Planta (1989) :178:334–341

Planta

Organization of cortical microtubules and microfibril deposition in response to blue-light-induced apical swelling in a tip-growing *Adiantum* protonema cell

Takashi Murata and Masamitsu Wada
Department of Biology, Faculty of Science, Tokyo Metropolitan University, Fukazawa, Tokyo 158, Japan

Abstract. The arrangements of cortical microtubules (MTs) in a tip-growing protonemal cell of *Adiantum capillus-veneris* L. and of cellulose microfibrils (MFs) in its wall were examined during blue-light (BL)-induced apical swelling. In most protonemal cells which had been growing in the longitudinal direction under red light, apical swelling was induced within 2 h of the onset of BL irradiation, and swelling continued for at least 8 h. During the longitudinal growth under red light, the arrangement of MFs around the base of the apical hemisphere (the subapical region) was perpendicular to the cell axis, while a random arrangement of MFs was found at the very tip, and a roughly axial arrangement was observed in the cylindrical region of most cells. This orientation of MFs corresponds to that of the cortical MTs reported previously (Murata et al. 1987, Protoplasma **141**, 135–138). In cells irradiated with BL, a random rather than transverse arrangement of both MTs and MFs was found in the subapical region. Time-course studies showed that this reorientation occurred within 1 h after the onset of the BL irradiation, i.e. it preceded the change in growth pattern. These results indicate that the orientation of cortical MTs and of cellulose MFs is involved in the regulation of cell diameter in a tip-growing *Adiantum* protonemal cell.

Key words: *Adiantum* – Blue light – Cell shape (tip-growing cell) – Microfibril – Microtubule – Protonema – Pteridophyta – Tip growth

Introduction

The direction of cell expansion in plants is determined by physical properties of the cell wall. The arrangement of cellulose microfibrils (MFs) within the cell wall is one of the major factors in the determination of these properties, and is considered to be influenced in many kinds of plant cells by the orientation of cortical microtubules (MTs) for a recent review, see Robinson and Quader 1982). In cells with diffuse longitudinal growth, the hypothesis has been advanced that transversely-arranged MFs are induced by transverse hoops of cortical MTs and restrict transverse expansion of the cell wall as a result of cell-wall reinforcement (for review, see Green 1980).

Tip-growing cells also regulate their diameter during growth and differentiation in a precise manner (Schnepf 1982; Kataoka 1981; Nagata 1973). Therefore, a mechanism regulating cell diameter in these cells must exist but its nature is not known. Considering the case of cells with diffuse longitudinal growth, it may be assumed that MF arrangement plays a role but its precise contribution to the regulation of cell diameter in tip-growing cells is not understood.

To study the regulation of the cell diameter in tip-growing cells the cell diameter should be controllable experimentally. In protonemal cells of ferns, spherical expansion at the tip (apical swelling) can easily be induced by irradiation with blue light (BL) (Howland 1972; Wada et al. 1978), indicating that these cells may be a good material for such studies. Miller and Stephani (1971) proposed that MF orientation controlled by MTs regulates the diameter of a protonemal cell under various light conditions. Recently, we reported the arrangement of the cortical MTs in an *Adiantum* protonema growing linearly under red light (Murata et al. 1987), and suggested the importance of MTs and MFs in the subapical region for the control of cell diameter. However, MF arrangements in fern protonemal cells have not been unequivocally demonstrated so far. In this study, we investigated

Abbreviations: BL = blue light; MF(s) = microfibril(s); MT(s) = microtubule(s)

the arrangement of MFs and MTs during BL-induced apical swelling in an *Adiantum* protonemal cell.

Material and methods

Plant material and culture. Spores of *Adiantum capillus-veneris* L. were collected in the summer of 1986 in a greenhouse of the Botanic Gardens of the University of Tokyo, and stored at approx. 5° C in the dark. The spores were sterilized with 0.1-strength of "Purelox" (approx. 4–6% solution of sodium hypochlorite; Oyalox Co., Tokyo) using Ito's (1970) aseptic technique, and were sown between two layers of a thin agar-gelatin film (Murata et al. 1987). The spores were then submerged under 3 ml of liquid medium, consisting of 0.1-strength modified Murashige and Skoog's mineral salt solution (Wada and Furuya 1970). For MF observation, spores were sown on the same medium solidified with 0.5% agar, and were covered with a coverslip ($3 \cdot 18 \cdot 0.3$ mm^3). The spores were imbibed for 1 d in the dark and then cultured under continuous red light (0.5 W·m^{-2}) at 25° C. Protonemal cells cultured under red light for 5 d were used for the experiments.

Light sources. Red light was obtained by passing white light from a fluorescent lamp (FL40SD/NL; Tokyo Shibaura Electric Co., Kawasaki, Japan) through a red acrylic-resin filter (Torayglas 130; Toray Co., Osaka, Japan). Blue light was obtained by passing light from fluorescent lamps (FL20BW; Tokyo Shibaura Electric Co.) through two layers of blue plastic film (Ryutate No. 63; Ryudensha, Tokyo, Japan). The fluences of the red light and BL used were 0.5 W·m^{-2} and 1.2 W·m^{-2}, respectively.

