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Cytomorphogenesis in Coenocytic Green Algae. IV. The Construction of Cortical Microtubules during Lenticular Cell Formation in *Valonia utricularis*

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The siphonocladalean alga *Valonia utricularis* consists of one or more giant multinucleate cells. In this alga, cell division occurred by the splitting off of protoplasm into a lenticular cell. Changes in the arrangement of cortical microtubules (MTs) and nuclei during lenticular cell formation were examined by an indirect immunofluorescent technique, using a confocal laser scanning microscope. Cortical MTs were oriented approximately parallel to the cell axis when chloroplasts were distributed evenly throughout the cytoplasm. The first visible sign of cell division was a small assembly of chloroplasts at a specific site in the cell. A radial array of cortical MTs was constructed at the center of the assembly and extended well beyond the assembly, but in other areas the MT orientation was unchanged. Further assembly of chloroplasts led to a larger disc-shaped aggregate of protoplasm, within which a number of mitoses occurred. A radial array of MTs disassembled into short random microtubules. The protoplasm then divided into a lenticular cell by a septum wall, which was produced inwardly from the cell wall at the periphery of the protoplasm. The lenticular cell grew out to develop into a mature obovoid cell, simultaneously developing a new parallel array of MTs. When local aggregation of protoplasm occurred, treatment with a microtubule inhibitor (amiprophos methyl, APM) made chloroplasts assembling in the protoplasm disperse, but did not cause the nuclei to migrate. These results suggest that a radial array of cortical MTs provides positional information for local aggregation of protoplasm and synchronous mitoses in this alga.

Key words: cell division – coenocytic green algae – cortical microtubules – nuclear division – Siphonocladales – *Valonia utricularis*

Coenocytic green algae consist of giant multinucleate cells, in which morphogenesis is involved in local growth and differentiation and is regulated in time and space. Cytoskeletal arrays including microtubules and actin filaments are present in coenocytic green algae, but they seem to be unique in appearance and regulation: cortical microtubules (MTs) are extremely long and parallel to each other and persist without depolymerization during mitosis (La Claire, 1987; Okuda *et al.*, 1990a); perinuclear MTs radiate from the peripheries of each interphase nucleus (La Claire, 1987); actin filaments appear when cells are wounded, and are thus involved in protoplasmic retraction (La Claire, 1989). In land plants and some fresh water green algae, cortical MTs help to form an array of cellulose microfibrils parallel to the MTs to regulate the direction of cell growth (Lloyd, 1984). However, such parallelism between cortical MTs and cellulose microfibrils does not always appear in coenocytic green algae (Okuda and Mizuta, 1987). Instead, cortical MTs in these algae are involved in the creation and maintenance of cellular polarity (Okuda *et al.*, 1993).

Valonia is a coenocytic green algal genus belonging to the Siphonocladales. The thallus cells are obovoid to clavate in shape, each of which has a huge central vacuole and a thin parietal layer of

This paper is dedicated to the late Professor Mitsuo Yamanaka in honor of his contributions to Kochi University.

protoplasm containing many nuclei and chloroplasts. A characteristic manner of cell division is known to occur in *Valonia* (van den Hoek *et al.*, 1995): During cell division, a local accumulation of nuclei and chloroplasts is cut off by a domed cell wall, forming a small lens-shaped daughter cell. The cell division is generally called lenticular cell formation (Bold and Wynne, 1985). So far, there has been no cell biological study on lenticular cell formation. Herein we describe dynamic changes in the arrangement of cortical microtubules and concomitant nuclear division occurring in the processes of lenticular cell formation in *Valonia utricularis*.

MATERIALS AND METHODS

Valonia utricularis (strain LB 2357) was obtained from UTEX, the algal culture collection at the University of Texas at Austin. The thalli were cultured in nutrient-enriched sea water medium (PES medium) (Starr and Zeikus, 1987), under 22 °C and a 14:10 h light : dark cycle. Cool white fluorescent lamps, with an intensity of ca. 1 W/m^2, were used. For induction of lenticular cell formation, thallus cells that had not produced any daughter cell were used. They were transferred into a petri-dish containing sterilized sea water without any enrichment and cultured for several days under the same temperature and photoregime conditions, except that the light intensity was 4 W/m^2.

