukaryotes (Miyagishima et al. 2001, H. Kuroiwa et al. 2002) and the mechanism that links chloroplast replication with mitosis might also be universal.

During the differentiational stage, chloroplast replication was not accompanied by cytokinesis. Chloroplasts replicated at an average of 2.2 times during this stage, resulting in about 60 chloroplasts in cells of fully expanded leaves. Endoreduplication occurred about 1.2 times and cell volume increased 60 times (Fig. 4). The pattern of increase in the number of chloroplasts was similar to that of ploidy increase and to that of cell growth, suggesting a correlation between them. These ideas are consistent with reports that have suggested a relationship between chloroplast numbers and ploidy level (Butterfass 1973, Tymms et al. 1982), ploidy level and cell volume (Butterfass 1987, Melaragno et al. 1993, Kudo and Kimura 2002), and cell size and chloroplast numbers (Ellis and Leech 1985, Pyke and Leech 1987). The slight delay seen in the increase in the number of chloroplasts in relation to ploidy or cell volume increases in leaf 5 might indicate that ploidy level or cell volume influence chloroplast numbers (Fig. 4). We postulate that the mechanism that links chloroplast replication,

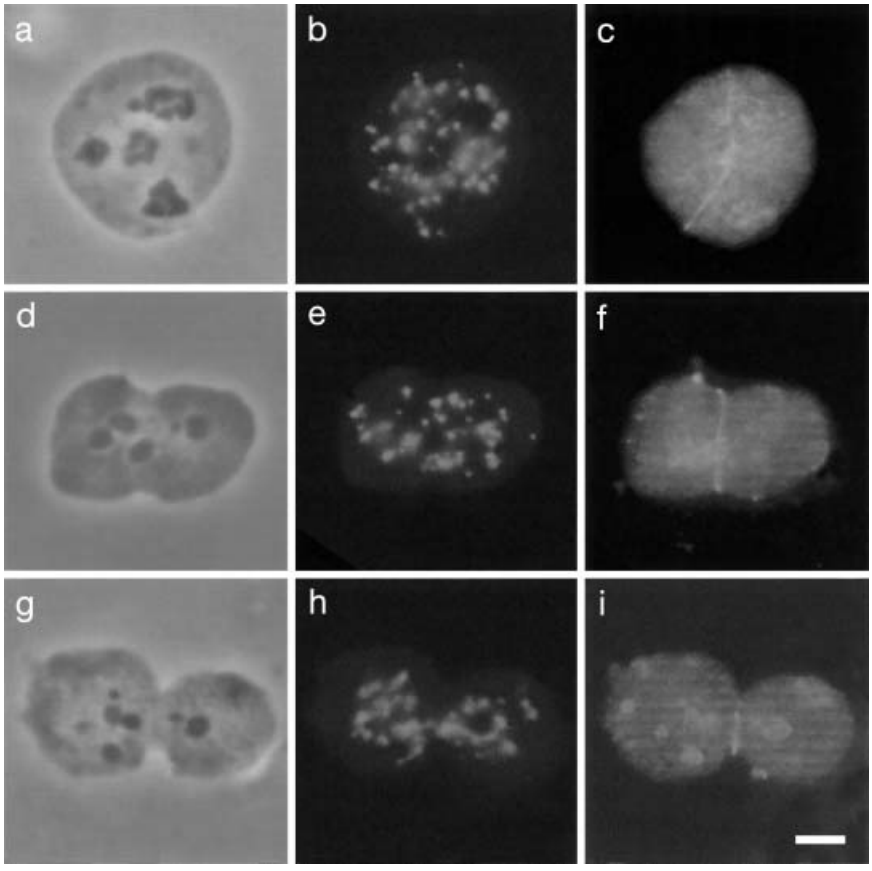

Fig. 8a–i. Immunofluorescence images of FtsZ. **a**, **d**, and **g** Phase contrast images. **b**, **e**, and **h** 4′,6-diamidino-2-phenylindole staining. **c**, **f**, and **i** Immunofluorescence images. A ringlike structure was observed at the division site in each chloroplast. Chloroplast DNA was distributed equally to each daughter chloroplast. Bar: 2 μm

nuclear DNA synthesis, nuclear division, cell growth, and cytokinesis at the proliferative stage is also used at the differentiational stage, though cytokinesis and nuclear division have diminished (Fig. 9b, d).

Cultures of leaf disks and effects of benzyladenine

Leaf disks cut from leaves at the differentiational stage were cultured on nutrient medium. The number of chloroplasts per cell in these disks was higher than in in vivo cells. The larger final number of chloroplasts in the leaf 5 cultures, as compared to those of leaves 1 and 3 (Fig. 5), is thought to have been due to the difference in the developmental program at day zero. In leaf 5 cultures, endoreduplication and cell growth were also enhanced by culturing (Fig. 6b, c). It is reported that sucrose induces the production of CycD, a D-type cyclin, which is necessary for the G_1–S transition (Riou-Khamilichi et al. 2000). Although the factor responsible for this phenomenon remains to be determined, the sucrose in the medium might be responsible for the endoreduplication.

BA enhanced chloroplast replication in our system (Fig. 6a). Although the difference between the number of chloroplasts per cell in media with and without BA was not clear at day 8, it became distinct by day 16. We think that the difference results from endogenous cytokinins being sufficient to promote plastid division in 8-day cultures, whereas older cultures either need exogenous cytokinin to supplement the endogenous cytokinins that have decomposed or require higher levels of cytokinins for large numbers of plastids to divide. This is consistent with a report that added BA had little effect in 7-day spinach leaf disk cultures but a measurable effect in 14-day cultures, which included a 7-day preculture in the dark (Possingham 1976).