Time-lapse observations. The apical parts of protonemal cells were continually recorded with a time-lapse video system (Kadota and Wada 1986) combined with an inverted microscope (CKC-Tr-I; Olympus Optical Co., Tokyo) under BL. The video system was controlled by a time-lapse controller (VTG-88; For-A Co., Tokyo) and the real time was recorded and shown on a monitor screen. The image on the monitor screen was printed by a video printer (SCT-P60; Mitsubishi Electric Corp., Tokyo) and the cell shape was traced with a sheet of tracing paper on the printed image.

For measurements of cell diameter at higher resolution the shapes of protonemal cells were recorded photomicrographically at scheduled times with an infrared film (HIE 135-36; Eastman Kodak Co., Rochester, N.Y., USA) under infrared light obtained by passing light from a tungsten lamp (JC12V100W; Iwasaki Electric Co., Tokyo) through an infrared filter (IR85; Hoya Corp., Akishima, Japan). The images of the protonemal cells in the negatives were directly measured under a stereomicroscope.

Indirect immunofluorescence microscopy. The method of immunofluorescence was basically the same as described in Murata et al. (1987). Protonemal cells were fixed in 8% paraformaldehyde, 0.2% picric acid, 5 mM ethyleneglycol-bis-(β-aminoethylether)N,N,N′,N′-tetraacetic acid (EGTA) dissolved in P-buffer (0.1 M sodium-phosphate buffer, pH 7.0, containing 0.5 mM phenylmethylsulfonyl fluoride and 1% dimethylsulfoxide) for 30 min at room temperature. The fixed cells were cut transversely with a razor blade and washed three times with P-buffer. The cells were then treated with 1% Nonidet P-40 (Sigma Chemical Co., St. Louis, Mo., USA) in P-buffer for 4 h, and again washed three times with P-buffer. After this, the buffer was replaced with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM NaH_2PO_4, pH 7.3), and the protonemal cells were incubated overnight at 37° C with 20 µl of anti-tubulin antibody comprised of a mixture (1:1, v/v) of mouse monoclonal anti-chicken-α-tubulin and anti-chicken-β-tubulin diluted 1:500 with PBS. After rinsing three times with PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Wako Pure Chemical Industries, Osaka, Japan) and one washing with PBS, 10 min each, the cells were incubated at 37° C with fluorescein-labeled second antibody diluted 1:10 with PBS for 6 h. All antibodies were purchased from Amersham Japan Co. (Tokyo). The immunostained cells were mounted on a glass slide with 0.1% *p*-phenylenediamine and observed under an epi-fluorescence microscope.

Observation of MFs on the inner surface of the cell wall. A method modified from Hogetsu and Shibaoka (1978) was used. Protonemata were transferred from culture medium under appropriate light conditions onto an acetyl-cellulose film (0.08 mm in thickness; Nisshin EM Co., Tokyo), dipped immediately into liquid nitrogen for freezing, and stored in a freezer at approx. −20° C until use. For analysis, they were thawed and cut longitudinally on the film with a razor blade. The cytoplasm was removed with a fine paint brush moistened with 0.1% polyoxyethylene octylphenol ether (Triton X-100) and the cell wall was spread, inner side up, on the film with the same paint brush moistened with 0.1% Triton X-100, and then washed with the paint brush moistened with water. Samples thus treated on the film were shadowed with platinum at an angle of 30°, and coated with carbon at room temperature using a freeze-fracture device (FD5A; Eiko Engineering Co., Mito, Japan). The resulting replica was ionized using an ion coater (IB-3; Eiko Engineering Co.) and reinforced with 1% polyvinylalcohol as follows. The polyvinylalcohol solution (Wako Pure Chemical Industries) was dropped on the pre-heated replica and excess solution was immediately shaken from the preparation, the remainder drying quickly. Then a grid was placed exactly on the sample and affixed with Epoxy glue (Araldite Rapid; Ciba-Geigy, Basel, Switzerland). The reinforced replica on acetyl-cellulose was cut along the edge of the grid, and the acetyl-cellulose film was dissolved in methyl acetate. After being rinsed twice with methyl acetate, the sample on a grid (Fig. 6) was dried and observed with a Hitachi (Tokyo) H-300 electron microscope.

Results

Induction of apical swelling by BL. Under red light applied horizontally, protonemal cells grew toward the red light source, with a constant diameter (approx. 20 µm) and without cell division (Fig. 1a). When instead of red light BL was applied vertically onto the protonemata apical swelling was induced (Fig. 1b) after a certain lag period (Fig. 2). The cylindrical region that had been formed under the red light did not swell (Fig. 2). The lag period was found to be about 1–2 h (Fig. 3), the percentage of protonemal cells with swollen tips reaching more than 90% about 2 h after the onset of BL (Fig. 5, open circle). The apical swelling continued at least for 8 h. Cell division occurred later on.

