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# Cytomorphogenesis in cenocytic green algae. V. Segregative cell division and cortical microtubules in *Dictyosphaeria cavernosa* (Siphonocladales, Chlorophyceae)*

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## SUMMARY

Disassembly and reassembly of cortical microtubules (MT) during and after segregative cell division (SCD) in *Dictyosphaeria cavernosa* (Forssk.) Børgesen were observed using fluorescence microscopy. Parallel cortical MT in a mother cell were intact just after the initiation of SCD, but soon circular, MT-free patches appeared. Protoplasmic contraction enlarged the patches, and in these areas, the protoplasm eventually became perforated. Long and undulating cortical MT were arranged densely in the reticulate protoplasm. During further protoplasmic contraction, cortical MT appeared to be random and decreased in density. Finally, short and random cortical MT were present in the segregated protoplasts. Parallel cortical MT reassembled in the expanding daughter cells. After the daughter cells came in contact with one another, a radial system of cortical MT was constructed at the side that faced the inside of the mother cell wall. A microtubule inhibitor (amiprophos methyl, APM) had no effect on SCD. Segregative cell division was not induced directly by mechanical wounding. A comparison between SCD and wound-induced protoplasmic contraction was made.

Key words: cenocytic green alga, cortical microtubules, *Dictyosphaeria cavernosa*, protoplasmic contraction, segregative cell division, Siphonocladales, wound responses.

## INTRODUCTION

Børgesen (1905) first described a distinctive cell division in the marine cenocytic green alga *Siphonocladus*, in which the whole protoplasm simultaneously cleaves into spherical portions, which later expand and develop into new cells. This mode of cell division was also found in other siphonocladalean algae such as *Dictyosphaeria, Struvea, Chamaedoris, Cladophoropsis* and *Boodlea*, and termed segregative cell division (SCD; Børgesen 1913). Enomoto *et al.* (1982) reported in detail the processes of SCD in *Dictyosphaeria*: during the night period, the protoplasm contracts into small granular portions, which then fuse together to form a network. The network breaks down and separates into several spherical protoplasts on the inside of the mother cell wall. In the following light period, the individual protoplasts produce their own cell walls and increase in size. After the next 3 days, the young daughter cells connect circumferentially with each other to organize a hollow, globe-shaped thallus.

The course of SCD superficially resembles that of aplanospore formation in *Boergesenia*. According to Enomoto and Hirose (1972), mechanical wounding by cutting or puncturing the *Boergesenia* thallus cells induces a reticulation of protoplasm, which leads to cleavage into hundreds of spherical protoplasts. These protoplasts produce their own cell wall and develop into aplanospores. La Claire (1982) reported that protoplasmic contraction induced by wounding is a common characteristic of most siphonocladalean algal members including *Boergesenia* and may involve the same mechanisms that regulate SCD. The behaviors and functions of microtubule and actin cytoskeletons in wound-induced protoplasmic contraction in *Ernodesmis* and *Boergesenia* have been examined in detail (La Claire 1987, 1989, 1991). Cortical microtubules (MT) do not appear to be involved directly in the contraction (La Claire 1987). However, actin appears during wound-induced contraction and is involved directly in the protoplasmic motility in these algae (La Claire 1989). La Claire (1991) reported that myosin also appears concomitantly with wound-induced contraction and becomes co-localized with actin, and suggested that an actin-myosin interaction may mediate the contraction.

As La Claire (1987) pointed out, cortical MT in siphonocladalean algae are extremely long and parallel to each other and persist without depolymerization during mitosis. Okuda *et al.* (1993) reported that such parallel cortical MT radiate from the tip to the base of tip-growing thallus cells in *Chamaedoris*. A radial arrangement of

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cortical MT also appears during lenticular cell formation in *Valonia* (Okuda *et al.* 1997). Although the radial arrays of cortical MT constructed in these algal cells may act as an important cytoskeleton for cytomorphogenesis, the function of the cortical MT has not been fully elucidated. In the present study, we describe patterns of the disassembly and reassembly of cortical MT during and after SCD in *Dictyosphaeria cavernosa* (Forssk.) Børgesen.