The procedures for indirect immunofluorescent staining of microtubules were essentially those of Okuda *et al.* (1990b), except for the concentration of fixative. Cells were fixed for 10-15 min with a mixture of 1.6 ml 15% paraformaldehyde, 0.75 ml 8% glutaraldehyde, 1.65 ml H_2O and 2 ml buffer with a twofold concentration of MTSB (MTSB containing 587 mM NaCl, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ethylene glycol-bis-(ß-aminoethyl ether)- N,N,N',N'-tetraacetic acid and 50 mM piperazine-N,N'-bis-(2-ethanesulfonic acid), pH 7.0). The fixed cells were immersed in fresh MTSB and cut into several pieces with a fine razor to remove vacuolar inclusions. These pieces were then immersed in phosphate buffered saline (PBS). In PBS, thin protoplasmic layers of the pieces were removed from the cell wall, mounted on poly-L-lysine-coated coverslips, and then incubated in PBS containing 2% Nonidet P-40 and 0.5 mg/ml $NaBH_4$ for 1 h. Anti-ß-tubulin (1:200 dilution of stock solution from YL1/2, Sera Labs, Crawley Down, England) was applied to the specimens on the coverslips. This was followed by application of FITC-labelled anti-rat IgG (1:150 dilution of stock solution from Miles Scientific, Naperville, Illinois, USA). Nuclei were stained with 1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI). Fluorescence from FITC and DAPI was observed with a confocal laser scanning microscope (MRC-600, Bio-Rad) and an Olympus epifluorescence microscope (BHF-BH2-RFL), respectively.

For electron microscopy, thallus cells were fixed in 60% sea water containing 4% glutaraldehyde, 1 mg/ml ruthenium red and 0.05 M sodium cacodylate (pH 7.2) at 5 °C for 2 h. The fixed cells were rinsed with 70% sea water, post-fixed in 0.05 M cacodylate buffer (pH 7.2) containing 1% OsO_4 and 0.5 mg/ml ruthenium red at 5 °C for 2 h, and then dehydrated with acetone. The dehydrated specimens were finally embedded in Spurr resin. Thin sections were made on a Reichert Om U2 ultra-microtome, mounted on Formvar-coated grids, and then stained with 1% uranyl acetate followed by lead citrate. Sections were observed with a JEOL JEM 100U electron microscope.

Amiprophos-methyl (APM) is a potent inhibitor of plant microtubule polymerization (Morejohn and Fosket, 1991). The APM used in the present study was kindly donated by Nihon Bayer Agrochem K. K. Tokyo. It was dissolved in sterilized sea water containing 0.1% dimethylsulfoxide to a final concentrations of 10 µM as described by Okuda *et al.* (1993).

RESULTS

In the thallus of *Valonia utricularis*, a mother cell produced some lenticular cells on its lateral cell wall (Fig. 1A). The lenticular cells developed into mature clavate cylindrical cells. In the mature cell before lenticular cell formation, chloroplasts were distributed evenly throughout the cytoplasm. Nuclei