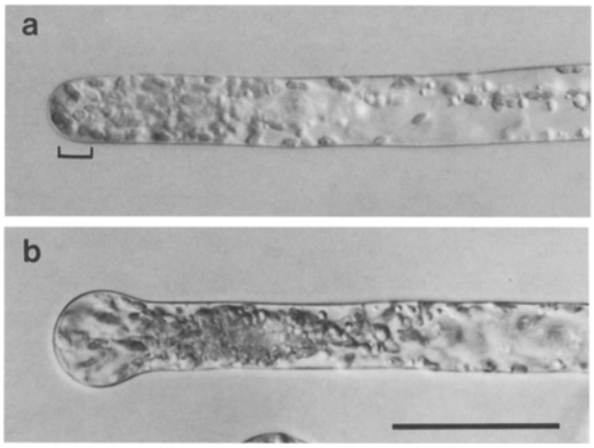


Fig. 1a, b. Photomicrographs of *Adiantum* protonemal cells growing under red light (**a**) and irradiated with BL for 4 h (**b**). The *bracket* in **a** indicates the subapical region. Nomarski optics. Bar = 50 μm; × 440

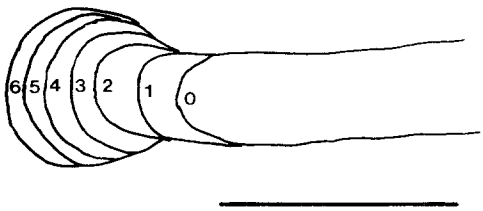


Fig. 2. Time-lapse sequence of apical swelling of a protonemal cell of *Adiantum* irradiated with BL. Cell outlines at 1-h intervals are shown. Numbers within the drawing are the time in hours after the onset of BL. Bar = 50 μm; × 620

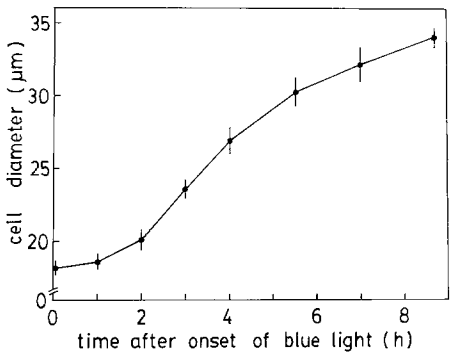


Fig. 3. Increment of cell diameter of *Adiantum* protonemal cells irradiated with BL. Cell diameter was measured at the widest part of the apical spherical region. Each point represents the mean value ± SE from three cells

Disappearance of transverse MTs following BL irradiation. In protonemal cells growing under red light, a transverse arrangement of cortical MTs around the base of the apical hemisphere (subapical region, about 5–15 μm from the tip; see Fig. 1a) was evident (Fig. 4a), as described previously (Murata et al. 1987). At 30 min after the onset of BL, the number of cells without a transverse arrangement of MTs in the subapical region increased greatly (Figs. 4b, 5), and after 2–4 h of irradiation with BL, the transverse arrangement of MTs in the subapical region was no longer found; instead, randomly arranged MTs were observed (Fig. 4c). A longitudinal arrangement of MTs in the cylindrical region was observed under both red-light and BL conditions. Dark incubation for 2 h or a change of the direction of red light for 1 h, as a control experiment, did not cause a disappearance of the transverse arrangement of MTs (data not shown).