## MATERIALS AND METHODS

Vegetative thalli of *D. cavernosa* were collected at Tei, Yasu-cho, Kochi Prefecture, Japan in July 1982. Isolation and identification of reproductive cells discharged from these thalli were performed as described by Enomoto and Okuda (1981). All of the thalli collected at Tei at this time produced only biflagellate male gametes. In the present study, gametophytic thalli which developed parthenogenetically from male gametes were used, which carried out SCD and developed into multicellular thalli with normal morphology as described by Enomoto *et al.* (1982). The gametophytic thalli were cultured in PES medium (Starr and Zeikus 1993) under 22°C and a 14:10 h light:dark cycle. Cool white fluorescent lamps with an intensity of *ca* 4 W/m$^2$ were used. Segregative cell division was induced and observed as described by Enomoto *et al.* (1982). Single cells about 3 mm in diameter with slender rhizoidal filaments, were transferred into Petri dishes containing fresh PES medium and cultured for a week under the same temperature conditions and light regime.

Amiprophos-methyl (APM), which is a potent inhibitor of plant microtubule polymerization (Morejohn and Fosket 1984), was kindly donated by Nihon Bayer Agrochem K.K., Tokyo. Amiprophos-methyl was dissolved in PES medium containing 0.1% dimethylsulfoxide to a final concentration of 10 μmol/L. To examine the effects of APM on SCD, groups of about ten cells were incubated in the presence and absence of APM for a week. In preliminary experiments, cortical MT almost completely disassembled in cells incubated with APM for 24 h.

To examine whether wound responses occur in *Dictyosphaeria*, intact cells were either cut into two fragments with a razor or punctured with a fine needle in the medium and then cultured for a week.

The procedures for indirect immunofluorescent staining of microtubules were essentially those of Okuda *et al.* (1997), except for the fixative concentration. The fixative consisted of 10% paraformaldehyde and 0.5% glutaraldehyde dissolved in buffer containing 587 mmol/L NaCl, 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 5 mmol/L ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid and 50 mmol/L piperazine-N,N′-bis(2-ethanesulfonic acid), pH 7.0. Nuclei were stained with 1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI). Fluorescence was observed with a confocal laser scanning microscope (MCR-600, Bio-Rad) or an Olympus epifluorescence microscope (BHF–BH2–RFL).

