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Cytoskeleton in cell morphogenesis of the coenocytic green alga *Valonia ventricosa* I. Two microtubule systems and their roles in positioning of chloroplasts and nuclei[1)]

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In vegetative cells of the coenocytic green alga, *Valonia ventricosa*, the spatial organization of microtubules in a thin cytoplasmic layer located between a huge central vacuole and the spherical cell wall was observed by using indirect immunofluorescence microscopy.

The cortical microtubules ran parallel to each other on the inner surface of the plasmalemma. The cortical microtubule system adhered on one side to the plasmalemma and on the other to surface of each chloroplast, anchoring the chloroplasts immediately next to the plasmalemma, forming a monolayered chloroplast sheet.

The nuclear-associated microtubule systems consisted of two different types of bundles: one that randomely surrounded the nuclear envelope, and another extending radially from the nucleus. The radial microtubules adhered to the chloroplast surface anchoring the nucleus to the chloroplast sheet, maintaining discreet distances between nuclei.

From the disorganization and reorganization of these microtubule systems, their roles in positioning chloroplasts and nuclei is discussed.

Key Index Words: coenocytic alga, cytoskeleton organization, Valonia, *cortical microtubules, nuclear -associated microtubules, indirect immunofluorescence.*

In the giant coenocytic cell *Valonia ventricosa*, the cytoplasm can be cleaved in response to mechanical stimulation and is converted into many protoplasts called aplanospores (KOPAC 1933). In culture these protoplasts develop into vacuolated coenocytic cells within 40 hrs like those of *Boergesenia forbesii* (ENOMOTO and HIROSE 1972) and *Bryopsis plumosa* (TATEWAKI and NAGATA 1970). Preliminary observations have shown that the dynamic behavior of the cytoplasm of *Valonia* proceeds under the control of the cytoskeleton (Shihira-Ishikawa unpublished). The study presented here describes the spatial organization of microtubules in a thin cytoplasmic layer inside the spherical cell wall of the vegetative phase of *Valonia ventricosa*. Indirect immunofluorescence has been used in analyzing the role of the cytoskeleton in the positioning of the cytoplasmic constituents.

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Material and Methods

Cell culture

Valonia ventricosa was cellected in Okinawa in 1983 and has been maintained in artificial sea water (Jamarin sea water, Jamarin Lab. Japan) under dim light at 20–25°C. For the experiments, the plants were transferred to Müller's synthetic medium (MÜLLER

1962) and cultured for 2–3 weeks at 22°C under a light-dark cycle of 12/12 hr (2000 lux, fluorescent lamps). Parts of the observations reported here were made on plants derived from a plant collected at Palau, Western Caroline Islands, in 1986.

Indirect immunofluorescence

Whole plants were immersed in prechilled methanol (−10°C) for 10 min and immediately transferred into phosphate buffered saline (PBS) at room temperature. This caused the cytoplasmic layer to detach from the cell wall and collapse into the central vacuole; the cell wall also cracked and the vacuolar contents leaked away. The cytoplasm was removed from the cell through the broken wall in PBS. Pieces of cytoplasmic layer were placed on a drop of PBS on a glass slide (previously coated with poly-lysine). After removal of the buffer, the samples were treated with 20 μl of the primary antibody (mouse ascites fluid containing monoclonal anti-α tubulin IgG which was raised against native chick brain microtubules) and incubated for 30 min at 37°C. After washing with 0.03 % Tween 20 in PBS for 15 min, fluorescein isothiocyanate (FITC)-labeled sheep anti-mouse IgG was applied and the samples were incubated for 30 min at 37°C. The samples were washed again with Tween 20 in PBS for 15 min and were mounted in Glycerin containing the anti-fade, p-phenylene-diamine (1 mg/ml, pH 9.0). Fluorescence micrographs were taken using an Olympus epifluorescence microscope (BH2-RFK) loaded with Ektachrome ASA 400 color positive film or Kodak Tri-X pan film.

Chemicals

Monoclonal anti-α tubulin (Amersham International, England) was diluted to 1/500, and fluorescein linked sheep anti-mouse IgG (Amersham International, England) were diluted to 1/10 with 1% of BSA and 0.1% of NaN_3. DAPI (4′-6-diamidino-2-phenylindole) was dissolved in S-buffer (NISHIBAYASHI and KUROIWA 1980) to make a solution of 1 μg/ml and a drop of this solution was used for nuclei staining.