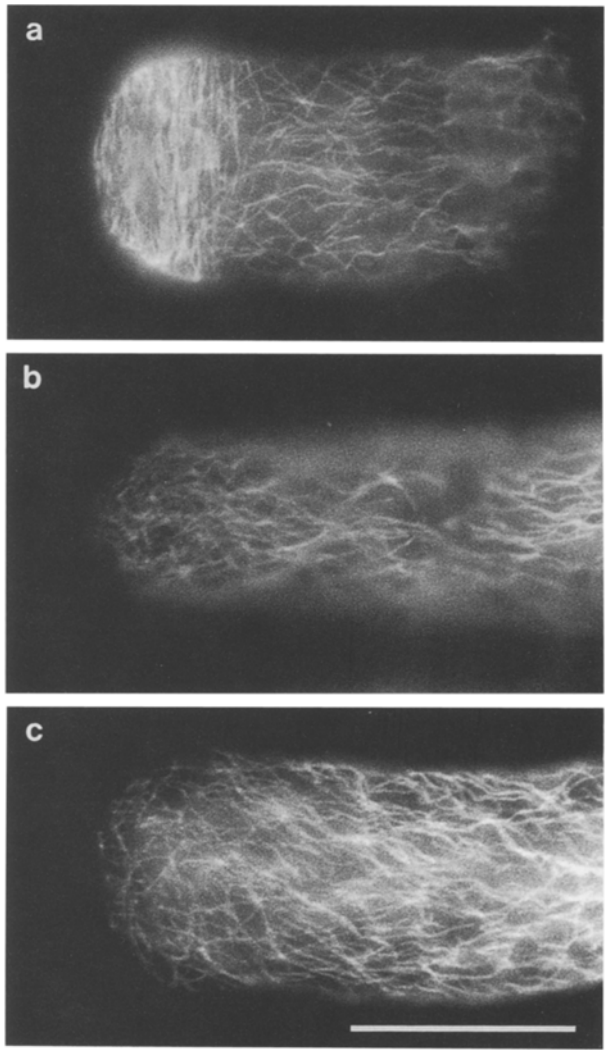


Fig. 4a–c. Immunofluorescence micrographs of the MT arrangements in an *Adiantum* protonemal cell under the influence of BL. Protonemal cells cultured under red light (**a**) were irradiated with BL for 30 min (**b**) or 3 h (**c**). Note that the transverse arrangement, seen in **a**, of MTs at the subapical region is not found in **b** and **c**. Bar = 20 μm; × 1450

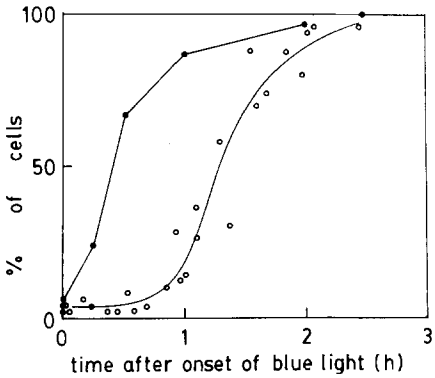


Fig. 5. Time-course of disappearance of the transverse arrangement of MTs (●—●) and development of apical swelling as detected under a light microscope (○—○) during BL irradiation of *Adiantum* protonemata. Each point was obtained from more than 30 protonemata (●) and from 50 protonemata (○)

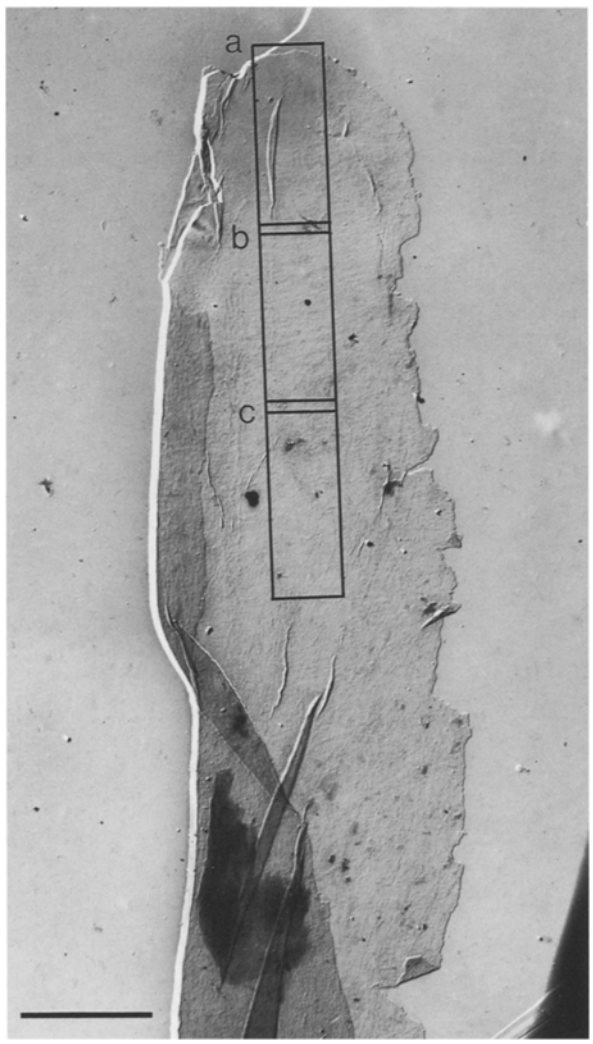


Fig. 6. An electron micrograph of a cell-wall sample of an *Adiantum* protonemal cell prepared for microfibril observation. The cell outline can easily be distinguished. The *boxed regions* (*a*, *b*, *c*) are magnified and shown in Fig. 7. Bar = 10 μm; × 1700