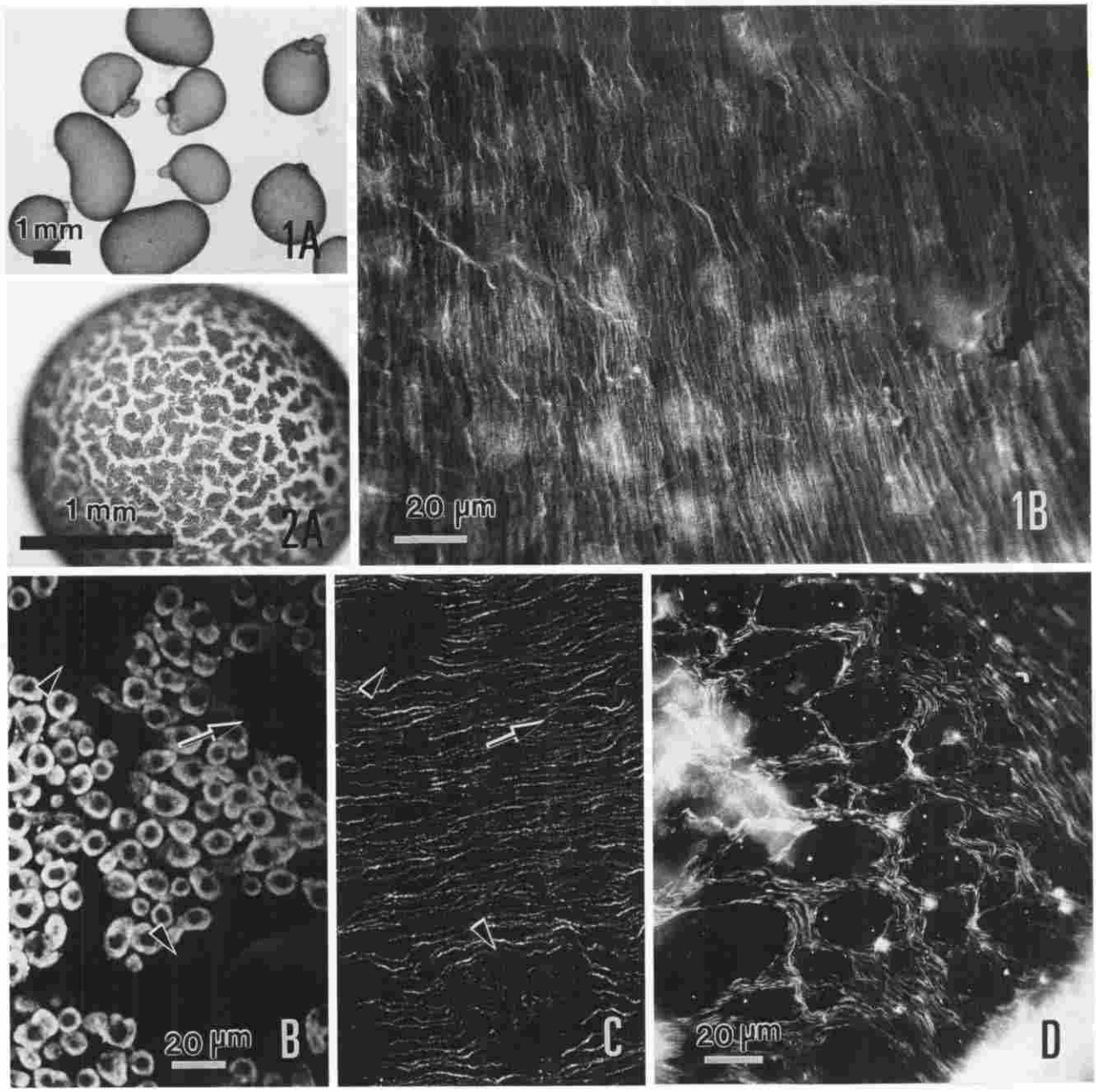
## RESULTS

### Cortical microtubules before segregative cell division

A parthenogenetically developed single cell had a large central vacuole and a thin parietal layer of protoplasm containing many nuclei and chloroplasts (Fig. 1A). Chloroplasts were distributed evenly throughout the cytoplasm. Nuclei were also distributed at an almost uniform density but positioned below the chloroplasts. Cortical MT were located just on the inner side of the plasma membrane and were arranged approximately in parallel in most parts of the cell (Fig. 1B). However, these MT seemed to extend from an indefinite site of the cell.

