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Real-time analyses of chloroplast and mitochondrial division and differences in the behavior of their dividing rings during contraction

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Abstract. The time courses of chloroplast and mitochondrial division and the morphological changes in the plastid-dividing ring (PD ring) and mitochondrion-dividing ring (MD ring) during chloroplast and mitochondrial division were studied in *Cyanidioschyzon merolae* De Luca, Taddei and Varano. To accomplish this, chloroplast and cell division of living cells were continuously video-recorded under light microscopy, and the morphological changes in the PD and MD rings were analyzed quantitatively and three-dimensionally by transmission electron microscopy (TEM). Under the light microscope, the diameters of the chloroplast and the cell decreased at uniform velocities, the speed depending on the temperature. To study in detail the sequential morphological change of the mitochondrion in M phase and the contractile mechanism in the divisional planes of the chloroplast and the mitochondrion, we observed the PD and MD rings, which are believed to promote contraction, under TEM, using the diameter of the chloroplast as an index of the time. Three PD rings (an outer PD ring on the cytoplasmic face of the outer envelope, a middle PD ring in the intermembrane space, and an inner PD ring on the stromal face of the inner envelope) were clearly observed, but only the outer MD ring could be observed. The PD ring started to contract soon after it formed, while the contraction of the MD ring did not occur immediately after formation, but was delayed until the contraction of the PD ring was almost complete. Once the MD ring began to contract, the rate of decrease of its circumference was 4 times as high as that of the PD ring. As the outer PD and MD rings contracted, they grew thicker and maintained a constant volume, while the thickness of the inner PD ring did not change and its volume decreased at a constant rate with contraction. In the early stage of contraction, the widths of the three PD rings increased in order, from the outer to the inner ring. With contraction, their widths changed at different rates until they came to have much the same width. In cross-section, the MD ring was wider where it was next to the chloroplast than at the opposite side, adjacent to the nucleus in the early stage of contraction. By the late stage, the widths of the two sides became equal. In our observations, the microbody elongated along the outer MD ring and touched the outer PD ring during contraction of the PD and MD rings. These results clearly revealed differences between the mode of contraction of the outer, middle, and inner PD rings, and between the PD and the MD rings. They also revealed the coordinated widening of the three PD rings, and suggested that the microbody plays a role in the contraction of the PD and MD rings.

Key words: *Cyanidioschyzon* – Microbody – Mitochondrion-dividing ring – Organelle division – Plastid-dividing ring

Introduction

Cell division is an essential and ubiquitous event for living organisms and one of the defining characteristics of life. It consists of nuclear division and cytokinesis. Previous studies have revealed that eukaryotic cytokinesis is driven by a contractile ring that lies on the cytoplasmic face of the cleavage furrow and is mainly composed of an actomyosin system. The bacterial cell division protein FtsZ, which is homologous to tubulin in eukaryotes, forms the dividing ring during bacterial cytokinesis (Bi and Lutkenhaus 1991; for review, see Rothfield and Justice 1997)

Mitochondria and plastids, which have their own genomes and ribosomes, and are believed to be descendants of bacterial endosymbionts, are never synthesized de novo, but proliferate by binary fission. This organelle

Abbreviations: $DiOC_6$ = 3,3′-dihexyloxacarbocyanine iodide; MD ring = mitochondrion-dividing ring; PD ring = plastid-dividing ring; TEM = transmission electron microscopy

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division consists of nucleoid division and division of the envelopes (for review, see Possingham and Lawrence 1983; Kuroiwa 1991). Many workers have looked for a mechanical apparatus equivalent to the contractile ring of cytokinesis or formation of septa inside organelles (Suzuki and Ueda 1975; Chaly and Possingham 1981; Leech et al. 1981). Such rings were first identified in the cleavage furrow outside the outer envelopes of plastids and mitochondria in the red algae *Cyanidium caldarium* RK-1 (Mita et al. 1986) and *Cyanidioschyzon merolae* (Kuroiwa et al. 1993, 1995), respectively, and these were thought to pinch-off envelopes. They were called the plastid-dividing ring (PD ring) and the mitochondrion-dividing ring (MD ring). Subsequently, the PD ring was revealed to consist of an outer ring on the cytoplasmic face of the outer envelope membrane and an inner ring on the stromal face of the inner envelope membrane (Hashimoto 1986). Recently, we showed that the PD ring has a triple-ring structure, identifying a middle ring in *C. merolae* that exists in the intermembrane space (Miyagishima et al. 1998b). The PD ring has been detected in a variety of terrestrial plants (Hashimoto 1986; Tewinkel and Volkmann 1987; Hashimoto and Possingham 1989; Oross and Possingham 1989; Duckett and Ligrone 1993a, b) and algae (Mita et al. 1986; Mita and Kuroiwa 1988; Chida and Ueda 1991; Kuroiwa et al. 1993; Ogawa et al. 1995; Hashimoto 1997), and is thought to be ubiquitous in plants (Kuroiwa 1998; Kuroiwa et al. 1998).

The MD ring and an MD-ring-like structure have been detected only in *C. merolae,* and *Cyanidium caldarium* RK-1 (Kuroiwa et al. 1998). It is thought that the MD ring in other eukaryotes may be too small to be made visible by current techniques. This seems to be analogous to the general trend in the PD ring, which is larger in primitive algae than in land plants. Furthermore, it is difficult to determine the divisional plane of mitochondria in most organisms, because the morphology of the mitochondrion is complex. Inner MD rings have sometimes been detected, but they can not be observed clearly, while the existence of the middle MD ring is not certain (Kuroiwa et al. 1995; Miyagishima et al. 1998b, c).

It is known that the PD and MD rings exist, but the mechanisms of their formation and the ultrastructural changes during their contraction are still unclear. The difficulty in elucidating these details arises because the PD rings of most plants are small and only detected during the late stage of plastid division, and the MD rings have only been detected in two species at present.

We chose *C. merolae* to study the formation and contraction of the PD and MD rings. In *C. merolae*, the PD and MD rings can be observed clearly from the onset of chloroplast and mitochondrial division. Moreover, the cells are very small, only 2–3 μm in length, each cell only has a single chloroplast and mitochondrion, and the mitotic and organelle division cycles can be synchronized on a 12-h light/12-h dark regimen (Suzuki et al. 1994). These features permit repeated, detailed observations of dividing chloroplasts and mitochondria in serial thin sections cut at different angles.

We recently revealed the order of some division events in organelles. The inner PD ring forms before the outer ring. The outer MD ring forms immediately after the outer PD ring has formed. Then the microbody moves from its remote location to the plane of division of the mitochondrion and contraction of the PD and MD rings commences (Miyagishima et al. 1998c).