Results

1. Microtubule organization in interphase cells.

The cytoplasm is a sheet, being placed between the cell wall and the huge central vacuole. At the plasmalemma, flat chloroplasts spread mostly in a single layer, although in older, non-dividing plants they can lie one upon another. All the nuclei are located adjacent to the central vacuole and are arranged between the chloroplast layer and the tonoplast (Fig. 1, a, b). The nuclei are placed roughly equidistant from each other (Fig. 2, a, b).

a) Cortical microtubules.

The cortical microtubules run in parallel on the inner surface of the plasmalemma (Fig. 3). The distances between adjacent bundles of the microtubules are approximately the same, but can differ depending on the age or condition of the plant. After the cold methanol treatment, the plasmalemma plus cortical microtubules separates from other constituents of the cytoplasm (Fig. 4), suggesting that one side of the bundles tightly adheres to the plasmalemma. Bundles of microtubules also adhere to the surface of each chloroplast and link the chloroplasts with each other in a sheet, suggesting that the other side of the bundles adheres to each chloroplast and anchors them immediately next to the plasmalemma (Fig. 5). The parts of the cortical microtubules located between the chloroplasts are liable to damage and are easily lost during the preparation of the sample (arrows in Fig. 5).

b) Nuclear associated microtubules.

Bundles of microtubules randomly surround the surface of every nucleus (Fig. 6) and large bundles extend radially from the nuclei to the chloroplast layer (Fig. 7). Those two types of bundles are connected

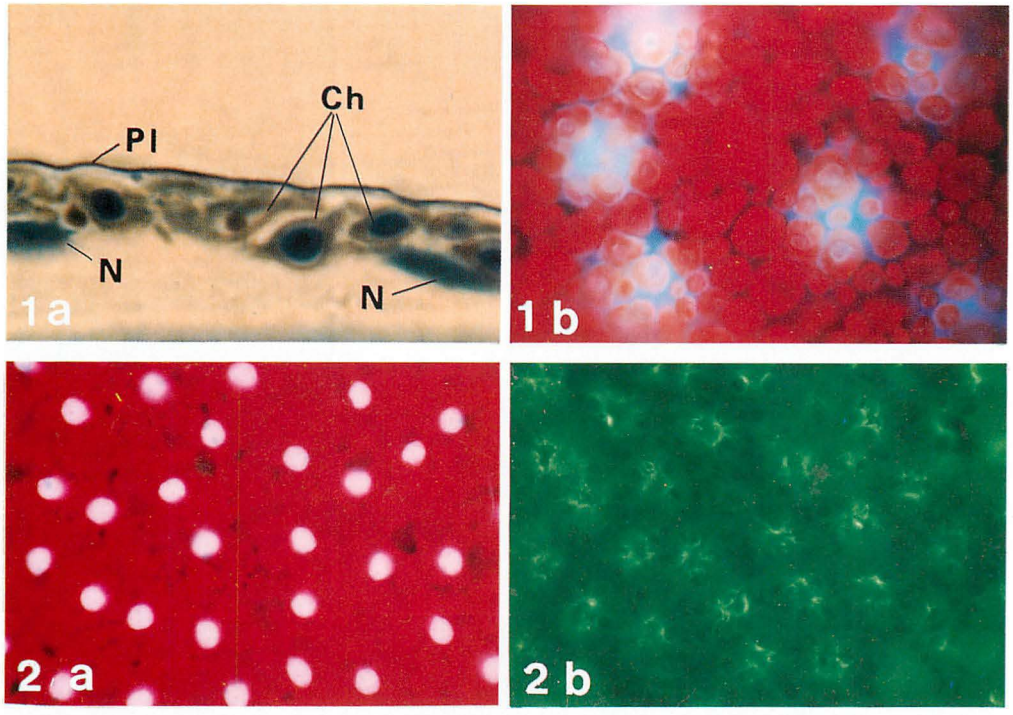


Fig. 1. Positional relationship of nuclei and chloroplasts in the thin cytoplasmic layer. (a) Section of the cytoplasmic layer. Methylene blue-staining. N: nucleus, Ch: chloroplast, Pl: plasmalemma. (b) Isolated cytoplasmic layer. DAPI-staining. Nuclei (white) are located underneath the chloroplast monolayer (red.). ×1000.