### Disassembly of cortical microtubules during segregative cell division

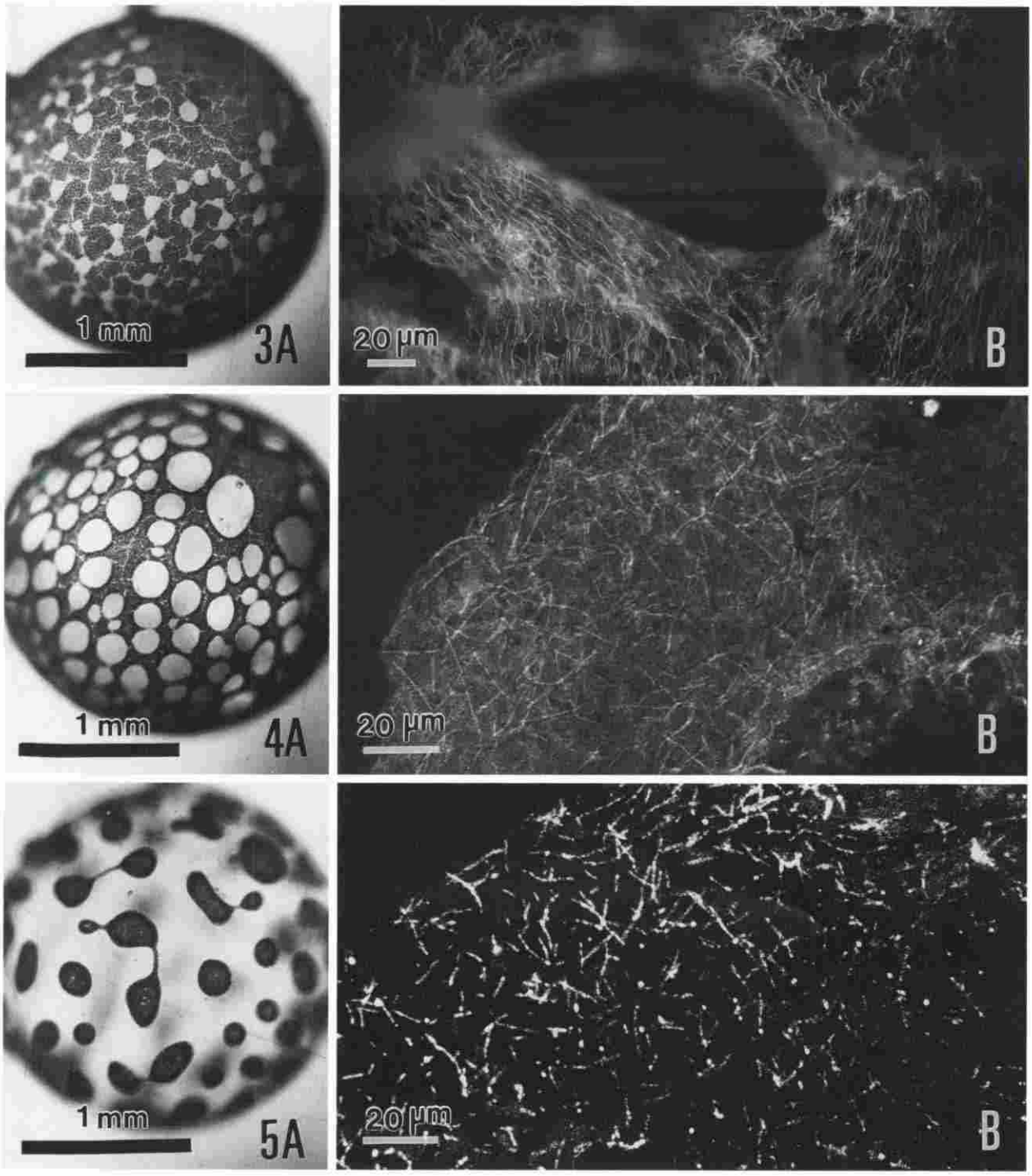
After the cells were transferred to fresh medium, 10–30% of them were committed to SCD within a week. SCD began 6–7 h after the initiation of the dark period. The protoplasm first contracted into many, small granular portions (Fig. 2A) in which chloroplasts and nuclei were shared. The plasma membrane seemed to remain intact in this initial stage, since cortical MT were often observed in hyaline gap areas between the granular portions (Figs 2B, C). The parallel arrangement of cortical MT was unchanged. However, 1 h after the initiation of SCD, small circular patches devoid of cortical MT appeared and increased in number (Figs 2C, D). These MT-free patches increased in size and fused with each other. Two hours after the initiation, further protoplasmic contraction led to the retraction of the plasma membrane back into the fused patches, so that the protoplasm of the mother cell became perforated in places (Fig. 3A). The peripheral protoplasm along the holes was convoluted towards the inner side of the mother cell, and long cortical MT were arranged densely but curved along the peripheries of the holes (Fig. 3B). Three hours after the initiation, the whole protoplasm of the mother cell formed a reticulate globe (Fig. 4A), where random cortical MT appeared at a lower density than before (Fig. 4B). Four to 5 h after the initiation (around the onset of the next light period), the reticulum broke down and eventually was segregated into almost spherical protoplasts on the inside of the mother cell wall (Fig. 5A). Short, random MT were distributed in the cortical area of the protoplasts (Fig. 5B). The protoplasts soon began to produce cell walls. No synchronous nuclear division was observed during SCD.




**Figs 1, 2.** *Dictyosphaeria* cells and cortical microtubules (MT) before and after the initiation of segregative cell division. 1. Single cells cultured for 3 months (A) and cortical MT parallel to each other (B). 2. Segregative cell division initiated by contraction into granular portions (A). Cortical MT (C), which are intact (arrow) or begin to open up (arrowheads) in gap areas between granular portions consisting of chloroplasts (B). B and C are in the same regions. MT-free patches (D) increasing in number and size.