To elucidate the real-time behavior of the PD and MD rings during their contraction, chloroplast and cell division were continuously video-recorded in living cells under light microscopy, and the three-dimensional morphology of the two rings was analyzed quantitatively by transmission electron microscopy (TEM). Our results revealed that the PD and MD rings contract at a uniform velocity, and that the volumes of the outer PD and MD rings do not change during contraction, while that of the inner PD ring decreases. In this paper, differences in the behavior of the PD and MD rings and the coordinated widenings in the triple PD ring structure are also described.

Materials and methods

Synchronous culture. Cells of Cyanidioschyzon merolae De Luca Taddei and Varano were synchronized using the method of Suzuki et al. (1994). Cells were cultured in Allen's medium (Allen 1959) at pH 2.5. L-shaped culture tubes or flasks containing 1×10^8–2×10^8 cells/mL were shaken under continuous light (40 W/m^2), at 42 °C. The cells were subcultured to give a concentration of 1×10^7 cells/mL and then synchronized by subjecting them to a 12-h light/12-h dark regimen at 42 °C, while the medium was aerated with 5% CO_2.

Time-lapse video recording of chloroplast and cell division. To follow the process of division, a synchronized early M-phase cell culture was collected 6 h after the initiation of the second dark period, and a sample was sandwiched between two coverslips with a 0.15-mm spacer to avoid crushing cells. The margins of the coverslips were sealed with liquid paraffin to protect the cells from drying. The sandwich was left upside down for 15 min to allow cells to stick onto the upper coverslip, and then put on a Thermo Plate (Tokai Hit, Japan) so that observations could be made at different temperatures. Light-field images of cells dividing at different temperatures were recorded with a video camera (C2400–01; Hamamatsu Photonics, Hamamatsu, Japan) and the diameters of the chloroplasts and cells at the equator were measured on the TV monitor at 10,000× magnification.

Fluorescence staining of mitochondria with DiOC$_6$. 3,3′-Dihexyloxacarbocyanine iodide (DiOC$_6$; 100 mg/mL stock in ethanol) was added to the culture medium to give a final concentration of 0.1 mg/mL, according to Suzuki et al. (1994). After staining for 10 min, the cells were washed with fresh medium. The stained samples were examined under an epifluorescence microscope (BHS-RFC; Olympus, Tokyo, Japan) with blue-light excitation. To determine the position of chloroplasts in the same fields, the autofluorescence of chloroplasts was observed with green-light excitation.

Transmission electron microscopy (TEM). Cells were collected by centrifugation and then rapidly frozen in liquid propane that had been cooled with liquid nitrogen (−195 °C). The frozen cells were transferred to 1% OsO_4, dissolved in dry acetone at −80 °C, and incubated for 48 h. Subsequently, the samples were warmed gradually by holding them for 2 h at −20 °C, then for 2 h at 0 °C, and finally for 1 h at room temperature. The samples were

washed with dry acetone at room temperature and embedded in Spurr's resin. Thin sections (90 nm thick) were stained with uranyl acetate and lead citrate, and examined with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

Three-dimensional reconstruction of whole cells. Tracings of the nucleus, chloroplast, mitochondrion, microbody, PD ring and MD ring of each cell were made from photographic prints of serial thin sections. These images were digitized with the program Cosmozone 2 SA (Nikon, Tokyo) and the profiles were entered into a computer (PC9801 DA; NEC, Tokyo) using a digitizing tablet. A three-dimensional reconstruction built by the computer was viewed on a color display and the image was rotated in space until a useful angle of view was obtained. Each three-dimensional image of a whole cell was constructed from 20–25 serial thin sections.

Results

Time courses of chloroplast and cell division, and observation of the mitochondrion. In a previous study, we determined that chloroplast division starts in early M-phase cells around the sixth hour of the second dark period in a synchronous culture (Miyagishima et al. 1998c). The culture was harvested at this point and a video recording of chloroplast and cell division was made at different temperatures. The video images in Fig. 1A show a cell every 20 min for the first 220 min after the onset of chloroplast division, and Fig. 1B shows a cell every 10 min from 340 min after the onset of chloroplast division. These cells were recorded at 45 °C. The chloroplast appears dark grey, while the other parts of the cell appear white. The chloroplast divided in the middle after passing through trapezoidal and dumbbell-shaped forms. Cytokinesis finished in the middle between two daughter nuclei (in the middle of the white part of the cell in Fig. 1B).

The mitochondrion of M-phase cells was not visible with this setup and could not be observed in real time. Cells were stained with $DiOC_6$ and observed by fluorescence microscopy to compare mitochondrial division with the images of cell division shown in Fig. 1A, and the chloroplast was also observed by its autofluorescence (Fig. 1C). Images a1-c1 in Fig. 1C show a chloroplast, a2-c2 show the front view of a mitochondrion, and a3-c3 show the top view of a mitochondrion. In the front view, the mitochondrion, which is on top of the chloroplast, appears to be rod-shaped and forms a V-shape as it