Cytokinin has been reported to have a wide range of effects on chloroplast development. For example, it induces the expression of light-harvesting chlorophyll *a/b* protein (LHCP) in watermelon cotyledons grown in the dark (Longo et al. 1990) and promotes greening in the *pac* (pale cress) mutant of *Arabidopsis thaliana*, which has white leaves (Grevelding et al. 1996). To our knowledge, the only report that suggests a clear promotion of chloroplast division by cytokinin is one that describes a mutant of the moss *Physcomitrella patens* (Kasten et al. 1997). Cytokinin induces chloroplast division to some degree in

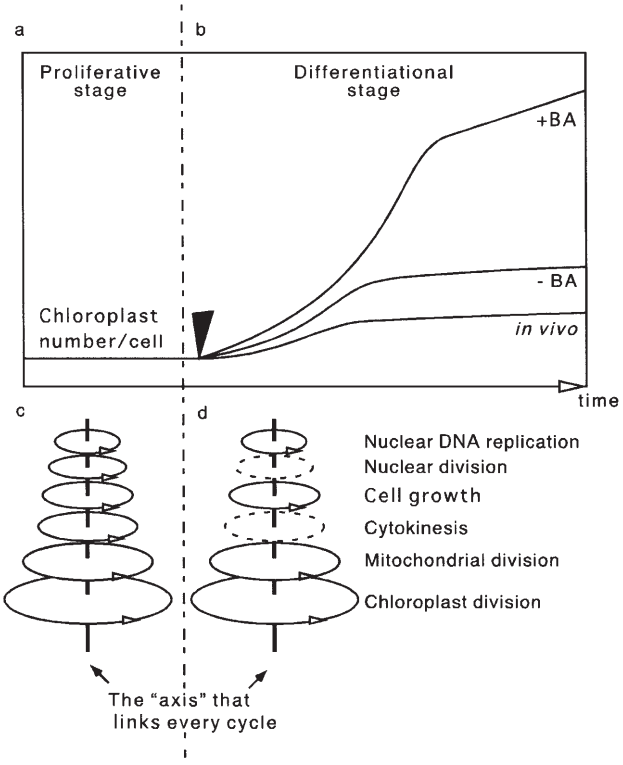

Fig. 9. A model for chloroplast proliferation in vivo and in cultures with and without BA. From leaf emergence to full expansion in whole plants, leaf cells undergo an early proliferative stage, in which mitosis occurs, and a differentiational stage after mitosis has diminished. In the proliferative stage, the number of chloroplasts per cell is constant (**a**) and chloroplast replication keeps pace with nuclear DNA replication, nuclear division, cell growth, and cytokinesis (**c**). We postulate the existence of an "axis" that synchronizes these events. In the differentiational stage, the number of chloroplasts per cell increases (**b**). Chloroplast replication continues to keep pace with nuclear DNA replication and cell growth, although nuclear division and cytokinesis do not occur (**d**). We suppose that the axis in the proliferative stage continues to work in the differentiational stage in the absence of nucleokinesis and cytokinesis. Mitochondrial replication is probably under the same regulation. The arrowhead indicates the point from which leaf disks were cut. Chloroplast replication is enhanced by culturing and further enhanced by the addition of BA, and with nuclear DNA replication and cell growth. This is thought to indicate that the regulation described in **d** is present in cells of leaf disks cultured with or without BA

PC22, a mutant of *Physcomitrella patens*, the cells of which contain a single large chloroplast. We believe that cytokinin promotes chloroplast development and division in our system and results in extensive chloroplast replication.

It has been reported that chloroplast division is accelerated in somatic cell hybrids between mesophyll and cell culture protoplasts (Kamata et al. 1989) and the number of chloroplasts per cell increases in the unicellular red alga *Cyanidioschyzon merolae* treated with aphidicolin, a specific inhibitor of nuclear DNA synthesis (Itoh et al. 1996). Although the chloroplast size decreased in these cases, the size of the chloroplasts in the leaf disks showed no changes (Fig. 7). FtsZ visualized with fluorescein isothiocyanate was observed as a ringlike structure at the chloroplast division site as reported in many plants (Mori et al. 2001a, b; Vitha et al. 2001) (Fig. 8). It was also shown that chloroplast DNA was also equally distributed to both daughter chloroplasts (Fig. 8). These results suggest that the chloroplast replication in this culture is normal.

Cell growth and endoreduplication were further enhanced by BA in our system (Fig. 6b, c). Because the cell size and ploidy level are thought to affect chloroplast numbers, the effect of BA on chloroplast replication might be through these factors. Cell volume and ploidy level increased in a very similar manner to the number of chloroplasts, same as in vivo, suggesting a relationship between these phenomena. Since by electron microscopy, chloroplasts were observed to be arranged in tightly packed monolayers just below the cell surface and most cells were occupied by vacuoles, the number of chloroplasts might be related to the extent of the cell surface area (data not shown). Chloroplast replication continues to keep pace with nuclear DNA replication and cell growth, although nuclear division and cytokinesis do not occur. We speculate that the same mechanism that links chloroplast replication, nuclear DNA replication, and cell growth working in the differentiational stage in vivo works in cultured leaf disks (Fig. 9b, d). These facts suggest that a system in which the number of chloroplasts per cell increases significantly in the same manner as in vivo was established. If it is used together with the recent information on chloroplast division and various biological methods, this system will become a powerful tool to reveal the regulatory mechanisms of chloroplast replication.

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