## Reassembly of cortical microtubules in daughter cells

Discrete daughter cells rapidly increased in size (Fig. 6A). About 6 h after segregation of the protoplasm, long cortical MT, which overlapped each other, appeared (Fig. 6B). By the beginning of the next dark period, daughter cells further expanded and came in contact with one another, although without losing their spherical shape, at the lateral sides of the neighboring cells. The daughter cells assembled parallel cortical MT, which were often observed to extend radially from a single site on the daughter cell surface (Fig. 6C). Three days after that, daughter cells beneath the mother cell wall expanded further and took on a polygonal shape as they pressed against each other (Fig. 7A). A radial array of cortical MT was constructed in each of these daughter cells (Fig. 7B). The center, from which cortical MT extended, was always positioned at the outermost side of the daughter cell facing the inside of the mother cell wall.



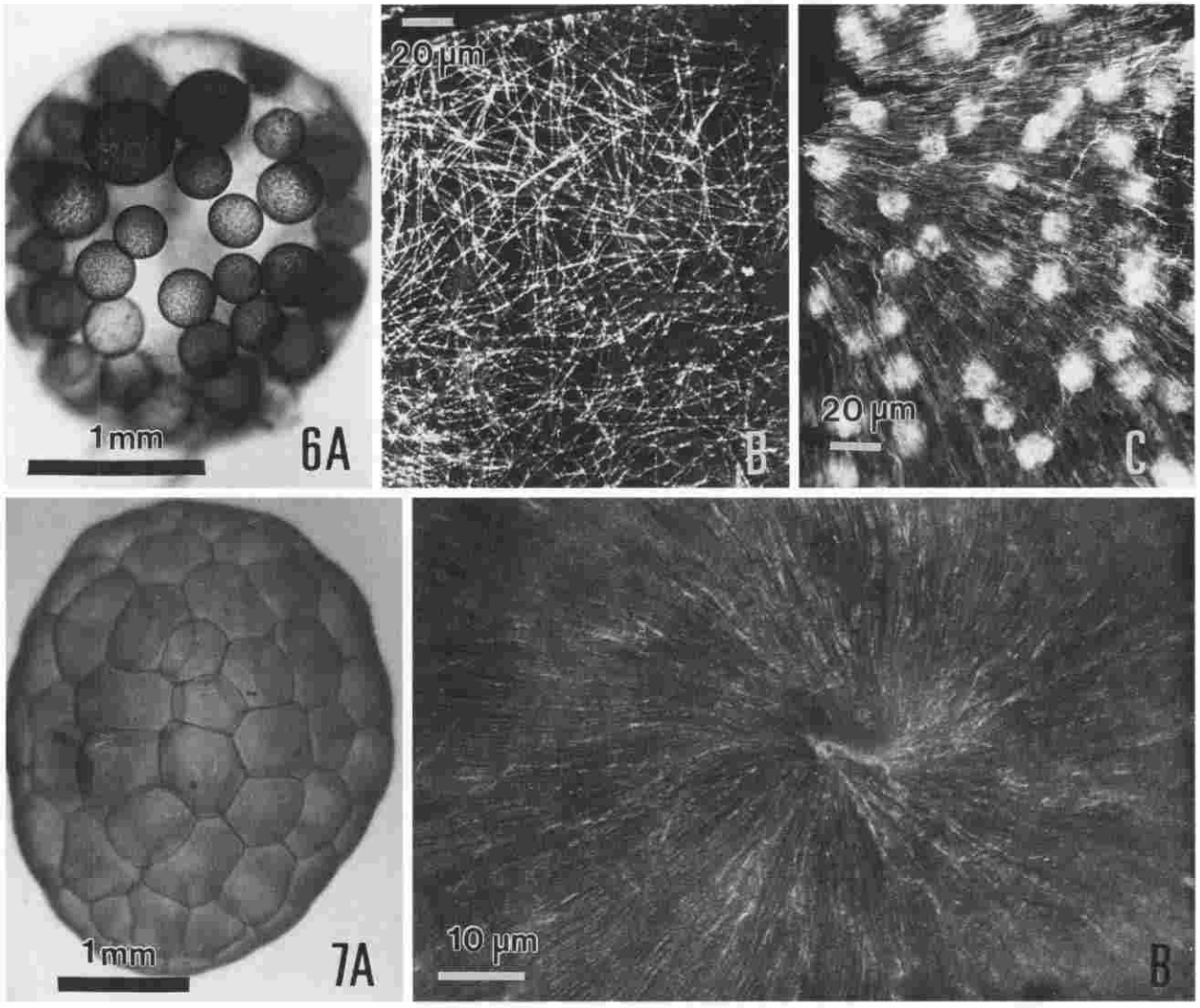

**Figs 3–5.** Disassembly of cortical microtubules (MT) during segregative cell division. 3. Protoplasm with several perforations (A), along which cortical MT curved (B). 4. Reticulate protoplasm (A), where random cortical MT are distributed (B). 5. Segregation into almost spherical protoplasts (A), where short, random cortical MT are present (B).

## Effect of amiprophos-methyl on segregative cell division

Incubation of cells with APM even for more than 2 days had no effect on SCD. Cortical MT in these cells were considered to disassemble completely, based on the results of preliminary experiments. The percentage of cells committed to SCD in the presence of APM ranged from 10–30%, which was almost the same as in the controls. Amiprophos-methyl also had no effect on the expansion of the daughter cells.

## Responses to mechanical wounding

When cells were punctured with a fine needle, two distinctive healing processes were observed. Some cells