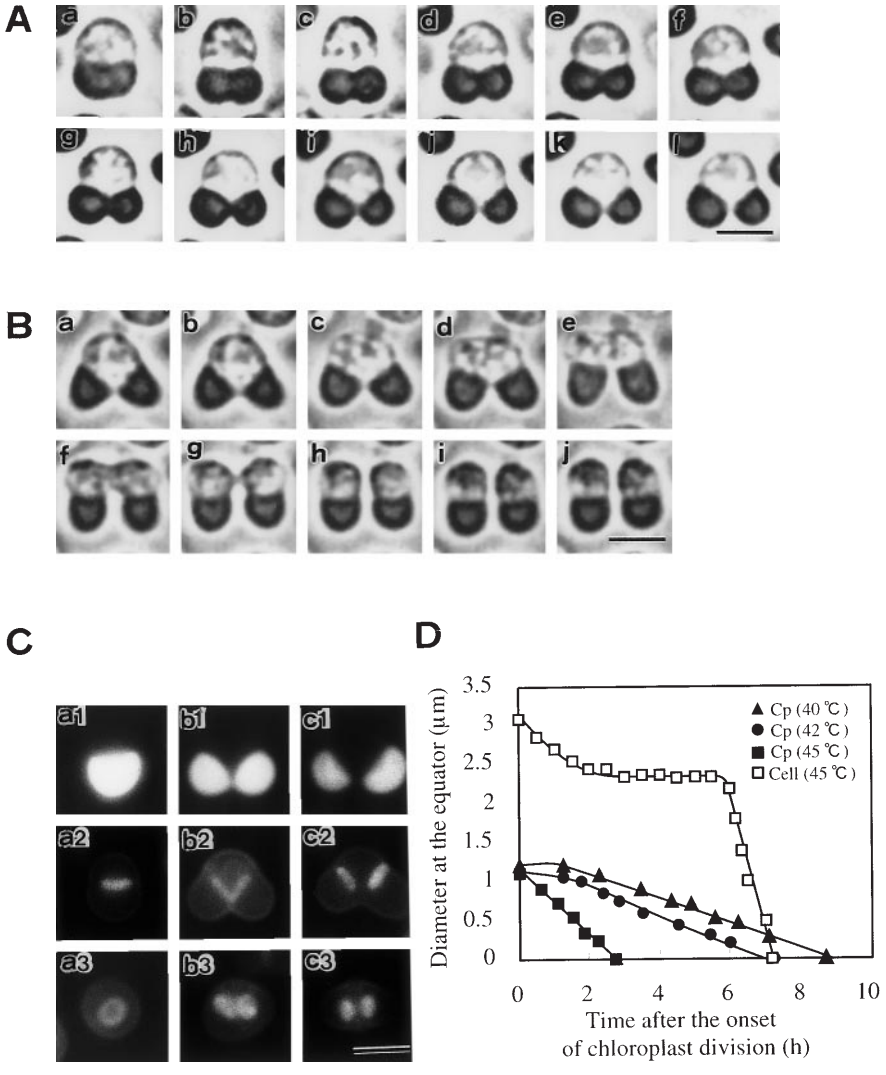

Fig. 1A,B. Time-lapse video images of *Cyanidioschyzon merolae* during the M phase under light microscopy at 450 °C, in which the chloroplasts appear black. The images are shown every 20 min for the first 220 min after the onset of the chloroplast division (**A**), and every 10 min from 340 min after the onset of the chloroplast division (**B**). **C** Images of a cultured M-phase cell, stained with $DiOC_6$. **a1–c1** Autofluorescence of chloroplasts with green-light excitation. **a2–c2** Fluorescent images of mitochondria after staining with $DiOC_6$ showing the same views as **a1–c1**, respectively. **a3–c3** Top views of mitochondria stained with $DiOC_6$. **a1–a3** A-red-blood-cell-shaped mitochondrion in the early M phase. **b1–b3** A dividing mitochondrion that is folded in two at the equator. **c1–c3** Divided mitochondria. **D** Time-course of chloroplast (*C*p) and cell division. The diameters of the chloroplast and cell at the equator are plotted versus time. Chloroplast division at 40 °C, 42 °C and 45 °C, and cell division at 45 °C are shown. Bars = 2 μm (**A–C**)

divides (Fig. 1C a2-c2), while in the top view it appears to have the shape of a red blood cell, and divides via a dumbbell shape (Fig. 1C a3-c3). These images indicated that in three-dimensions the red-blood-cell-shaped mitochondrion divides by bending and constricting at the equator. The cells shown in a1-a3, b1-b3, and c1-c3 correspond to the cells in Fig. 1Aa, Fig. 1Ai, and Fig. 1Aj, respectively, indicating that division of the mitochondrion is completed just after the chloroplast divides, as mentioned by Suzuki et al. (1994).

To obtain details of the changes with time, the diameters of the chloroplast and cell at their equators (constricted furrow) were plotted versus time (Fig. 1D). The speed of chloroplast division depended on the temperature, and chloroplasts divided faster at higher temperatures. However, when the temperature was increased to 50 °C, chloroplast and cell division stopped (data not shown). At any temperature, the diameter of the chloroplast decreased in proportion to time, after a short lag. In other words, the diameter at the equator, which is proportional to the circumference of the PD ring, decreases at a uniform velocity. Initially, the diameter of the cell decreased in step with chloroplast division. After the chloroplast had divided, the diameter of the cell did not change for about 3 h and then decreased at maximum speed for 1 h until cell division was complete. The timing of these events also depended on the temperature (only the data for 45 °C are shown). This indicates that the initial reduction in the cell diameter is due to the reduction in the diameter of the chloroplast at the equator, while the second contraction at a constant speed actively depends on the mechanism of cytokinesis. At any temperature, the speed of cytokinesis was about three times that of chloroplast division. These results reveal that chloroplast division progresses at a constant speed and, for cells observed by TEM, enabled us to estimate the time since the onset of chloroplast division from the diameter of the chloroplast.

Observation by TEM of morphological changes in the PD and MD rings during contraction. Our previous study showed that the PD and MD rings are formed in this order in cells with a trapezoidal chloroplast, and that contraction in the equatorial regions of the chloroplast and mitochondrion starts after the microbody moves into the divisional plane of the mitochondrion (Miyagishima et al. 1998c). The PD and MD rings are believed to cause the contraction at the equator of the chloroplast and mitochondrion. To elucidate the subsequent changes in the PD and MD rings during contraction, a synchronous culture that contained cells with dividing chloroplasts at several stages was harvested at the eighth hour of the second dark period, fixed by the rapid-freeze-substitution method, and observed by TEM (Figs. 2, 3).

Figure 2A shows a serial thin section of a cell at the onset of the contraction of the PD ring. This cell was cut perpendicularly to the equator at the center of the cell, chloroplast and mitochondrion, and gives the diameter of the equator. The PD and MD rings were observed at the equators of the chloroplast and mitochondrion, respectively. The chloroplast is trapezoidal in shape, while the mitochondrion is concave where it is next to the cell nucleus, causing the red-blood-cell shape. The microbody attaches to the mitochondrion at the equator. At a higher magnification of a serial thin section of the same cell, the outer, middle, and inner PD rings and outer MD ring were clearly seen, but as for MD rings, structures on the stromal face or in the intermembrane space were not detected. The outer, middle, and inner PD rings were 30×10 nm (width × thickness), $50 \times < 2$ nm, and 80×5 nm, respectively. The middle PD ring was less than 2 nm thick and an accurate measurement could not be made. There is a difference in the width of the outer MD ring, between where it is adjacent to the cell nucleus and where it is adjacent to the chloroplast. The former width was 80 nm and the latter 30 nm (Fig. 2B, C). Figure 2D shows a section of a cell at the same stage, cut parallel to and at the equator. The mitochondrion is concave in shape. The microbody, which is spherical in interphase (data not shown), starts to elongate at the equator of the mitochondrion. Only a part of the PD and MD rings can be seen in this section and a complete image was constructed from three serial sections. The equator of the chloroplast was elliptical and its circumference was 3,300 nm, while that of the mitochondrion was 2,200 nm.