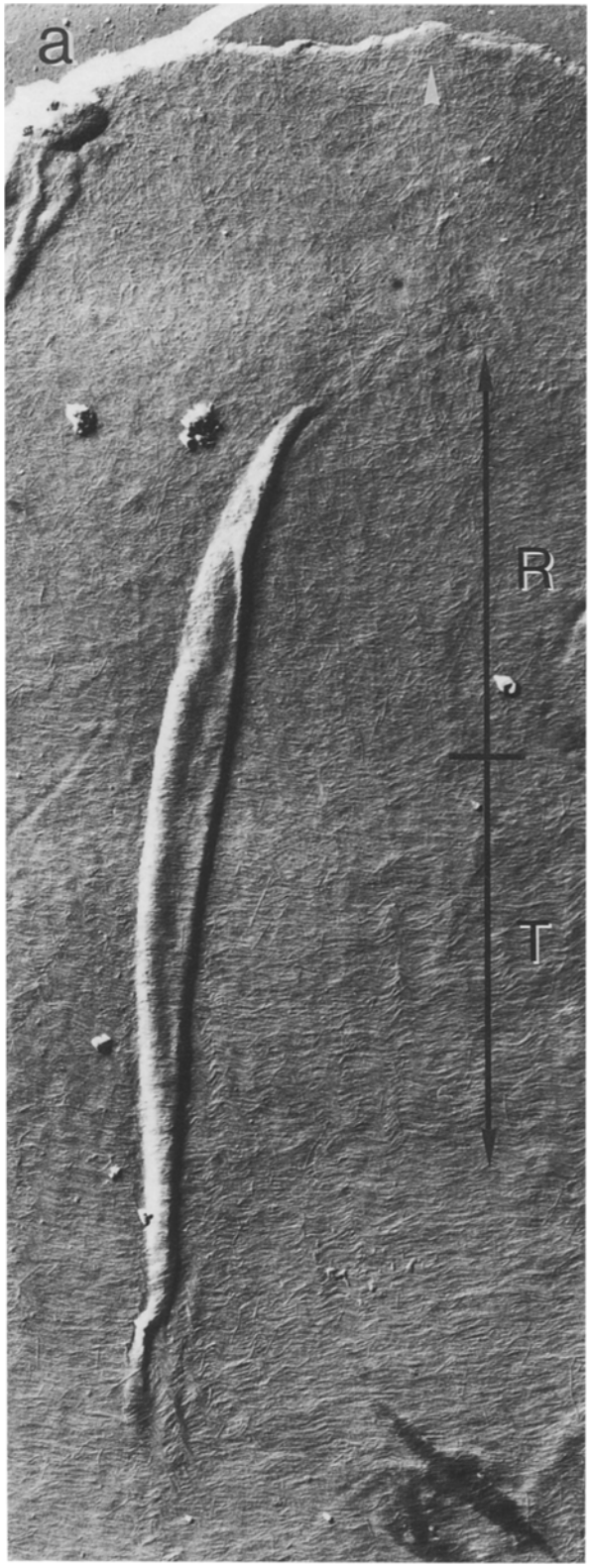
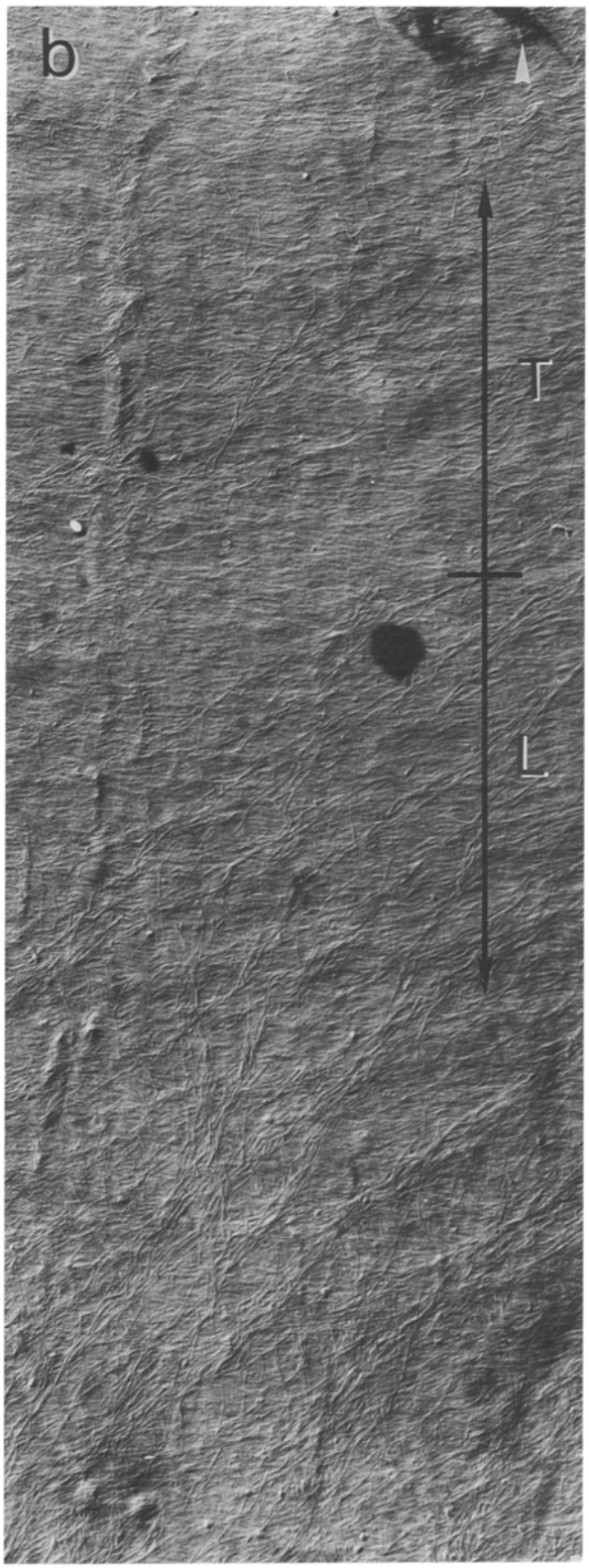