**Figs 6, 7.** Reassembly of cortical microtubules (MT) in daughter cells. 6. Daughter cells increasing in size (A), where long cortical MT appear in random orientation first (B) and then orient parallel to each other (C). 7. Daughter cells pressed against each other at the lateral sides to form a hollow, globular thallus (A) and a radial system of cortical MT is constructed at the side which is tangential to the thallus (B).

resumed turgor pressure, after discharging a small amount of protoplasm through the wound, and recovered completely within 2 days. After recovery, some of these cells eventually carried out SCD, identical to that of normal growing cells. However, SCD was never induced directly by puncturing cells. In other punctured cells, the parietal protoplasm contracted into a small protoplast, which produced a new cell wall in a day and regenerated into a small cell. However, in this case, protoplasmic contraction never led to reticulate protoplasm and cleavage into many protoplasts. In the case where cells were cut into two fragments, the protoplasm disintegrated in the course of 3 days and finally no surviving portion was observed.

## DISCUSSION

In the present study, SCD in *Dictyosphaeria* started 6–7 h after the dark period, and segregation of the protoplasm was completed 4–5 h after the initiation of SCD. Daughter cells that were produced expanded and tightly attached to neighboring cells in the next few days. These results and the morphological changes in the protoplasm during SCD observed in the present study correspond well with the description reported by Enomoto *et al.* (1982). The following morphological and physiological differences between SCD in *Dictyosphaeria* and wound-induced protoplasmic contraction (aplanospore formation) in *Boergesenia* were noticed: (i) Contraction of the protoplasm into small granular portions is followed by reticulation of the protoplasm in *Dictyosphaeria*, whereas the first event is reticulation of the protoplasm in *Boergesenia* (Enomoto and Hirose 1972; La Claire 1982); (ii) spherical cells formed in a mother cell rapidly enlarge in *Dictyosphaeria*, but they hardly increase in size in *Boergesenia* (La Claire 1982); (iii) SCD is inhibited by light in *Dictyosphaeria* (Enomoto *et al.* 1982), while wound-