Figure 2E shows a section of a cell halfway through chloroplast division, cut perpendicularly to the equator at the center of the chloroplast, mitochondrion and cell. As contraction progresses, the chloroplast becomes dumbbell-shaped, and the red-blood-cell-shaped mitochondrion bends into a V-shape (Fig. 2E, G). At a higher magnification of a serial thin section of the same cell, the outer, middle, and inner PD rings were 35×15 nm (width × thickness), $65 \times < 2$ nm, and 90×5 nm. The outer MD ring was 15 nm thick and 80 nm wide adjacent to the cell nucleus and 28 nm wide adjacent to the chloroplast (Fig. 2F, G). In a section of a cell at the same stage cut parallel to and at the equator, the circumferences of the PD and MD rings measured from two serial thin sections were about 2,300 nm and 2,200 nm, respectively (Fig. 2H). Although, in this section, the elongated microbody appears to be split into two parts, three-dimensional observations of serial thin sections showed that the microbody did not split, but elongated along the outer MD ring. The length of the MD ring is much the same as it was in Fig. 2D, indicating that the PD ring starts to contract before the MD ring.

At the late stage of chloroplast division (when its diameter was 150 nm), the mitochondrion was bent even more, and the three PD rings became considerably wider and the outer PD ring became thicker, while the dimensions of the MD ring did not change much (Fig. 2I). At a higher magnification, the dimensions of the outer, middle, and inner PD rings were 100×15 nm (width × thickness), $110 \times < 2$ nm, and 120×5 nm (Fig. 2J), indicating that the widths of the three PD rings, which differed in the early stage of contraction,

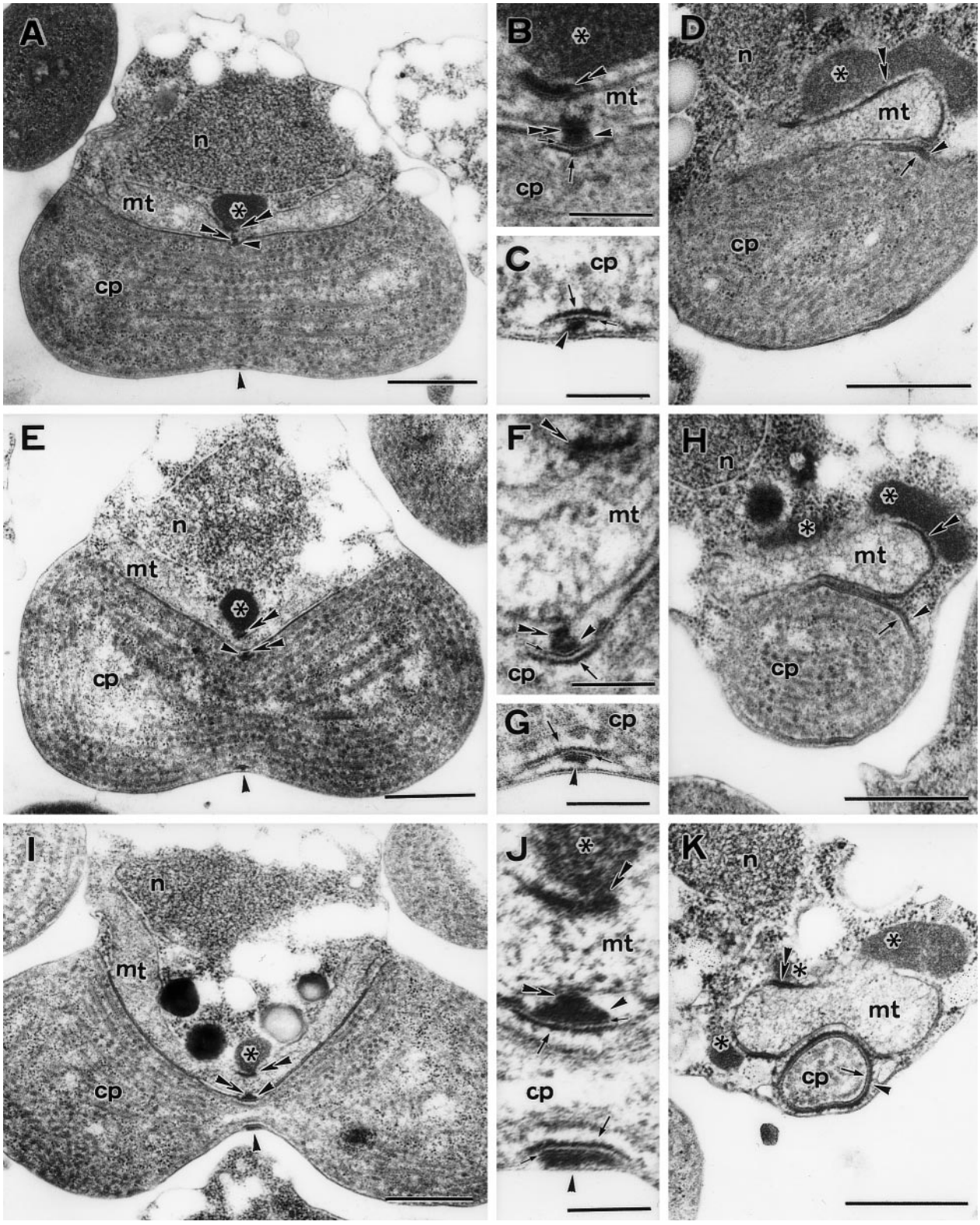

Fig. 2A–K. Electron micrographs of thin sections of M-phase *C. merolae* cells cut perpendicular to (**A–C, E–G, I, J**) and parallel to the divisional plane (**D, H, K**) at the center of the chloroplast, mitochondrion and cell, showing the early (**A–D**), middle (**E–H**), and late (**I–K**) stages of chloroplast division. The high-magnification images of the PD and MD rings from serial thin sections of the same cell in **A** (**B, C**), **E** (**F, G**) and **I** (**J**) are also shown. *Arrowhead*, the outer PD ring; *small arrow*, the middle PD ring; *large arrow*, the inner PD ring; *double-arrowhead*, the outer MD ring; *cp*, chloroplast; *mt*, mitochondrion; *n*, nucleus; *, microbody. Bars = 500 nm (**A, D, E, H, I, K**) and 100 nm (**B, C, F, G, J**)