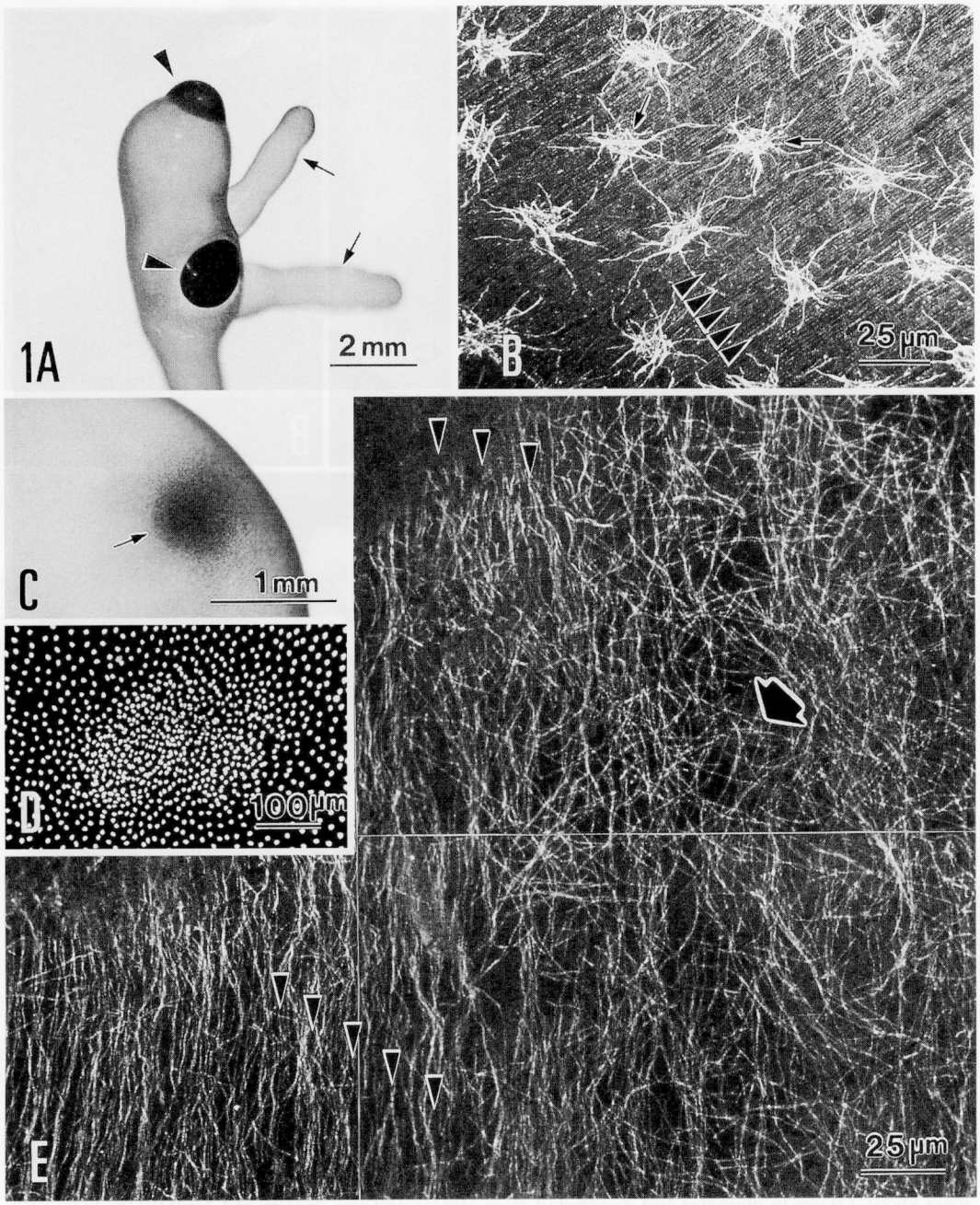

Fig. 1 Lenticular cell formation of *Valonia utricularis*. A. A part of vegetative thallus. Young (arrowheads) and developing (arrows) lenticular cells. B. Indirect immunofluorescence images of cortical (arrowheads) and perinuclear (arrows) MTs. C-E. Stage I in lenticular cell formation. C. Surface view of a local chloroplast assembly (arrow). D. Fluorescence images of nuclei in the area of a chloroplast assembly. E. Immunofluorescence images of cortical MTs. Disoriented MTs (arrow) in the area of a chloroplast assembly and parallel MTs (arrowheads) in the outside of the area.