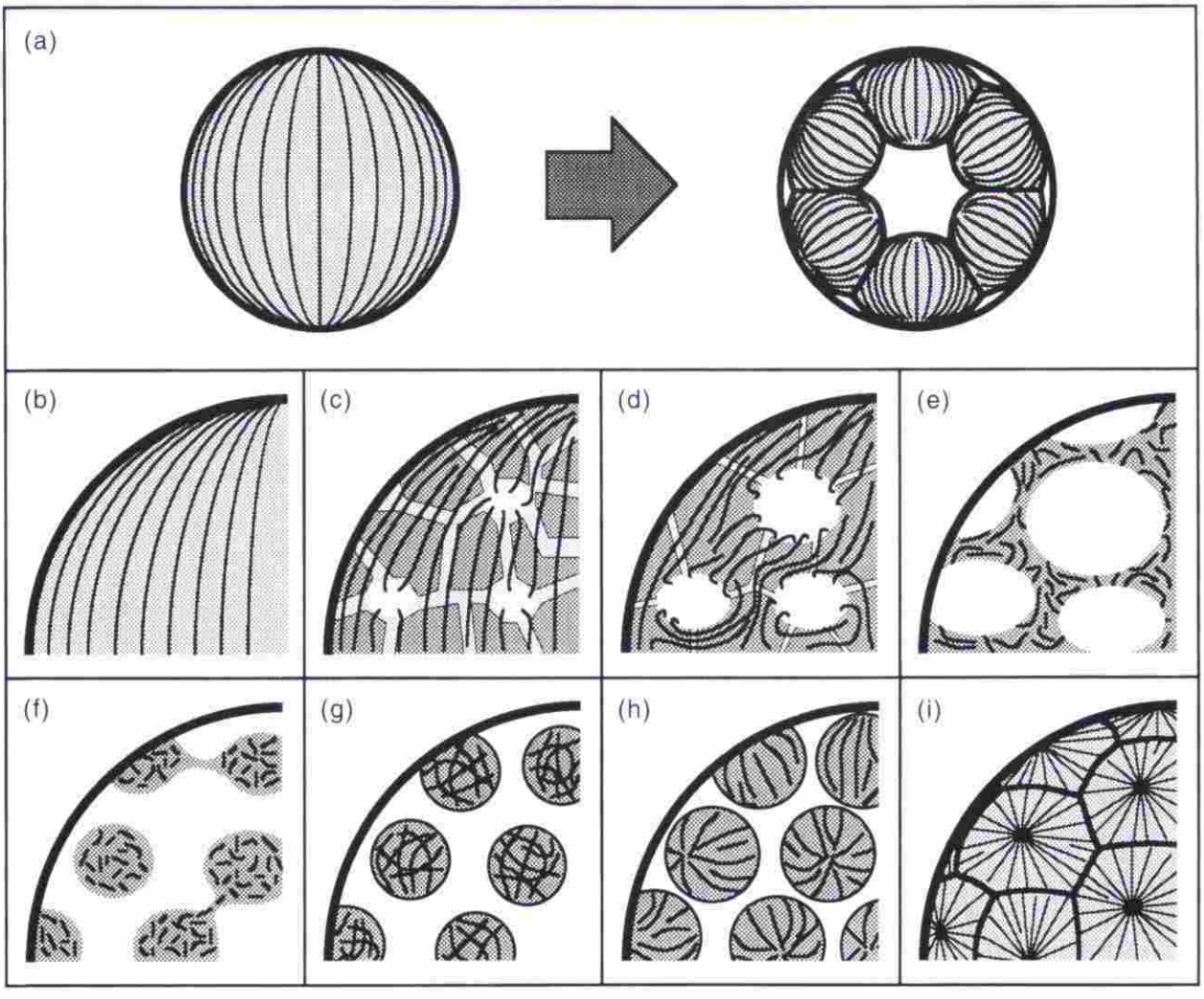



**Fig. 8.** Schematic representation of changes in the arrangement of cortical microtubules (MT) during and after segregative cell division (SCD) in *Dictyosphaeria*. (a) Lateral views of cortical MT in a mother cell and daughter cells produced in the mother cell wall. (b–i) Surface views of cortical MT. Disassembly of cortical MT during SCD (b–f) and reassembly of cortical MT after SCD (g–i).

induced protoplasmic contraction does not seem to be affected by light conditions in *Boergesenia* (Enomoto and Hirose 1972; La Claire 1982); and (iv) SCD is not immediately induced by wounding in *Dictyosphaeria* as shown in the present study.