Fig. 2. Roughly equidistant distribution of nuclei. (a) DAPI-staining of cytoplasmic layer. White: nuclei, red: chloroplasts. (b) Indirect immunofluorescence. Microtubules around each nucleus are shown. ×300.

to each other at the surface of the nuclei; the bundles of peri-nuclear microtubules extend radially into the cytoplasm surrounding the nuclei (Fig. 8).

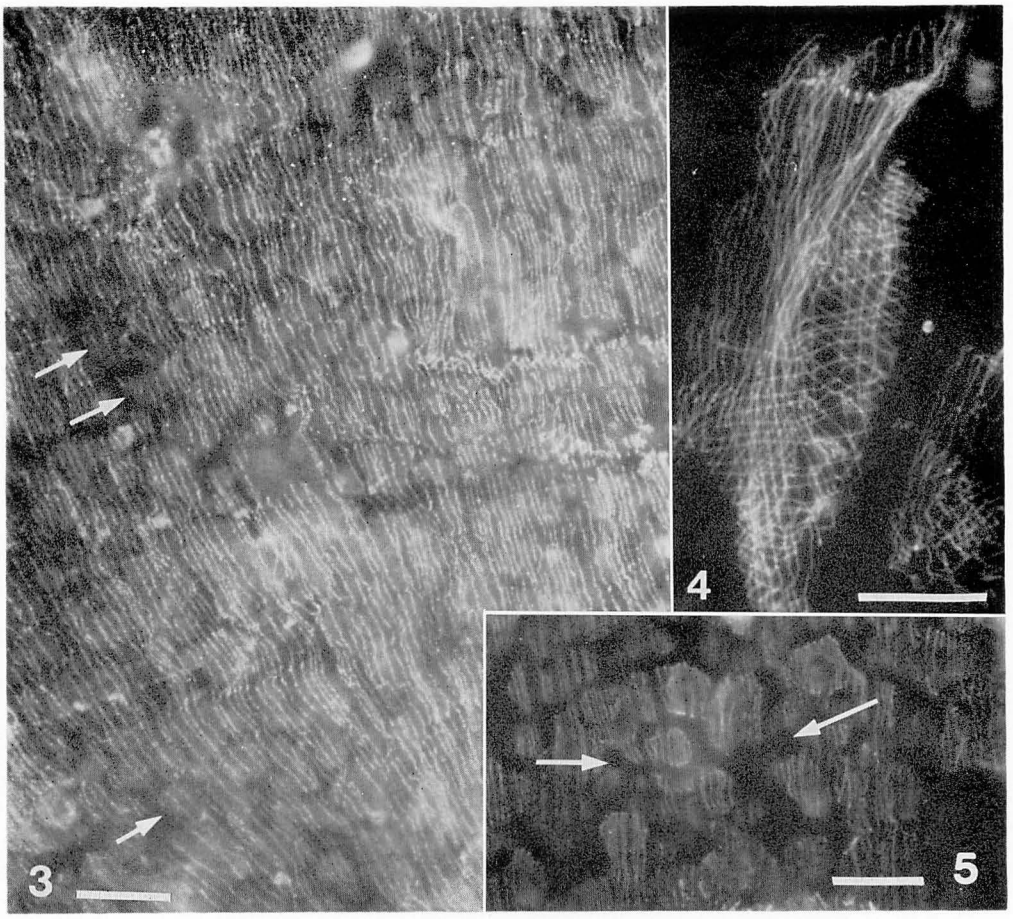
During preparation of the sample, pieces of the cytoplasmic layer became loose at the edges, and nuclei could frequently be observed with chloroplasts attached (Fig. 9). Microtubule links between nuclei and chloroplasts were observed, and it appeared that the ends of the radial microtubule bundles adhered to the chloroplast surface (arrows in Fig. 9).

The nuclear-associated microtubules and the cortical ones are not connected with each other.