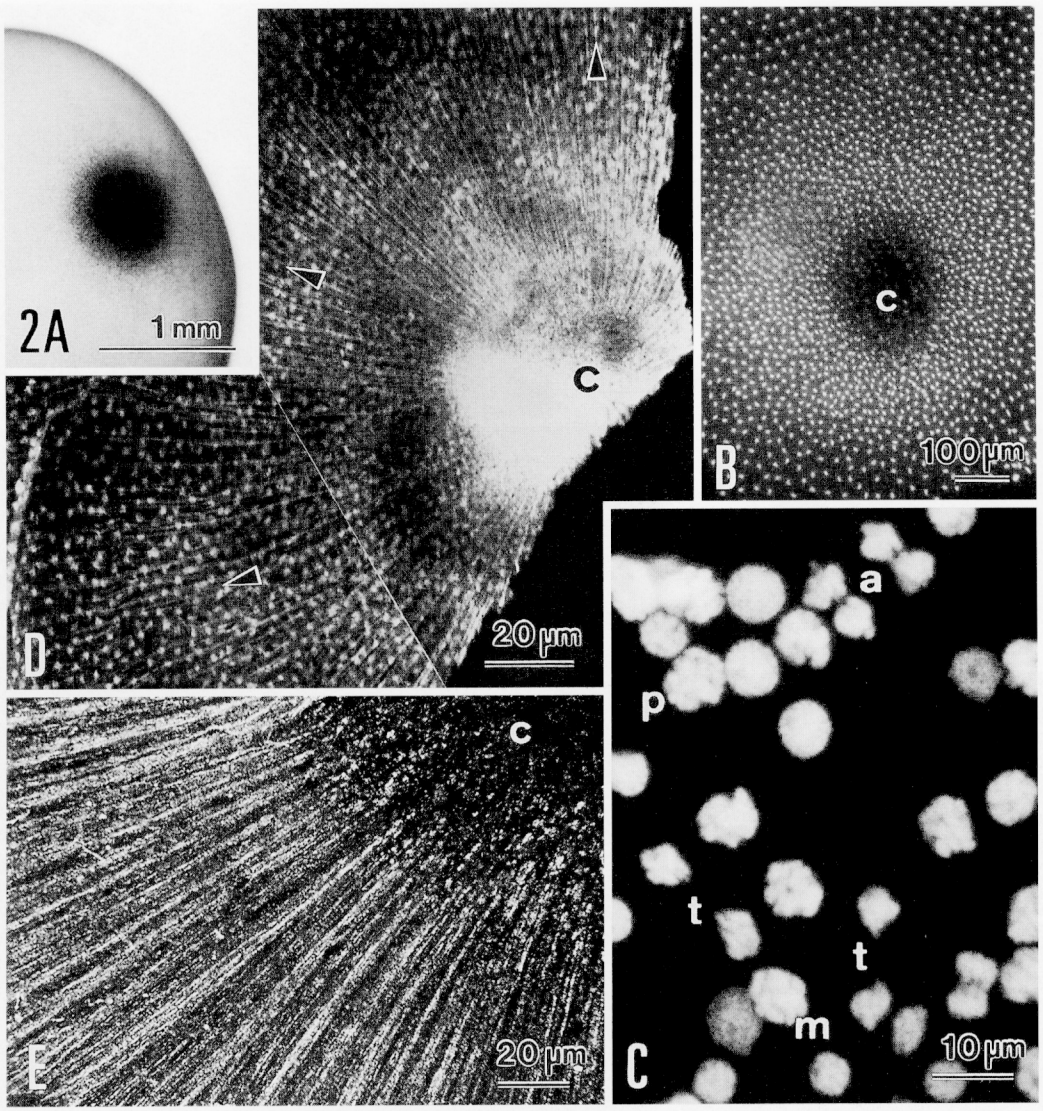

Fig. 2 Stage II in lenticular cell formation of *Valonia utricularis*. A. Surface view of a chloroplast assembly. B. Fluorescence images of nuclei in the area of a chloroplast assembly. c, center of a chloroplast assembly. C. Various stages of mitoses. Prophase (p), metaphase (m) and anaphase (a) nuclei. D. A radial array of cortical MTs. Direction of MTs (arrowheads) from the center of a chloroplast assembly (c). E. High magnification of a radial array of cortical MTs. c, center of a chloroplast assembly.

were about 7.9 μm in diameter and distributed at an almost uniform density ($12.2 \pm 0.9/10^4$ μm^2) in the zone of protoplasm below the zone containing the chloroplasts. Perinuclear MTs surrounded the nucleus and some of them extended outward (Fig. 1B). Cortical MTs lay just on the inner side of the plasma membrane and were oriented approximately parallel to the cell axis (Fig. 1B). In the present study, we divided the processes of lenticular cell formation into five stages, based on changes in the distribution of chloroplasts.

Stage I: In this stage, non-enriched sea water under high light intensity caused the induction of