Fig. 7a–c. Arrangements of microfibrils on the innermost wall of a protonemal cell of *Adiantum* growing under red light. The distances from the tip are 0–13 (**a**), 13–27 (**b**) and 27–41 μm



(c). Boundaries between each pattern of the MF arrangement are indicated by a bar crossing a vertical double-headed arrow. R=random, T=transverse, L=longitudinal arrangement of MFs. *White arrowheads* indicate the direction of cell apex. *White arrows* in **c** indicate bundles of MFs. Bar=1 μm; ×13000

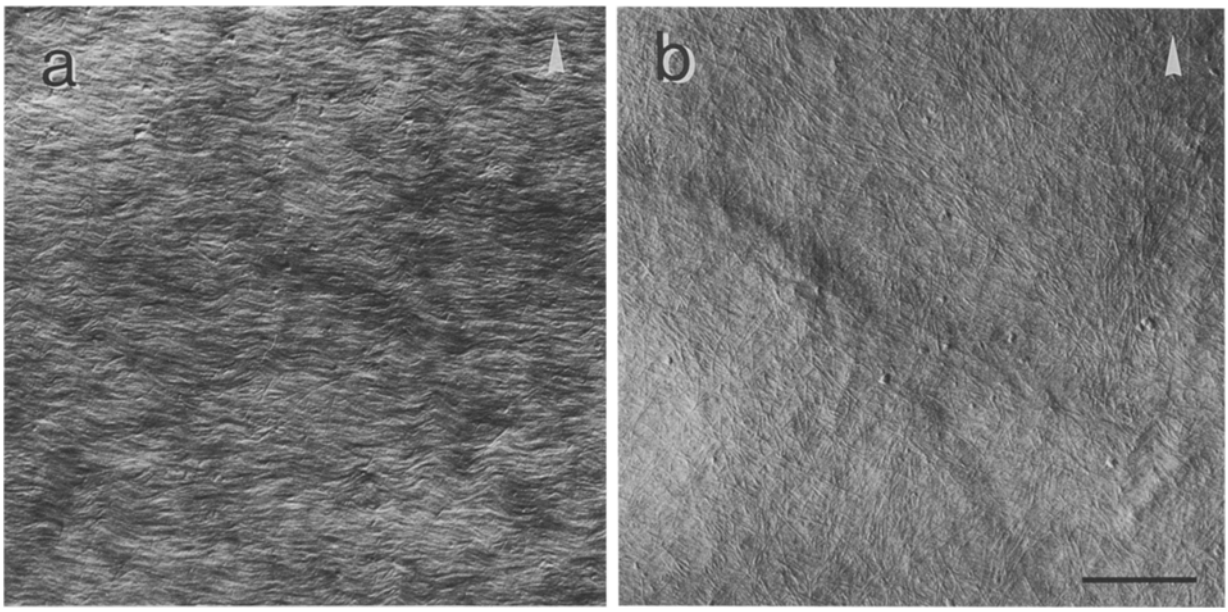


Fig. 8a, b. Electron micrographs of MFs in the subapical region of an *Adiantum* protonemal cell under the influence of BL. **a** Red-light control; **b** BL for 2 h. In **b**, a nearly random arrangement of MFs is observed. *Arrowheads* indicate the direction of cell apex. Bar = 1 μm; × 15000

A comparison of the time-courses of the apical swelling and of the disappearance of the transverse arrangement of MTs showed that the latter occurred approx. 50 min prior to the former (Fig. 5).

Arrangements of MFs on the innermost layer of the cell wall. The innermost layer of the cell wall at approx. 0–60 μm from the tip was observed (Fig. 6). In protonemal cells grown under red light, the MFs were deposited randomly in the apical region (Fig. 7a) and transversely in the subapical one (Fig. 7a, b). Microfibrils in the apical and subapical region seemed to be embedded in a non-fibrillar matrix. Longitudinally arranged bundles of MFs were observed in the cylindrical region (Fig. 7c). In some cases (2 out of 11 cells), the bundles of MFs in the cylindrical region ran in various directions (data not shown). Boundaries of each arrangement were determined as shown in Fig. 7. Microfibrils turning their orientation from transverse to longitudinal were observed near the boundary between transverse and longitudinal arrangements. When the range of each arrangement of MFs was measured (Table 1) the boundary between random and transverse arrangements of MFs was found to be at 5.5 ± 0.6 μm (mean ± SE from nine cells) from the tip; this is the middle of the apical hemisphere.