2. Disorganization and reconstruction of microtubule systems.

a) Cortical microtubules

The organization of cortical microtubules was destroyed by mechanical stimulation. Immersion of cells in pure water at room temperature induced the destruction in 15 min (Fig. 10). Under the dissection microscope, many crater-like holes were observed in the cytoplasmic layer, which had partially collapsed into the central vacuole as a result of the destruction of cortical microtubules (Fig. 11). This treatment did not cause the destruction of nuclear-associated microtubules. The cortical microtubules reassembled after the cells were transferred into the culture medium and the cytoplasmic layer became smooth in appearance again. The crater-like holes were also induced by stimulation with a needle to the cytoplasm, in which case the cell proceeded to aplanospore formation without the recovery of cortical microtubule

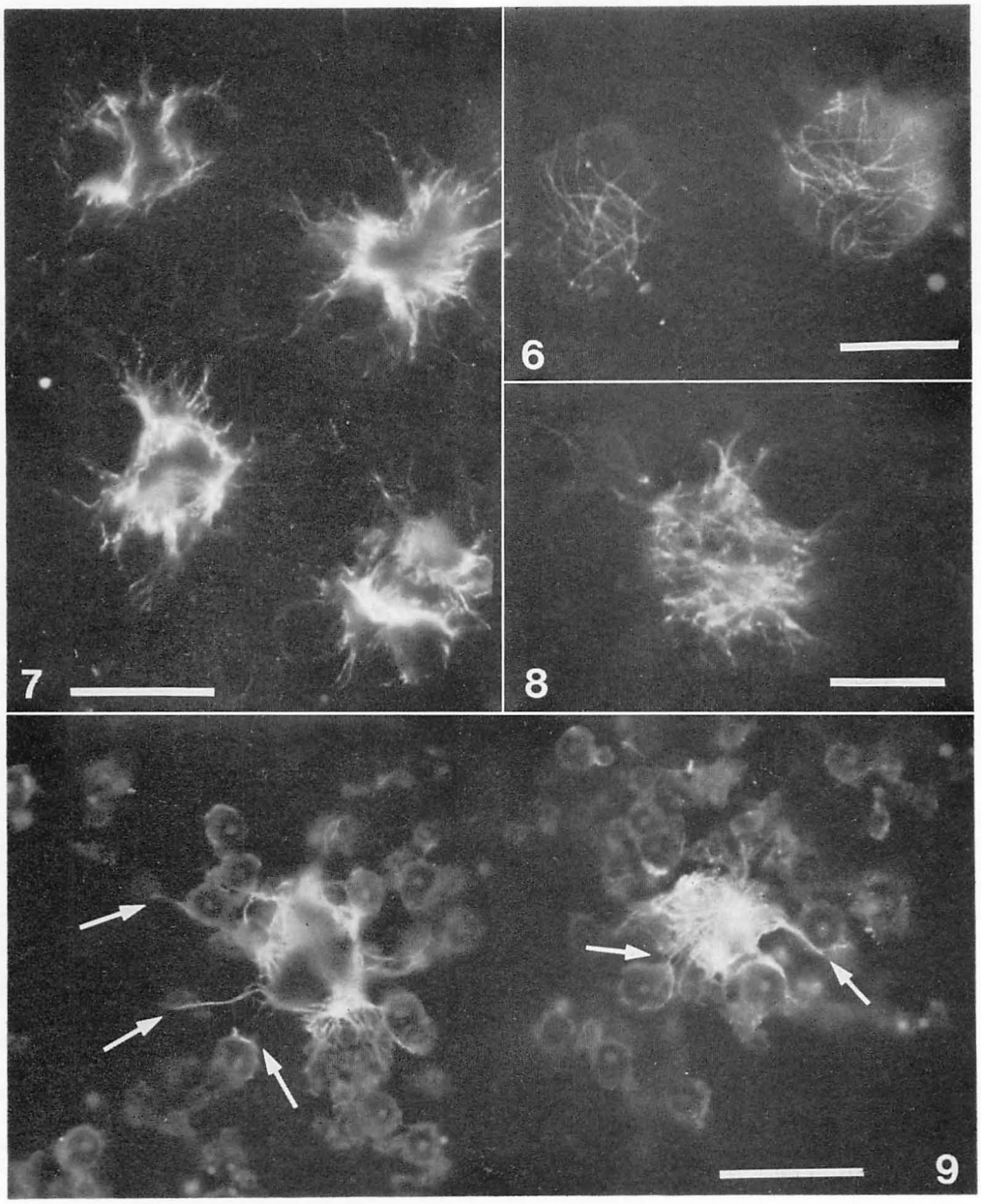


Figs. 3–5. Cortical microtubules. 3. Parallel arrangement of cortical microtubules covering the total inner surface of the plasmalemma. Chloroplasts are faintly seen under the cortical microtubules (arrow). 4. Cortical microtubules adhering to the plasmalemma which has been separated from other cytoplasmic constituents. The rolled pieces of plasmalemma were pressed by a cover glass onto a glass slide. The parallel structure of cortical microtubules are seen as being folded. 5. Cortical microtubules adhering to chloroplast surfaces. The parts of the cortical microtubules between chloroplasts are liable to be lost (arrows). (Scale bar: 10 μm).