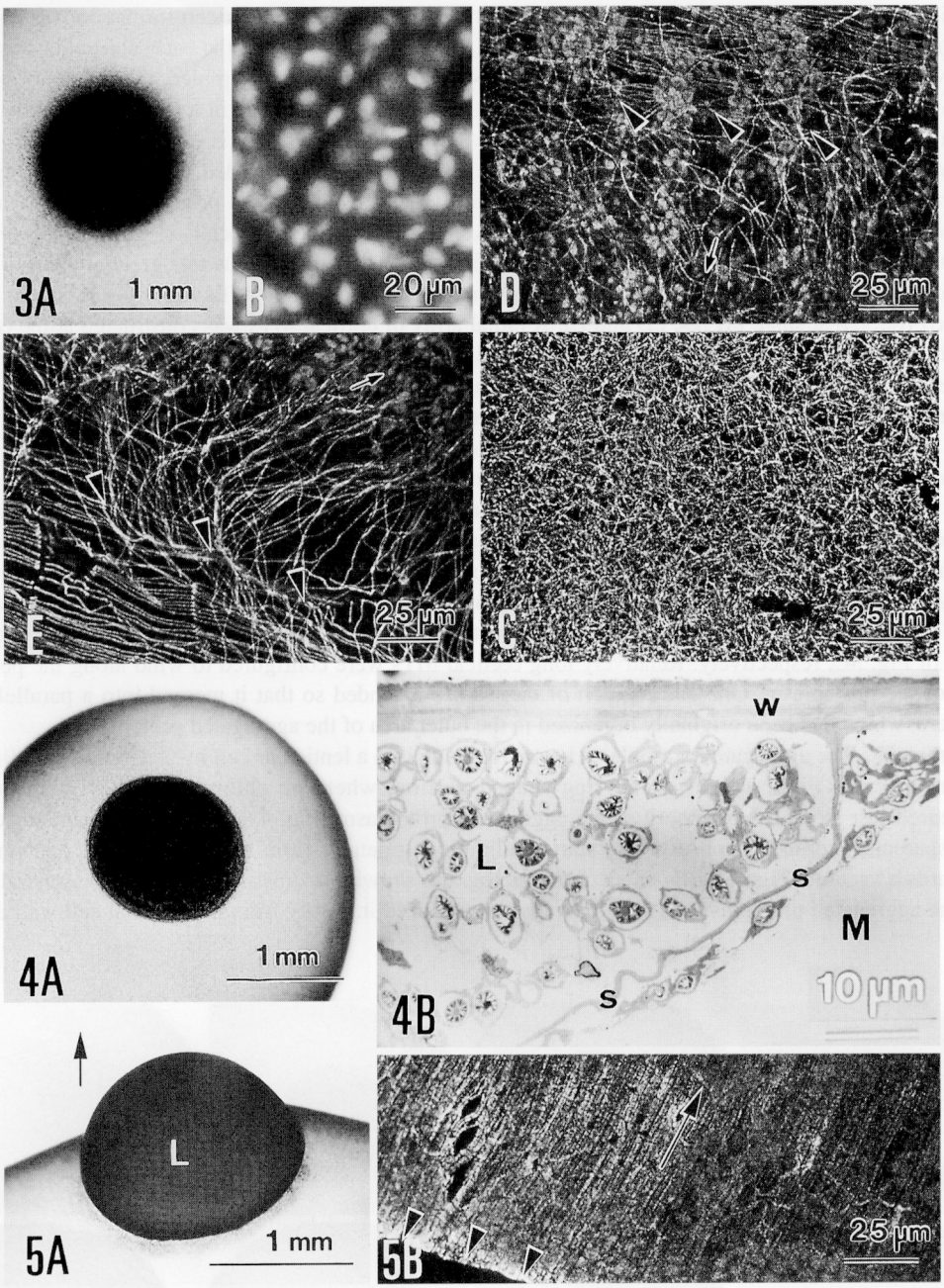

Fig. 3-5 Lenticular cell formation of *Valonia utricularis*.
Fig. 3 Stage III. A. Surface view of a chloroplast assembly. B. Fluorescence images of nuclei in the area of a chloroplast assembly. C. A random system of MTs at the center of a chloroplast assembly. D. Immunofluorescence images of cortical MTs in the periphery (arrowheads) of a chloroplast assembly. Arrow, direction of Fig. 3C. E. Immunofluorescence images of cortical MTs in the periphery (arrowheads) of a chloroplast assembly. Arrow, direction of Fig. 3C.
Fig. 4 Stage IV. A. Surface view of a juvenile lenticular cell. B. Ultrastructure of a cross section of a lenticular cell. A septum (S) between mother (M) and lenticular (L) cells extended from mother cell wall (W).
Fig. 5 Stage V. A. Lateral view of a developing lenticular cell. B. Parallel cortical MTs in the lateral side of a lenticular cell. Arrow, the direction of growth of a lenticular cell. Arrowheads, boundary between mother and lenticular cells.

lenticular cell formation in cultured cells. The first visible sign of lenticular cell formation was a small assembly of chloroplasts at a specific site in a cell (Fig. 1C). The shape of the early chloroplast assembly varied from cell to cell. In the cytoplasmic area where chloroplasts assembled, the density of nuclei increased ($28.1 \pm 5.8/10^4$ μm^2) (Fig. 1D). Cortical MTs appeared to curve in and overlap in this area, but they were arranged parallel to each other in the surrounding area (Fig. 1E).

Stage II: About 12 h after stage I, further assembly of chloroplasts led to a larger disc-shaped aggregate of protoplasm (Fig. 2A). Nuclei confined within the aggregated protoplasm, except for the center area, remarkably increased in number and were also distributed vertically (Fig. 2B). The density of nuclei rose to $51.2 \pm 9.2/10^4$ μm^2. This increase in nuclear density was due to a great number of synchronous mitoses (Fig. 2C). The features of the arrangement of cortical MTs were very different from those observed at stage I (Figs. 2D and E). A radial array of cortical MTs was constructed at the center of the aggregated protoplasm and extended well beyond the protoplasm. However, random MTs appeared at the very center. In other areas, the parallel MT orientation appeared to be unchanged.

Stage III: Aggregation of protoplasm began to stop about 15 h after stage II (Fig. 3A). The average size of the aggregated protoplasm was 1.27 ± 0.16 mm in diameter. The density of nuclei in the protoplasm further increased (to $66 \pm 3/10^4$ μm^2), and nuclei were spindle-shaped rather than spherical (Fig. 3B). Nuclear divisions were almost completed. A radial array of cortical MTs depolymerized from the center of the protoplasm towards the periphery during the period from stage II to stage III. As a result of the disassembly of the MTs, random MT systems appeared to cover the area of the aggregated protoplasm (Fig. 3C). The random MTs were short and densely distributed. Figures 3D and E show the MT arrangement in the peripheral areas of the protoplasm, which are located in the upper part and left part of Fig. 3C, respectively. Relatively long cortical MTs were configured to wind along the periphery of the protoplasm. One end of each of these MTs extended so that it merged into a parallel MT system, which had been originally organized in the outer area of the aggregated protoplasm.