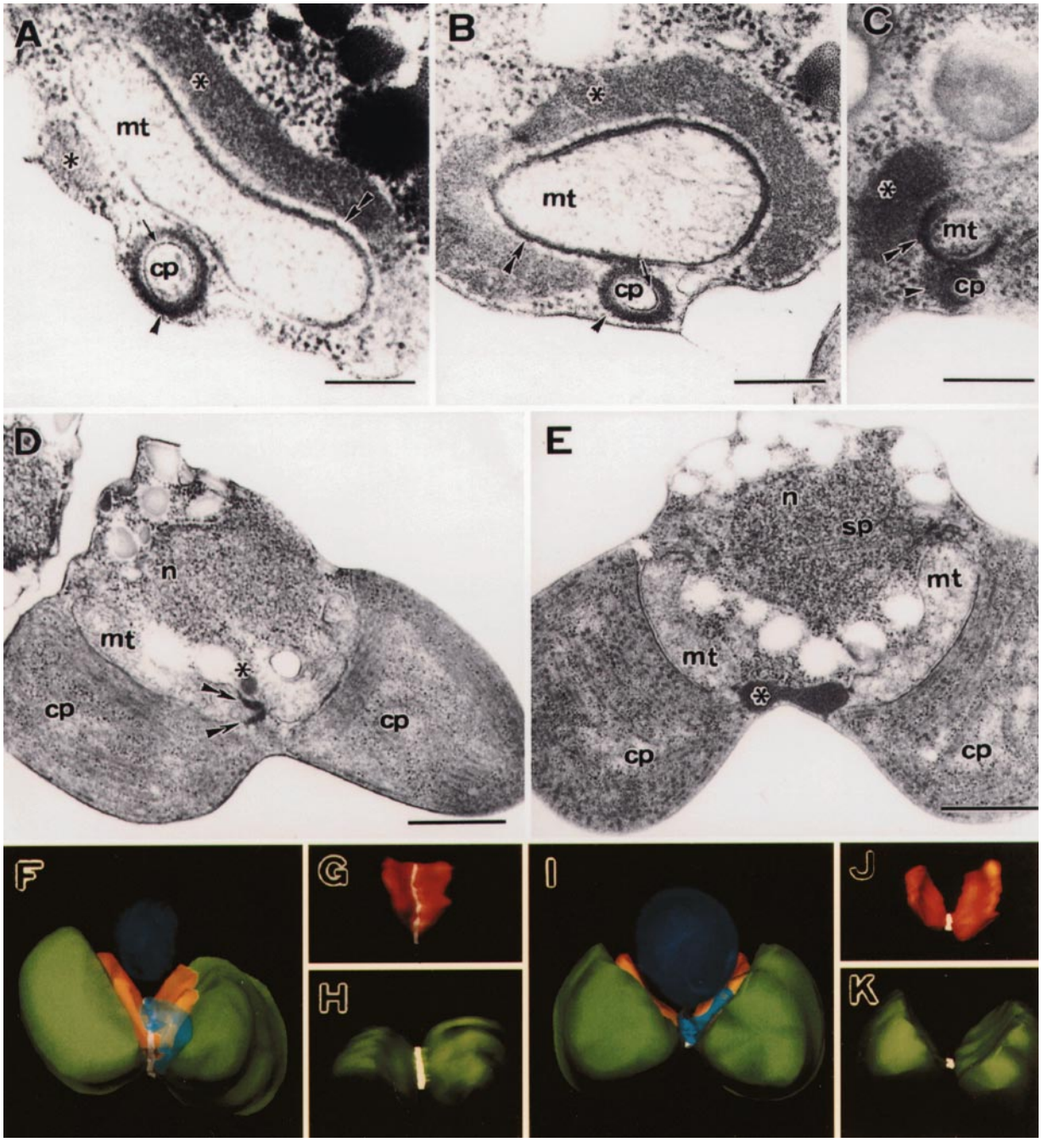

Fig. 3A–E. Electron micrographs of thin sections of *C. merolae* cells cut parallel to (**A–C**) and perpendicular to (**D, E**) the divisional plane at the center of the chloroplast, mitochondrion and cell. **A–C** Cells at the last stage of chloroplast division. **D** A cell in which the chloroplast has divided while the mitochondrion is dividing. **E** A cell in which the chloroplast and mitochondrion have both divided. **F–K** Three-dimensional images of whole cells reconstructed from serial thin sections, showing a cell halfway through chloroplast division in which the MD ring has not started to contract (**F–H**) and a cell in the last phase of chloroplast division in which the MD ring is contracting (**I–K**). **G** and **J** are the mitochondria of the cells shown in **F** and **I**, respectively, and **H** and **K** are the chloroplasts of the same respective cells. *Arrowhead*, the outer PD ring; *arrow*, the inner PD ring; *double-arrowhead*, the outer MD ring; *cp*, chloroplast; *mt*, mitochondrion; *n*, nucleus; *, microbody. Bars = 200 nm (**A–C**) and 500 nm (**D, E**). *Semitransparent blue*, nucleus; *green*, chloroplast; *brown*, mitochondrion; *light blue*, microbody; *white*, outer PD and MD rings

subsequently became similar during contraction. The MD ring was 18 nm thick and 35 nm wide adjacent to the chloroplast, but 110 nm wide adjacent to the cell nucleus. Figure 2K shows a section of a cell (the diameter of the chloroplast is 260 nm) at a stage just before those shown in Fig. 2I, J. The circumferences of the PD and MD rings were 1,100 nm and 2,200 nm respectively. The MD ring had not yet started to contract. As in Fig. 2H, while the microbody appears to be split into three parts in this section, in a three-dimensional view it is not split.

Figure 3A–C shows sections of cells in the last stage of chloroplast division, cut just parallel to and at the equator. In these sections, elongated microbodies surround the MD ring and touch the PD ring (Fig. 3B). In

Fig. 3A and 3C, only a part of the microbody can be observed, but the complete structure determined from serial thin sections is much the same as in Fig. 3B. In the cell shown in Fig. 3A, the chloroplast was 110 nm in diameter and the circumferences of the PD and MD rings were 480 nm and 1,950 nm, respectively. The circumference of the MD ring in Fig. 3A is smaller than in Fig. 2D, 2H, and 2K, indicating that the MD ring has started contraction in the cell shown in Fig. 3A. In Fig. 3B, the chloroplast was 70 nm in diameter and the circumferences of the PD and MD rings were 260 nm and 1,500 nm respectively. In the final phase of chloroplast division, the circumferences of the PD and MD rings were 180 nm and 200 nm (Fig. 3C). These figures illustrate that the onset of contraction of the MD ring is delayed relative to that of the PD ring, but the contraction of the MD ring subsequently catches up with that of the PD ring, although the chloroplast completes its division before the mitochondrion. The widths of the MD ring adjacent to the chloroplast and to cell nucleus become equal. The MD ring was 140 nm long and 40 nm wide (Fig. 3D). When the mitochondrion divided, the microbody elongated perpendicular to the equator at the bottom of the cell, and the mitotic spindle formed (Fig. 3E).