Table 1. The ranges of the random and transverse arrangements of MFs along the cell axis in the apical region of protonemal cells of *Adiantum*. Mean ± SE. Cells without a longitudinal arrangement of MFs in the cylindrical region were not included in the data. N = number of cells examined

Light treatment	Random arrangement (μm)	Transverse arrangement (μm)	*N*
Red	5.5 ± 0.6	15.6 ± 1.5	9
Blue, 1 h	10.0 ± 1.3	3.1 ± 1.2	7
Blue, 2 h	12.3 ± 1.3	0.2 ± 0.2	8

After 2 h or 4 h of BL irradiation, a random arrangement of MFs was observed in the subapical region instead of the transverse arrangement (Fig. 8), while no apparent change of MF arrangement was found in other regions, i.e., random and longitudinal arrangements were found in the apical and cylindrical region, respectively. In protonemata irradiated with BL for 1 h, MF arrangement exhibited an intermediate pattern (data not shown); the area showing transverse arrangement was smaller than that of a red-light-growing protonema, while the area showing random arrangement was greater (Table 1).

Arrangements of MTs and MFs during apical swelling are summarized diagrammatically in Fig. 9.

Discussion

Microfibrils and cell-diameter control. In contrast to cells with diffuse longitudinal growth, tip-

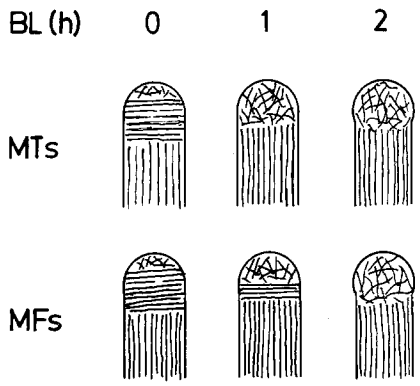


Fig. 9. Diagrams of changes in arrangement of cortical MTs and MFs in an *Adiantum* protonemal cell. The numbers indicate hours after the onset of BL. Note that longitudinal cell growth during the period has been disregarded

growing cells expand in their apical hemisphere only. In the regulation of the cell diameter of such cells, therefore, directionality of expansion in the growth zone and the gradient in the local rate of area expansion at different distances from the apex are important (Green 1969). Cell-wall extension perpendicular to the cell axis (transverse extension) seems to be involved in regulating the cell diameter, increased transverse extension leading to an increase of cell circumference. Kataoka (1982) showed that transverse extension of the cell wall increases at the apical hemisphere during colchicine-induced apical swelling in *Vaucheria* cells.

We found a transverse arrangement of MFs around the base of the apical hemisphere (the subapical region) in linearly growing protonemal cells of *Adiantum*. Reinforcement of the cell wall by MFs would restrict the transverse extension in the subapical region as in the case of cells with diffuse longitudinal growth (e.g. Hogetsu and Shibaoka 1978). We further found that in the subapical region of protonemata exposed to BL a reorientation of the MFs from transverse to random occurs which precedes the apical swelling. The reorientation of the MFs may increase the rate of transverse extension and cause apical swelling as a result. Thus, orientation of MF deposition could play an important role in the control of cell diameter in fern protonemal cells.

In addition to the directionality of expansion, the gradient of the local rate of wall expansion in the apical hemisphere is also important. In protonemata of the fern *Pteridium*, growth activity, which is restricted to the tip region of the apical hemisphere during filamentous growth, is redistributed more evenly during apical swelling (Davis et al. 1974). Recently, Cooke and Racusen (1986) suggested that the change of pattern of proton efflux, which might cause loosening and expansion of the cell wall by acidification, is involved in the change of growth pattern by BL. Together with the change of direction of wall extension caused by the reorientation of MF deposition, redistribution of growth activity may participate in accomplishing the apical swelling. To demonstrate the importance of the directionality and gradient of rate of wall extension, precise twodimensional measurement of wall extension in the apical hemisphere should be performed.

Orientation of MTs and MFs. If the arrangement of MFs plays a role in the control of cell diameter, a mechanism for regulating the MF-deposition pattern is very important. In the *Adiantum* protonemal cell, the pattern of the cortical MTs corresponds to that of the innermost MFs before and after the BL irradiation, consistent with the general hypothesis that a MT system regulates MF deposition, and determines cell diameter as a result.

The question as to how MTs regulate the orientation of MF deposition is unresolved. The spatial relationship between MTs and plasma-membrane rosettes, which are considered to be sites of cellulose synthesis, has been investigated in *Closterium*, a green alga (Giddings and Staehelin 1988). In *Adiantum* protonemata, the rosettes have also been seen (Wada and Staehelin 1981). Observation of rosettes in relation to MT orientation in *Adiantum* may provide a clue for understanding the role of MTs in MF orientation in tip-growing fern protonemata.