Stage IV: The aggregated protoplasm began to divide into a lenticular cell by a septum wall 10-20 h after stage III. First, a transparent, ring-shaped portion, where no chloroplasts were distributed, appeared just along the periphery of the aggregated protoplasm (Fig. 4A). Under a light microscope, transparent material was observed to start swelling centripetally from the inside of the ring portion towards a vacuole. Figure 4B is an electron micrograph showing a cross section of the peripheral area of the aggregated protoplasm. A septum wall was produced inwardly from the original cell wall at the

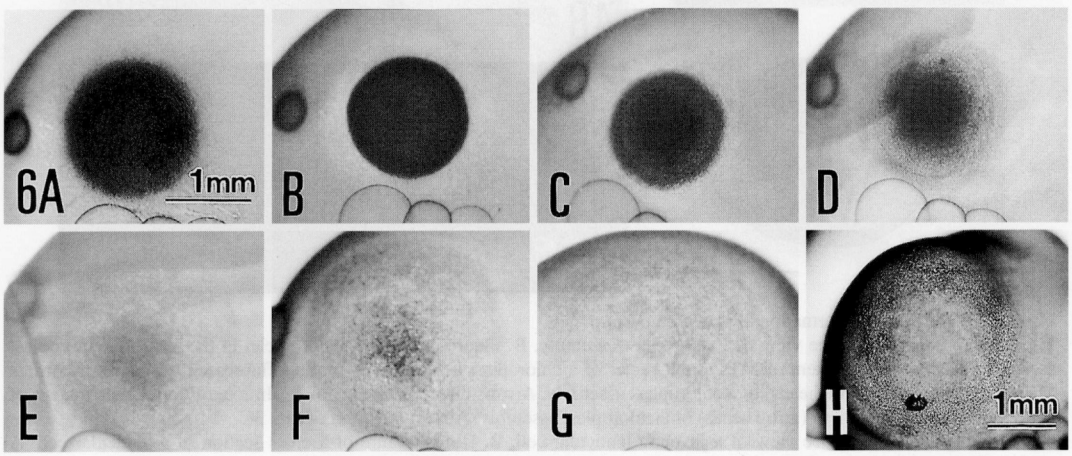

Fig. 6 Effect of APM on lenticular cell formation. Surface views of the area of chloroplasts assembling, 0 (A), 9 (B), 21 (C), 30 (D), 42 (E), 54 (F), 64 (G) and 305 h (H) after treatment with APM. Scale bar in A also applies to B-G.

periphery of the protoplasm, and it finally separated a lenticular cell from the mother cell. Near the septum, densely packed protoplasm was found on the lenticular cell side, whereas thin parietal protoplasm was found on the mother cell side. The completion of a septum took about 12 h.

Stage V: A newly formed, young lenticular cell began to grow out (Fig. 5A) and develop into a mature obovoid cell. A new parallel array of cortical MTs formed in the lateral side of the cell (Fig. 5B). The cortical MTs oriented towards the apex of the cell.

Effect of APM: Cells at stage III in lenticular cell formation were continuously incubated in PES medium containing the anti-microtubule agent APM for 305 h (Figs. 6A-H). Twenty-one hours after the beginning of treatment with APM, the assembly of chloroplasts began to disperse at the periphery of the chloroplast assembly (Fig. 6C). Chloroplasts continued to disperse away from the periphery as the duration of APM treatment increased (Figs. 6D-F). The area to which the chloroplasts dispersed was somewhat wider than the initial size of the chloroplast assembly. APM did not affect the distribution of chloroplasts in other areas of the cell. APM treatment for 64 h made the assembly almost disappear (Fig. 6G). After further treatment with APM, the part of the cell, within which chloroplasts had dispersed, protruded (Fig. 6H). In spite of the chloroplast dispersion, APM did not cause the nuclei to migrate. On the other hand, two to three days after the beginning of APM treatment, some cells formed new initial assemblies of chloroplasts, which were similar to those found in stage I in normal lenticular cell formation. However, these assemblies no longer developed, and they eventually disappeared.

DISCUSSION

The present study is the first report of the processes of cell division, i. e., lenticular cell formation, in a coenocytic green alga. In a normal growing cell of *Valonia*, chloroplasts and nuclei were distributed evenly throughout the protoplasm, and therefore they seemed to be stationary. The most prominent feature when the cell was committed to lenticular cell formation was the uneven distribution of protoplasm, especially the uneven distribution of chloroplasts. It is also important to note that the chloroplasts assembled at a specific locus. These results mean that there are at least two regulatory systems for the chloroplast assembly, one moving chloroplasts and the other controlling the direction of the movement. In a stationary cell, a parallel system of cortical MTs was organized throughout the cell. The disorganization of the MT system started locally at the site of a small assembly of chloroplasts, and a distinctive radial array of cortical MTs was eventually constructed, followed by further assembly of chloroplasts. The fact that APM did not inhibit the initial small assembly of chloroplasts suggests no involvement of cortical MTs in generating the motive force for chloroplasts. However, APM disturbed the further assembly of chloroplasts and also made chloroplasts that were assembling disperse. These observations indicate that the radial array of cortical MTs functions as a guiding ele-