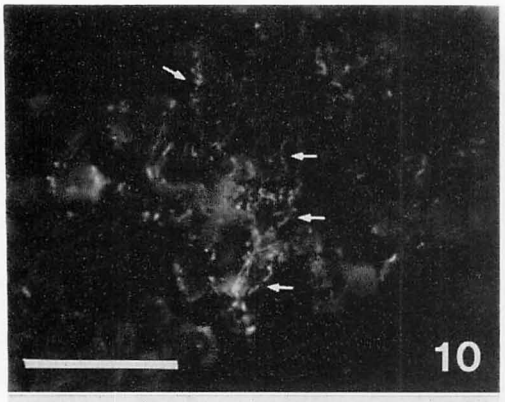
organization. These results suggest that the cortical microtubules stretch the protoplasm to a thin layer, sandwiching it between the huge central vacuole and cell wall.

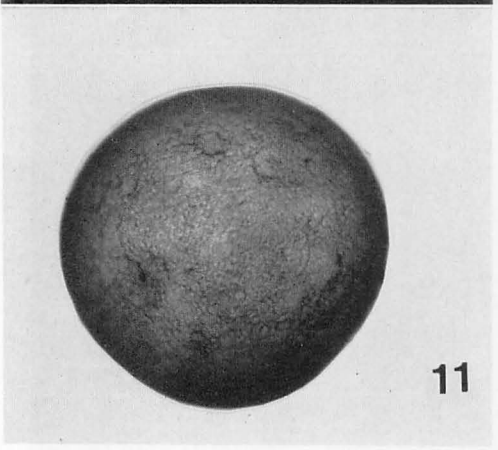
b) Nuclear-associated microtubules

Preceeding mitosis, polar microtubules appeared at both ends of the long axis of the nuclei (Fig. 12). The nuclear-associated microtubule bundles, either surrounding the nucleus or radiating from it, decreased at the same time. During nuclear division, the presence of microtubules increased, forming interzonal spindles (HORI and ENOMOTO 1980) suggesting the reorganization of previously depolymerized nuclear-associated microtubules (Fig. 13). At the final course of nuclear division, interzonal spindle microtubules were scattered, probably because of the break of the nuclear envelope. Each of the divided nuclei turned about 90°, in the same rotation. The scattered microtubule bundles wound about the nucleus and radially arranged microtubules were reorganized (Fig. 14). As the nuclear division took place syn-



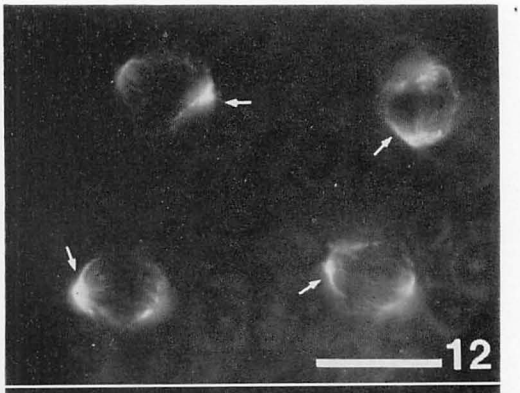
Figs. 6–9. Nuclear-associated microtubules. 6. Microtubules surrounding the surface of nuclei. 7. Radially-extending microtubules from the nuclear surface. 8. Peri-nuclear microtubules and radially-extending microtubules are continuous. (Peri-nuclear microtubules, radially-extending microtubules and the connection region of above lie at the slightly different focal levels.) 9. Radially-extending microtubules attached to the chloroplast surface (arrows) suggesting a positional linkage between nucleus and chloroplast. (Scale bars: 10 μm for Figs. 6 and 8; 20 μm for Figs. 7 and 9)