Reorganization of MTs coupled with that of MFs has also been demonstrated during leaf-primordia formation in *Graptopetalum* (Hardham et al. 1980). In comparison with that system, a feature of the fern system is its rapidity of reorganization. While several days are needed for the reorganization in *Graptopetalum*, reorganization in *Adiantum* is completed within 2 h after the onset of BL irradiation. Such a rapid reorientation of MTs has also been shown in epidermal cells of pea epicotyls and mung-bean hypocotyls in response to ethylene (Roberts et al. 1985), although MF orientation was not investigated in this case. Systems with rapid reorganization of MTs may be particularly useful for a better understanding of the mechanism by which MT arrangement is regulated.

We are grateful to Dr. T. Hogetsu (University of Tokyo) for his advice on observation of MFs, and Dr. T.H. Giddings (University of Colorado, Boulder, USA) for careful reading of the

manuscript. This work was supported by a fund for Special Research Project from the Tokyo Metropolitan Government and by a Grant-in-Aid for Scientific Research on Priority Areas (62621003).

References

Cooke, T.J., Racusen, R.H. (1986) The role of electrical phenomena in tip growth, with special reference to the developmental plasticity of filamentous fern gametophytes. Symp. Soc. Exp. Biol. **40**, 307–328

Davis, B.D., Chen, J.C.W., Philpott, M. (1974) The transition from filamentous to two-dimensional growth in fern gametophytes. IV. Initial events. Am. J. Bot. **61**, 722–729

Giddings, T.H., Staehelin, L.A. (1988) Spatial relationship between microtubules and plasma-membrane rosettes during the deposition of primary wall microfibrils in *Closterium* sp. Planta **173**, 22–30

Green, P.B. (1969) Cell morphogenesis. Annu. Rev. Plant Physiol. **20**, 365–394

Green, P.B. (1980) Organogenesis – a biophysical view. Annu. Rev. Plant Physiol. **31**, 51–82

Hardham, A.R., Green, P.B., Lang, J.M. (1980) Reorganization of cortical microtubules and cellulose deposition during leaf formation in *Graptopetalum paraguayense*. Planta **149**, 181–195

Hogetsu, T., Shibaoka, H. (1978) The change of pattern in microfibril arrangement on the inner surface of the cell wall of *Closterium acerosum* during cell growth. Planta **140**, 7–14

Howland, G.P. (1972) Changes in amounts of chloroplast and cytoplasmic ribosomal-RNAs and photomorphogenesis in *Dryopteris* gametophytes. Physiol. Plant. **26**, 264–270

Ito, M. (1970) Light-induced synchrony of cell division in the protonema of the fern, *Pteris vittata*. Planta **90**, 22–31

Kadota, A., Wada, M. (1986) Heart-shaped prothallia of the fern *Adiantum capillus-veneris* L. develop in the polarization plane of white light. Plant Cell Physiol. **27**, 903–910

Kataoka, H. (1981) Expansion of *Vaucheria* cell apex caused by blue or red light. Plant Cell Physiol. **22**, 583–595

Kataoka, H. (1982) Colchicine-induced expansion of *Vaucheria* cell apex. Alteration from isotropic to transversally anisotropic growth. Bot. Mag. Tokyo **95**, 317–330

Miller, J.H., Stephani, M.C. (1971) Effects of cholchicine and light on cell form in fern gametophytes. Implications for a mechanism of light-induced cell elongation. Physiol. Plant. **24**, 264–271

Murata, T., Kadota, A., Hogetsu, T., Wada, M. (1987) Circular arrangement of cortical microtubules around the subapical part of a tip-growing fern protonema. Protoplasma **141**, 135–138

Nagata, Y. (1973) Rhizoid differentiation in *Spirogyra*. I. Basic features of rhizoid formation. Plant Cell Physiol. **14**, 531–541

Roberts, I.N., Lloyd, C.W., Roberts, K. (1985) Ethylene-induced microtubule reorientations: mediation by helical arrays. Planta **164**, 439–447

Robinson, D.G., Quader, H. (1982) The microtubule-microfibril syndrome. In: The cytoskeleton in plant growth and development, pp. 109–126, Lloyd, C.W., ed. Academic Press, London

Schnepf, E. (1982) Morphogenesis in moss protonemata. In: The cytoskeleton in plant growth and development, pp. 321–344, Lloyd, C.W., ed. Academic Press, London

Wada, M., Furuya, M. (1970) Photocontrol of the orientation of cell division in *Adiantum*. I. Effects of the dark and red periods in the apical cell of gametophytes. Develop. Growth Different. **12**, 109–118

Wada, M., Kadota, A., Furuya, M. (1978) Apical growth of protonemata in *Adiantum capillus-veneris*. II. Action spectra for the induction of apical swelling and the intracellular photoreceptive site. Bot. Mag. Tokyo **91**, 113–120

Wada, M., Staehelin, L.A. (1981) Freeze-fracture observations on the plasma membrane, the cell wall and the cuticle of growing protonemata of *Adiantum capillus-veneris* L. Planta **151**, 462–468

Received 6 December 1988; accepted 3 February 1989