Patterns of the disassembly and reassembly of cortical MT during and after SCD in *Dictyosphaeria* are schematically summarized in Fig. 8. Parallel cortical MT in *Dictyosphaeria* were intact just after the initiation of SCD but soon small, MT-free areas began to appear. These areas subsequently enlarged to bring about a perforation of the protoplasm. Long but undulating cortical MT were arranged densely in the reticulate protoplasm. These initial changes in cortical MT distribution appear to be identical to those in wound-induced protoplasmic contraction in *Boergesenia* (La Claire 1987). However, when the separation of the protoplasm into protoplasts was almost complete, one difference was noted: random, short cortical MT were observed in *Dictyosphaeria*, whereas nearly parallel cortical MT survive in *Boergesenia* (La Claire 1987). This may be attributed to the depolymerization and disassembly of cortical MT occurring during SCD in *Dictyosphaeria*. La Claire (1987) reported that APM does not inhibit wound-induced protoplasmic contraction at all in *Boergesenia* and *Ernodesmis*, and that changes in cortical MT distribution, which occur when intact cells are wounded, are only a passive phenomenon. In the present study, both SCD and expansion of daughter cells in *Dictyosphaeria* proceeded normally in the absence of MT. These facts indicate no involvement of cortical MT in SCD in *Dictyosphaeria* as well as in wound-induced protoplasmic contraction in *Boergesenia* and *Ernodesmis*. As mentioned below, SCD with *Dictyosphaeria* is assumed to involve the restructuring of cortical MT function in the cell division cycle. This suggests that the cortical MT cytoskeleton which had been organized in the mother cell must be disintegrated, so that a new cortical MT cytoskeleton can be formed to function for the daughter cells.

In *Dictyosphaeria,* cortical MT reassembled in the daughter cells that were produced by SCD. The arrangement of cortical MT changed from random to parallel, and eventually a radial system of cortical MT was constructed in the expanded daughter cells. The most remarkable feature is that the center from which cortical MT extend is exclusively localized at the outer side of each daughter cell (i.e. the side which is tangential to the globular thallus). Wilson (1951) reported the occurrence of two poles in the inner and outer walls of *Dictyosphaeria* thallus cells where orientation systems of cellulose microfibrils converge. Such poles have been observed at the tip walls of the tip-growing cylindrical thallus cells in *Chamaedoris* (Okuda *et al.* 1990). In addition, in *Chamaedoris,* cortical MT radiate from the growing tips and thus may localize growth activities at definite sites of the cells (Okuda *et al.* 1990, 1993). According to Enomoto and Okuda (1981), in well developed *Dictyosphaeria* thalli, the basal cells elongate outward to develop into cylindrical rhizoidal cells. Thus, a radial system of cortical MT constructed in daughter cells of *Dictyosphaeria* may be a manifestation of a potential growth site together with a pole site for cellulose microfibril orientation systems. However, how the center position of the radial system of cortical MT is determined remains unknown. It is speculated that either connections between daughter cells and a mother cell wall or lateral connection between expanding daughter cells may provide positional information for the construction of the radial array of cortical MT.

In *Dictyosphaeria,* there were three kinds of responses to mechanical wounding. In the case of cells punctured with a fine needle, some cells completely recovered, and in others, the entire protoplasm contracted, resulting in the formation of a small surviving cell. However, contraction into many protoplasts such as is seen in *Boergesenia* was never induced by puncturing. Thus, healing activities and the ability to contract and thus survive wounding in *Dictyosphaeria* cells appear to be very limited, since no survival was observed when cells were cut into two pieces. If both wound-induced protoplasmic contraction and SCD in *Dictyosphaeria* are based on a common mechanism, the above observations suggest that cells are not committed to SCD before the ability of contraction has attained a certain level that is sufficient for the completion of SCD.

Okuda *et al.* (1997) reported that, in the siphonocladalean alga *Valonia,* contraction and aggregation of protoplasm occur at a specific site of the cell, the site where a lenticular cell is produced. A radial system of cortical MT is constructed in the area of the aggregation of protoplasm, and APM makes the aggregation disperse and thus inhibits the formation of a lenticular cell (Okuda *et al.* 1997). As mentioned above, cortical MT are not involved in SCD in *Dictyosphaeria* or in wound-induced protoplasmic contraction in *Boergesenia* and *Ernodesmis* (La Claire 1987). No radial system of cortical MT is constructed during these two processes. These results indicate that not all the types of protoplasmic contraction occurring in siphonocladalean algae are independent of cortical MT. If lenticular cell formation in *Valonia* is interpreted as a category of SCD as described by Bold and Wynne (1985), Lee (1989) and La Claire (1992), mechanisms regulating SCD must be distinct between the siphonocladalean algae, *Dictyosphaeria* and *Valonia.*

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