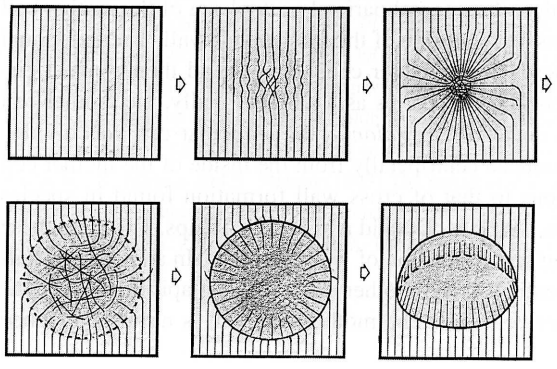

Fig. 7 Schematic representation of changes in the arrangement of cortical MTs during lenticular cell formation in *Valonia utricularis*.

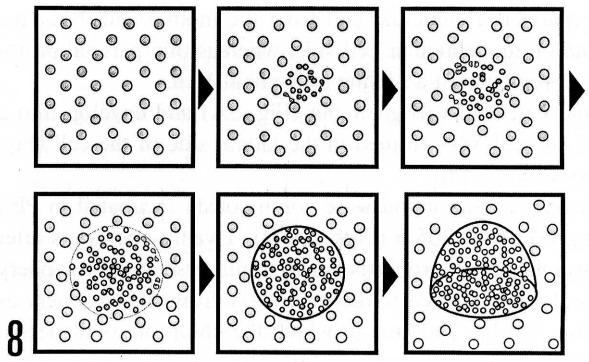

Fig. 8 Schematic representation of changes in the distribution of nuclei during lenticular cell formation in *Valonia utricularis*.

ment in the regulatory system to control the direction of chloroplast movement. It is also evident that MTs have a role in fixing or stabilizing the assembly of chloroplasts at a definite site. APM inhibited all the MTs of the parallel, radial and random systems. However, of these three MT systems, only the radial system is probably involved in stabilizing the chloroplast assemblies, since chloroplasts were radially dispersed in the presence of APM. Changes in the arrangement of cortical MTs during lenticular cell formation in *Valonia* are schematically summarized in Fig. 7.

Figure 8 is a schematic representation showing the distribution of nuclei during lenticular cell formation in *Valonia*. Synchronous mitoses were observed within the protoplasm where chloroplasts assembled in the present study. Motomura (1996) reported that, in *Boergesenia*, a genus closely related to *Valonia*, mitoses occur simultaneously in some protoplasmic areas of the cell, since the timing of transition from the G1 phase to the S phase in the nuclear division cycle is synchronized in these areas. *Boergesenia* thallus cells never produce lenticular cells. In *Valonia*, we also frequently observed synchronous mitoses within indefinite protoplasmic areas in the stationary cell, which were not committed to lenticular cell formation (not shown). No chloroplast assembly appeared in those areas in the cell, and a parallel system of cortical MTs was not affected at all in these areas. Therefore, the radial MT system associated with lenticular cell formation is not directly involved in the regulation of the nuclear division cycle. The remarkable increase in the density of nuclei suggests that nuclear divisions occur repeatedly in the same protoplasmic area where chloroplasts assembled. Thus, the radial MT system, rather than directly regulating the nuclear division cycle in lenticular cell formation, might contain other factors, if they exist, that regulate and accelerate the nuclear division cycle.

Segregative cell division is the most important criterion for designating the order Siphonocladales, and lenticular cell formation in *Valonia* is regarded as an example of segregative cell division (Bold and Wynne, 1985). Although there are several genera in the Siphonocladales, no universal character in the segregative cell division has been defined. Enomoto *et al.* (1982) demonstrated the course of segregative cell division in *Dictyosphaeria*. In this alga, the integral parietal protoplasm of the mother cell contracts to divide into many spherical protoplasts on the inside of the mother cell wall. The individual protoplasts form their own cell walls around them. The daughter cells expand and then connect circumferentially with each other to organize a globe-shaped thallus as a whole. Finally, the mother cell wall covering the thallus ruptures and becomes detached. In *Valonia*, the lenticular cell was divided from the mother cell by a septum, which was produced centripetally from the inside of the mother cell wall. This type of septum formation is analogous to that of cross wall formation found in species belonging to the order Cladophorales (Kornmann, 1969; McDonald and Pickett-Heaps, 1976). In these species the cross wall arises by annular furrowing and deposition of wall materials. In addition, in *Valonia*, the septum wall was shared between the lenticular and mother cells, and the apical dome of the lenticular cell was originally a part of the mother cell wall. The mother cell wall is never abandoned

after cell division. Thus, the pattern of wall formation is distinct between *Valonia* and *Dictyosphaeria*. In addition, individual daughter cells are completely apart from each other just after cell division in *Dictyosphaeria*, but this never happens in *Valonia*. However, protoplasmic assembly during lenticular cell formation in *Valonia* can be regarded as a specific contractile movement of protoplasm. Therefore, La Claire (1992) concluded that the contractile property of protoplasm that occurs in siphonocladalean algae including *Valonia* is a common feature of segregative cell division.