The contractile process was also verified by three-dimensional reconstruction of a whole cell from 20–25 serial thin sections (Fig. 3F–K). In the middle of chloroplast division, the chloroplast is dumbbell-shaped, while the mitochondrion looks like a bent red blood cell, and the microbody elongates along the outer MD ring. The PD ring contracts first (Fig. 3F,H). When chloroplast division is almost complete, the MD ring starts to contract, and it then catches up with the contraction of the PD ring. The two daughter chloroplasts become cup-shaped, while the two daughter mitochondria are concave on each side (Fig. 3I–K).

Quantitative analyses of the PD and MD rings during contraction. The results discussed above showed that the PD and MD rings contract in different ways, and that their widths and thickness also change in different ways. To follow their contraction over time more accurately, the circumferences of the rings were plotted versus the diameter of the chloroplast at the equator, which decreased in proportion to time as mentioned above (Fig. 4). Their circumferences were measured on sections cut parallel to and at the equator (e.g. Fig. 3A–C). We used the short axis of the ellipse of the PD ring as its diameter. In this graph, shown in Fig. 1D, the abscissa, which is the diameter of the chloroplast, can be regarded as the time axis. The result clearly shows the contractile process of the two rings and the difference between them. Their circumferences decrease at uniform velocities. At 45 °C, the contractile velocity of the PD ring is 25 nm/min and that of the MD ring is 90 nm/min. Although the MD ring forms immediately after the PD ring, it does not start to contract until after the circumference of the PD ring has decreased to 200 nm. Then the MD ring starts to contract at about 4 times the speed of the PD ring and catches with to the contraction of the PD ring.

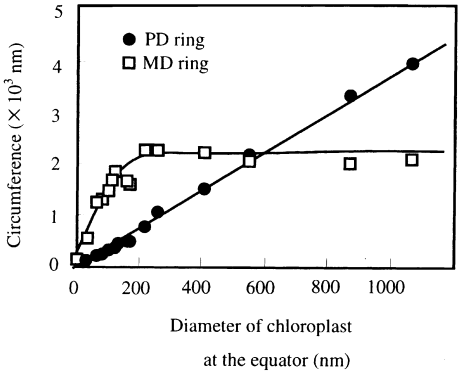

Fig. 4. Circumference of the PD (●) and MD rings (□) versus the diameter of the chloroplast. The circumferences were measured in thin sections of *C. merolae* cut parallel to the divisional plane at the equator. Since the diameter of chloroplast at the equator decreases linearly with time, the abscissa provides a time axis that can be read from right to left. Data points represent single measurements

To clarify the morphological changes of the PD and MD rings, the width, thickness, cross-sectional area, and volume of the outer, middle and inner PD rings, and the outer MD ring were plotted versus the diameter of the chloroplast at the equator in the same manner (Fig. 5). The width, thickness, and cross-sectional area were measured in serial thin sections cut perpendicularly to the rings in the middle of the cell. The volume was calculated by multiplying the cross-sectional area and the circumference for a given diameter of the chloroplast obtained from Fig. 4. Since the width of the MD ring varied, the volume was estimated approximately using the average cross-sectional area for the widths next to the cell nucleus and chloroplast.

In the early phase of contraction, the widths of the PD rings increase from the outer to the inner ring. As contraction progresses, the widths change at different rates and become roughly equal. Before contraction, the MD ring is wider next to the cell nucleus than it is next to the chloroplast. During contraction, the width of the MD ring becomes uniform. While the outer PD and MD rings become thicker during contraction, the width of the inner PD ring remains a constant 5 nm throughout contraction. As mentioned above, the middle PD ring was so thin that its thickness could not be measured, but observations showed that its thickness does not change during contraction. The cross-sectional areas of the outer PD and MD rings increase with contraction, while that of the inner PD ring increases slightly in the late phase of contraction. The width, thickness, and cross-sectional area of the MD ring barely change until the diameter of the chloroplast decreases to 200 nm, and then the MD ring starts to contract. The volumes of the rings were calculated by multiplying the cross-sectional area and circumference. The volumes of the outer PD and MD rings remain constant throughout their contraction, while that of the inner PD ring decreases constantly. These results reveal clear differences in the contraction of these ring structures, and the coordinated changes in the widths of the three PD rings.