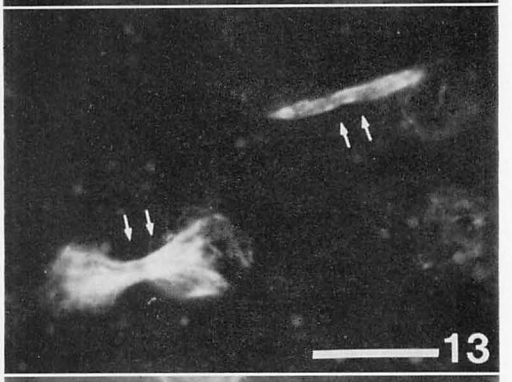


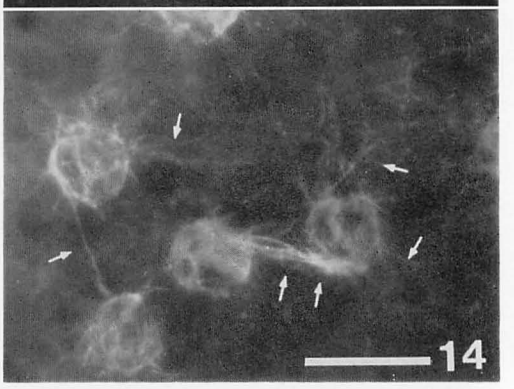


Figs. 10–11. Destruction of cortical microtubules. 10. Disorganized cortical microtubules (arrows) 15 min after immersion of whole cells in pure water. (Scale bar: 20 μm). 11. Crater-like holes on the cytoplasmic layer as a result of the destruction of cortical microtubules. $\times$20.

chronously, but the direction of axis of each nucleus was different, the equidistant distribution of nuclei was disordered at the end of mitosis. As the nuclei-associated radial microtubule system re-formed, chloroplasts became evenly distributed and gaps were filled by an increase of chloroplasts. The equidistant arrangement between each nuclei was restored again. The link between nuclei and chloroplasts became weak during nuclear division, however, the chloroplasts remained tightly linked with the cortical microtubules. Mitosis proceeded adjacent to the chloroplast monolayer.







Figs. 12–14. Probable conversion of nuclear-associated microtubules to spindles in mitosis. Microtubules are shown by arrows. 12. Initial step of mitosis. Nuclear-associated microtubules disappear. 13. Formation of interzonal spindles. 14. Final stage of mitosis. Nuclear microtubules are reorganized. (scale bar: 20 μm)

Discussion

In coenocytic green algae, the cytoskeleton is thought to be involved in cytoplasmic cleavage for spore formation. Actin and microtubule are known to be involved in cyst formation of *Acetabularia acetabulum*

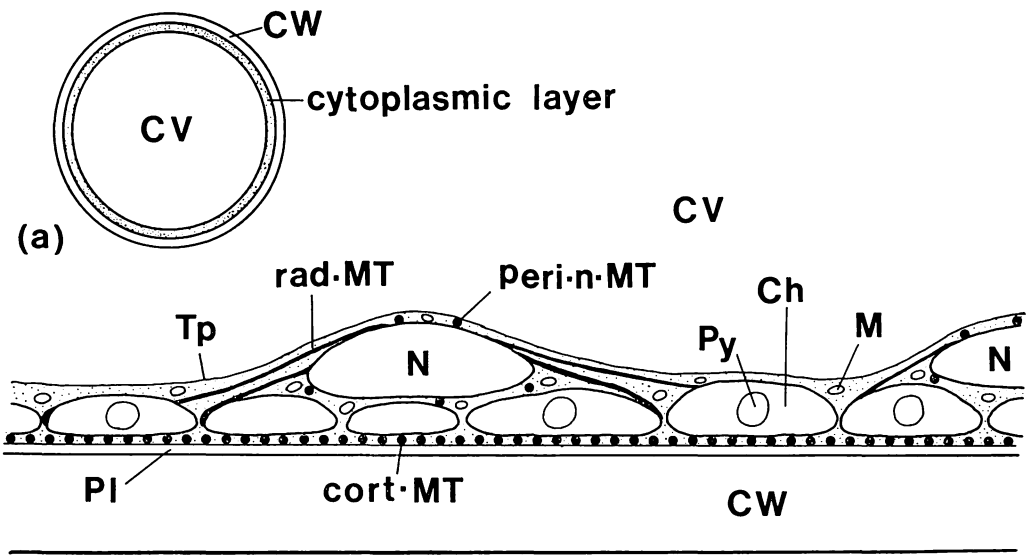


Fig. 15. The spatial organization of two microtubule systems and the relationships with other constituents of cytoplasmic layer. (a) Locality of the cytoplasmic layer in the whole plant of *Valonia ventricosa* is diagramatically presented. (b) Cross sectional view of cytoplasmic layer. (Relative size of each constituent is not exact) CW: cell wall, CV: central vacuole, Pl: plasmalemma, Tp: tonoplast, N: nucleus, Ch: chloroplast, Py: pyrenoid, M: mitochondrion, cort-MT: cortical microtubule bundle, rad-MT: radially-extending microtubule bundle, peri-n-MT: peri-nuclear microtubule bundle.