Lenticular cell formation in *Valonia* is a kind of local differentiation of the cell. A radial array of cortical MTs is constructed at a site that subsequently becomes the site of production of a lenticular cell, and the array is broken up before the lenticular cell is completed. The radial array is a temporal construction for coenocytic cell differentiation. Another example of the construction of a radial array of cortical MTs is found in the siphonocladalean alga *Chamaedoris* (Okuda *et al.*, 1990a; 1993). The radial array in *Chamaedoris* is localized at the apical dome of the cell where tip growth occurs. It is a permanent structure that is involved in continuous activities of coenocytic cell growth. Thus, a radial array of cortical MTs is an essential cytoskeleton for cytomorphogenesis in coenocytic green algae.

REFERENCES

Bold, H. C. and M. J. Wynne (1985) Introduction to the algae. 720 pp. Prentice-Hall, Inc., Englewood Cliffs, New Jersey, USA.

Enomoto, S., T. Hori and K. Okuda (1982) Culture studies of *Dictyosphaeria* (Chlorophyceae, Siphonocladales) II. Morphological analysis of segregative cell division in *Dictyosphaeria cavernosa*. *Jpn. J. Phycol.* **30**: 103-112.

Hoek, C. van den, D. G. Mann and H. M. Jahns (1995) Algae: an introduction to phycology. 623 pp. Cambridge University Press, Trumpington Street, Cambridge, Great Britain.

Kornmann, P. (1969) Gesetzmäßigkeiten des Wachstums und der Entwicklung von *Chaetomorpha darwinii* (Chlorophyta, Cladophorales). *Helgol. Wiss. Meeresunters.* 19: 335-354.

La Claire, J. W., II. (1987) Microtubule cytoskeleton in intact and wounded coenocytic green algae. *Planta* **171:** 30-42.

La Claire, J. W., II. (1989) Actin cytoskeleton in intact and wounded coenocytic green algae. *Planta* **177:** 47-57.

La Claire, J. W., II. (1992) Contractile movements in the algae: the siphonocladales as model systems. *In* The cytoskeleton of the algae. Edited by D. Menzel. pp. 239-253. CRC Press, Boca Raton, Florida, USA.

Lloyd, C. W. (1984) Toward a dynamic helical model for the influence of microtubules on wall patterns in plants. *Int. Rev. Cytol.* **86:** 1-51.

McDonald, K. L. and J. D. Pickett-Heaps (1976) Ultrastructure and differentiation in *Cladophora glomerata*. I. Cell division. *Amer. J. Bot.* **63:** 592-601.

Morejohn, L. C. and D. E. Fosket (1991) The biochemistry of compounds with anti-microtubule activity in plant cells. *Pharmac. Ther.* **51:** 217-230.

Motomura, T. (1996) Cell cycle analysis in a multinucleate green alga, *Boergesenia forbesii* (Siphonocladales, Chlorophyta). *Phycol. Res.* **44:** 11-17.

Okuda, K. and S. Mizuta (1987) Modification in cell shape unrelated to cortical microtubule orientation in growing thallus cells of *Chaetomorpha moniligera*. *Plant Cell Physiol.* **28:** 461-473.

Okuda, K., K. Matsuo and S. Mizuta (1990a) Characteristics of the deposition of microfibrils during formation of the polylamellate walls in the coenocytic green alga *Chamaedoris orientalis*. *Plant Cell Physiol.* **31:** 357-364.

Okuda, K., K. Matsuo and S. Mizuta (1990b) Preparation for immunofluorescence microscopy causes change in arrangements of cortical microtubules in some coenocytic green algae. *Mem. Fac. Sci. Kochi Univ., Ser. D (Biol.)* **11:** 5-10.

Okuda, K., K. Matsuo and S. Mizuta (1993) The meridional arrangement of cortical microtubules defines the site of tip growth in the coenocytic green alga, *Chamaedoris orientalis*. *Bot. Mar.* **36:** 53-62.

Starr, R. C. and J. A. Zeikus (1987) UTEX-The culture collection of algae at the University of Texas at Austin. *J. Phycol.* **23** (supplement): 1-47.

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