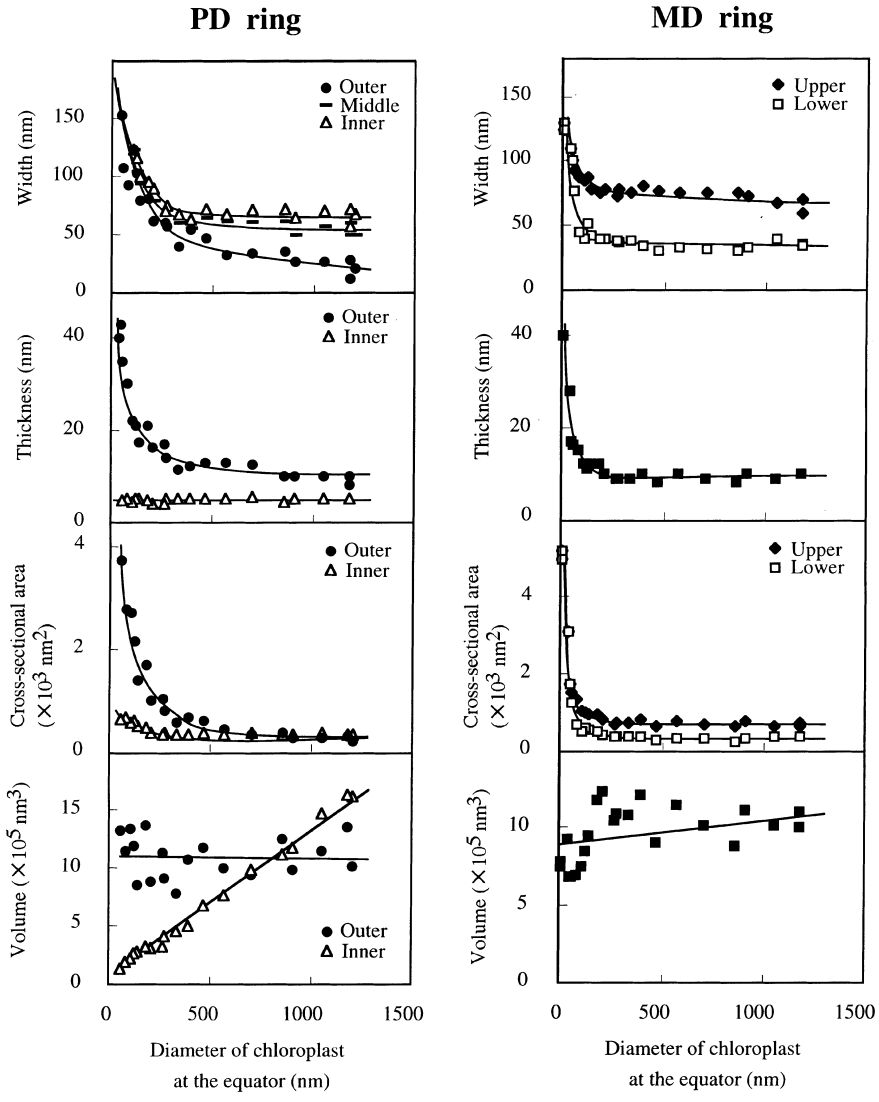

Fig. 5. Width, thickness, cross-sectional area, and volume of the PD and MD rings versus diameter of the chloroplast. The width, thickness, and cross-sectional area were measured in thin sections of *C. merolae* cut perpendicular to the divisional plane at the center of the chloroplast, mitochondrion and cell. The volumes were calculated from the cross-sectional area and the circumferences in Fig. 4.●, the outer PD ring; –, the middle PD ring; △, the inner PD ring; ■, MD ring; ◆, MD ring adjacent to the cell nucleus (*upper*); □, MD ring adjacent to the chloroplast (*lower*). Since the diameter of chloroplast at the equator decreases linearly with time, the abscissa provides a time axis that can be read from right to left. Data points represent single measurements

Discussion

Morphological changes in the cell and the chloroplast during the M phase. We observed the cell division of *Cyanidioschyzon merolae* by time-lapse video recording. Previously, the multiplication of chloroplasts in living cells has been observed in *Nitella* (Green 1964). Our system has several advantages. The chloroplast stands out under light microscopy, without the need for fluorescence microscopy and excitation light that may damage cells. Cells can live for at least 48 h with continuous video recording. With a synchronous culture, the division of many chloroplasts can be observed clearly and the time course defined. Furthermore, altering the temperature can change the time course. This feature allows this system to be used for several different analyses.

In *C. merolae*, the speed with which the M phase progresses largely depends on the temperature. The diameter of the cell at the equator decreased in proportion to the decreasing size of the chloroplast at the equator. After an interval, which was about 3 h at 45 °C, active contraction of the equator of the cell was observed. During this interval, mitotic spindles were observed in cells (Fig. 3E). When cells were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI), cells with either one or two cell nuclei were both observed during this interval under fluorescence microscopy (data not shown). This indicates that the separation of chromosomes and nuclear division occur during this interval, and that cytokinesis starts after cell nuclear division, as in other eukaryotes. Cytokinesis, chloroplast division, and mitochondrial division progress at constant speeds. In sea urchin eggs, it has been reported that during cytokinesis the diameter of the cell decreases at a constant rate after a certain delay (e.g. Mabuchi 1994). Therefore, the constant contraction is a characteristic common to the division apparatuses of the cell, mitochondrion and chloroplast.

Contraction of the inner, middle, and outer rings. To elucidate the morphological changes in the PD and MD rings, which are believed to cause chloroplast and mitochondrial division in the manner described above, these ring structures were observed quantitatively under TEM, using the diameter of the chloroplast at the

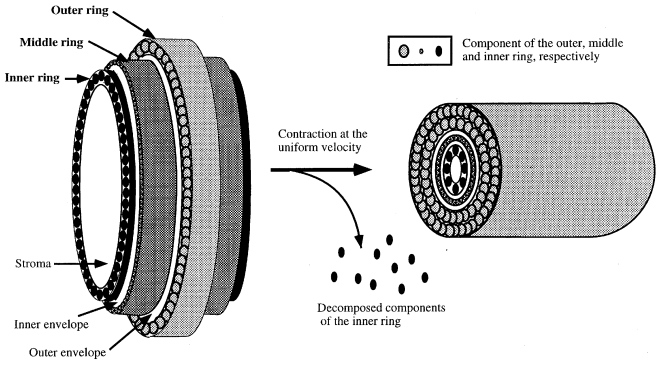

Fig. 6. Schematic representation of the morphological changes in the triple ring structure of the *C. merolae* PD ring. The actual components of the rings are unknown. The PD ring contracts at a uniform velocity. In the early stage of contraction, the widths of the rings increase from the outer to the inner ring (*left*). As the rings contract, the widths become equal (*right*). The outer ring grows thicker and contracts keeping its volume constant, while the thickness of the inner ring does not change and its volume decreases linearly

equator as the index of time. The changes in the PD ring are summarized in Fig. 6. In the early stage of contraction, the width of the PD rings increases from the outer to the inner ring (Fig. 6, left). Contraction occurs at a uniform velocity, and all three rings become wider. By the final stage of contraction, the widths of all three rings are roughly equal (Fig. 6, right). The outer ring grows thicker and keeps a constant volume while it contracts, while the thickness of the inner ring does not change and its volume decreases at a constant rate with contraction. The outer MD ring contracts in the same manner as the outer PD ring. Although the middle and inner MD rings were not observed clearly, they were sometimes observed faintly (data not shown) in sections cut parallel to the divisional plane. So a similar phenomenon to that seen with the PD ring likely occurs in the MD ring. Other fixation methods may be needed to make the MD rings visible. The densities of each PD and MD ring at all stages of their contraction appear to be constant, indicating that the material that makes up these rings does not become compressed during contraction. Although the components of the three PD rings and the outer MD ring have never been identified, the results indicate that the number of components of the outer ring does not change during contraction, while the constituents of the inner ring are decomposed or consumed with its contraction (Fig. 6). In *Cyanidium caldarium,* actin-like filaments approximately 5–7 nm in diameter have been observed in tangential sections of the outer PD ring (Mita and Kuroiwa 1988). This suggests that the filamentous outer PD ring contracts by the sliding of these filaments in the same way as the actomyosin system and that the components of the filaments would not change with contraction. The volume of the contractile ring of the sea urchin egg decreases as cytokinesis proceeds; its diameter decreases while the thickness and width remain unchanged (Schroeder 1972). The density of the contractile ring filaments also does not change during cytokinesis in HeLa cells (Schroeder 1970). These results suggest that the actin filaments decompose or disintegrate after they slide over the myosin filaments (Schroeder 1975). The outer PD ring is markedly different from these contractile rings for cytokinesis. As for the inner PD ring, although a filamentous structure has not been observed and it becomes slightly wider in the late stage of contraction, it seems to operate with a mechanism similar to that of cytokinesis during contraction. The volume of the middle ring could not be calculated because it is too thin to be measured precisely, but the thickness of the middle ring likely did not change during any stage of contraction, and its volume seemed to decrease with contraction. The differences between the inner, middle, and outer rings suggest that they have different roles in plastid division, and that they may have different origins. The inner PD ring may be a structure descended from cyanobacteria and composed of FtsZ or other proteins involved in bacterial division. *Arabidopsis* FtsZ translation product is imported into the chloroplast in vitro (Osteryoung and Vierling 1995). In *Physcomitrella patens*, the targeted disruption of *fts*Z (Pp*fts*Z) inhibits chloroplast division and leads to the formation of giant chloroplasts (Strepp et al. 1998). The middle and outer rings may have been gained during the process of evolution, from endosymbionts to plastids, to allow regulation by the cell nucleus.