(Menzel 1986). I have also studied the cytoskeletal organization in the multinucleate cap-ray of *A. calyculus* and observed an even cytoplasmic cleavage with a sole nucleus as the center of each piece of cleaved cytoplasm (unpubl. data). In *V. ventricosa,* the size of cleaved cytoplasm and the number of nuclei in a piece of the cytoplasm were uneven in the aplanospore formation, but on the other hand each swarmer was formed by an equal size of delimitted cytoplasm and a sole nucleus. In the search for general and spacific mechanisms of cytoskeletal organization in the cell differentiation of multinucleate cells, *V. ventricosa* is a useful material. In this report, the spatial organization of microtubules in vegetative phase was investigated which form the basic knowledge for the dynamic organization of cytoskeleton during the cell morphogenesis of *V. ventricosa.*

Coexistance of two microtubule systems was essential for the protoplasmic organization in the stationary phase of *V. ventricosa.* Cortical microtubules spread chloroplasts in a monolayer adjacent to the plasmalemma while nuclear-associated microtubules support an even distribution of nuclei next to chloroplast monolayer, adjacent to the inner surface of cytoplasmic layer, at the tonoplast. The two microtubule systems are illustrated in Figure 15.

The bundles of cortical microtubules were closely arranged in some samples but more loosely in others. Physiological significance of this difference is, however, not known. The rapid disorganization of the cortical microtubule system was caused by immersion of the plant in pure water, although the nuclear-associated microtubule system remained intact. The disorganization of the cortical microtubule system also occurred in the early step of aplanospore formation, which was started by wounding of the cytoplasm.

The terminal portion of the radial microtubule bundles adhered to the chloroplast surface. The positional relationships be-

tween nuclei and chloroplasts and also chloroplasts and plasmalemma were maintained by the organization of microtubule bundles that specifically bound to each cell component. The fine structure and molecular organization of these bindings are under investigation.

The nuclei are distributed roughly equidistantly on the chloroplasts sheet in *V. ventricosa*. The distances between adjacent nuclei are maintained by radial microtubules around the nuclei. The recovery of an even distribution of nuclei after synchronous division of nuclei suggests that the radially-extending microtubules shift the nuclei to certain positions, maintaining equidistance by binding with surrounding chloroplasts at what appears to be their terminal portion. At the same time, the chloroplasts increase in number by division which results in the enlargement of the chloroplasts sheet as well as the cytoplasmic layer, resulting in the enlargement of the whole cell.

LACLAIRE (1987) could not find a common function between the cortical microtubules of the two coenocytic algae, *E. verticillata* and *B. forbesii*. To know the generality of the roles of microtubules in coenocytic algal cells, further observation is now in progress using other coenocytic green algae.

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石川依久子：多核細胞性緑藻バロニアの形態形成における細胞骨格 I.
核・葉緑体配置に寄生する2つの微小管系

巨大細胞性緑藻バロニアの成長および形態分化の過程が細胞骨格の動的構成に導かれることを予備実験で確かめ一連の研究を計画した。本研究では栄養成長過程および休止期にある藻体中の微小管構成とその役割を間接蛍光抗体法により解析した。細胞質は巨大液胞と細胞壁にはさまれた薄層として存在し，細胞膜側に一層をなす葉緑体群と液胞側に分布する多くの核をもつ。微小管は細胞膜に接して密に平行配列し，細胞膜と葉緑体の双方に接着することによって葉緑体を細胞膜直下に固定している。一方，核の表面から微小管が放射状にひろがって葉緑体表面に接着し，葉緑体群上に核を固定し，同時に，一定数の葉緑体を核の周辺に確保することによって核の均等分布を助けている。(560 豊中市待兼山町1-1，大阪大学教養部生物学教室)