In *Cyanidium caldarium,* it has been reported that the width and thickness of the outer PD ring increase with contraction (Mita and Kuroiwa 1988). On the other hand, in spinach, in which the PD ring can only be detected in the late phase of plastid division, the cross-sectional area and volume of the outer plus inner PD rings decrease during the terminal phase of contraction (Oross and Possingham 1989). The decrease in the volume of the inner plus outer PD rings is consistent with this study, while the decrease in the cross-sectional area conflicts with our result. In ferns, it is reported that in the last phase of division, the outer PD ring becomes invisible, while the inner PD ring is clearly visible (Duckett and Ligrone 1993b). But in *C. merolae* (Fig. 3A–C) and *Cyanidium caldarium* (Kuroiwa et al. 1998), the width and thickness of the outer ring increase continuously until the final stage of contraction. The difference may be due to the difficulty in detecting the PD ring in higher plants, in which the PD ring is very small and can be detected only in the late phase of plastid division.

In the early stage of contraction, the widths of the PD rings increase from the outer to the inner ring, but the widths change at different rates with contraction, and eventually become equal. In the green alga *Nannochloris bacillaris*, the PD ring can be detected from the early stage of chloroplast division and the same phenomenon is reported for the outer and inner PD rings (Ogawa et al. 1995). In most plant cells the PD ring can only be detected during the last phase of plastid division and the outer and inner PD rings have essentially the same width. These facts suggested that this coordinated widening of the triple ring structure of the PD ring, at least for the inner and outer PD rings, is highly conserved throughout the plant kingdom, although at

present the middle ring has only been identified in *C. merolae* and the inner ring has not been identified in some plants (Tewinkel and Volkmann 1987; Chida and Ueda 1991; Hashimoto 1997). It is likely that the outer PD ring cannot be identified under TEM in higher plants because its cross-sectional area is so small in the early stage of contraction, since the plastids of higher plants are larger than those of primitive algae such as *C. merolae*, *Cyanidium caldarium* and *N. bacillari*s and the volume remains constant throughout contraction. However, the cross-sectional area of the inner ring does not change greatly, so the inner PD ring should be observed during the early stage of plastid division in higher plants. More observations are needed. At present, it is not clear how the three components of the triple ring structure cooperate during division. Nevertheless, three facts suggest that the inner PD ring may specialize in localizing the plane of division and play an essential role in the formation and widening of the outer and middle rings: the inner ring is formed before the outer ring; the inner ring is wider than the outer ring in the early phase of contraction; and the widths of the three rings become the same at the late phase of contraction.

Differences between the PD and MD rings. While any part of the PD ring has the same width in cross-section, the MD ring is wider adjacent to the cell nucleus than it is next to the chloroplast, except in the late stage of contraction. The difference in the width of the MD ring is thought to be connected to the morphology of the mitochondrion. During the M phase, the red-blood-cell-shaped mitochondrion bends. This likely results from a difference in stress between the side next to the cell nucleus and the side adjacent to the chloroplast. The width of the MD ring is not uniform between the two sides before this bending occurs (Fig. 2A,B), suggesting that the different widths of the MD ring on the two sides produce the stress that bends the dividing mitochondrion.

The onset and speed of contraction differ between the PD and MD rings. The MD ring is formed soon after the PD ring (Miyagishima et al. 1998c). The PD ring starts to contract just after it forms, while the MD ring starts to contract later, at about 4 times the speed of the PD ring, when contraction of the PD ring is almost complete (the diameter of chloroplast at the equator is 200 nm). This indicates that chloroplast and mitochondrial division result from different molecular mechanisms, and suggests that there is an unknown signal that prompts the MD ring to start contracting. When *C. merolae* cells are treated with aphidicolin, a DNA polymerase inhibitor, the duplication of nuclei and cytokinesis stop. In this condition, the PD and MD rings form and the chloroplast divides repeatedly, producing four or more daughter chloroplasts per cell, while the mitochondrion does not divide. It is suggested that the checkpoint control of the nucleus functions in mitochondrial division (Itoh et al. 1996, 1997). Considering these results together with our present results, perhaps when a cell passes the checkpoint, an unknown nuclear signal stimulates the MD ring to start its contraction. This also implies that mitochondrial division is more strongly controlled by the cell nucleus than chloroplast division.

Behavior of the microbody in relation to contraction of the PD and MD rings. Each *C. merolae* cell has a single microbody. When it is observed under fluorescence microscopy in interphase, the microbody is spherical. However, in M phase it changes position and shape several times in an orderly fashion (Miyagishima et al. 1998a). A previous study showed that when the PD and MD rings are formed, the rod-shaped microbody moves to the equator from its remote location and touches the MD ring, and then the PD ring start to contract (Miyagishima et al 1998c). In our study, the microbody elongated at the equator along the MD ring, and its edge reached the PD ring during the late phase of contraction. These observations suggest that the microbody may take part in the contractile process of the PD and MD rings. In the green alga *Trebouxia potteri* (Chida and Ueda 1991) and some terrestrial plants (Oross and Possingham 1989), ER profiles are seen in close association with the outer PD ring and are thought to participate in the process of contraction (Chida and Ueda 1991). Perhaps membranous structures such as the microbody of *C. merolae* and the ERs of some plants release a substance that leads to the contraction of the PD and MD rings. We are working to clarify the behavior of the microbody in detail, and the interaction between the microbody and the PD and MD rings.

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