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Nuclear Behavior during Branch Formation in a Centrifuged *Adiantum* Protonema and the Nuclear Polarity

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A new branch was induced on the side wall of fern protonema by cell centrifugation and subsequent polarized red light irradiation after the induction of cell division under white light. Nuclear behavior during the branch formation was analyzed.

Immediately after cell division, the two daughter nuclei moved away from the division site in both red and dark conditions. Under continuous irradiation with polarized red light, cell swelling occurred as an early step of branching near the cell dividing wall, even though the nucleus was localized far from the branching site at the beginning of the swelling. After a new branch started to grow, the nucleus returned to the branching site and moved into the new branch from its basipetal end. When a protonema incubated in the dark was centrifuged again acropetally or basipetally just before the irradiation of polarized red light, the rate of apical growth or branch formation was increased, respectively. Moreover, growth of a branched protonema was altered from its former apex or from the branch again by dislocating the nucleus acropetally or basipetally by centrifugation, respectively. These facts suggest that the nucleus has no polarity physiologically, i.e. head and tail, namely either end of the spindle-shaped nucleus can be the nuclear front in a tip-growing protonema.

Key words: *Adiantum* — Branching — Centrifugation — Nuclear behavior — Polarity (nucleus) — Protonema (fern)

Apical growth is one of the basic patterns of cell growth in plant cells. However, the mechanism of apical growth of tip-growing cells, such as pollen tubes, root hairs, and fern and moss protonemal cells, is largely unknown. In particular, the real nature of the growing point is not known, although the fine structure of the growing tips of various cells have been observed by light and electron microscopy (Wada and O'Brien 1975, Schmiedel and Schnepf 1980, Ridge 1988, Lancelle and Hepler 1992, Lloyd 1983, Murata *et al.* 1987, Kiss *et al.* 1995). Inward ionic current at the growing apex has also been observed in several organisms, such as hyphae of *Achlya* (Kropf *et al.* 1984), *Vaucheria* (Kataoka and Weisenseel 1988), *Palvetia* zygotes (Nuccitelli and Jaffe 1974), fern protonemata (Racusen and Cooke 1982), lily pollen tubes (Weisenseel and Jaffe 1976), wheat root hairs (Gassmann and Schroeder 1994). However, even if we study an apical cell in great detail, the nature of the apex and the mechanism of apical development might not be clarified. But in fucoidal zygote cells, since cell polarity can be induced in a desired orientation by various environmental factors (Weisenseel 1979), many novel experiments have been done and a model of induction and fixation of polarity as sequential phenomena has been proposed (Browley and Robinson 1985, Kropf 1992).

If we can observe apical development sequentially in a newly-induced side branch of a filamentous cell, it may be possible to answer questions about the cell apex and how it develops. Fern and moss protonemal cells are one of the best model systems for this purpose (Ootaki 1963, 1965, 1968, Schmiedel and Schnepf 1979a, b), because developmental processes of these cells can easily be controlled by light and observed under microscopy (Wada and Sugai 1994). *Adiantum* protonemata have a more complex system than other fern or moss protonemata concerning photoregulation of development (Wada and Sugai 1994) and structural knowledge of *Adiantum* includes cytoskeletal patterns (Wada and Murata 1991), and nuclear shape (Ootaki 1968, Wada and O'Brien 1975) (for review see Wada and Sugai 1994). Recently we have had a high rate of success in inducing side branches in *Adiantum* protonemata by cell centrifugation in combination with polarized red light treatment, using similar methods of Ootaki (1965) for *Pteris vittata*. In this report we describe the kinetics of new branch development and behavior of the nucleus during these processes, as a first step of further analyses in apical development.

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Abbreviations: DAPI, 4′, 6-diamidino-2-phenylindole; PPB, preprophase band

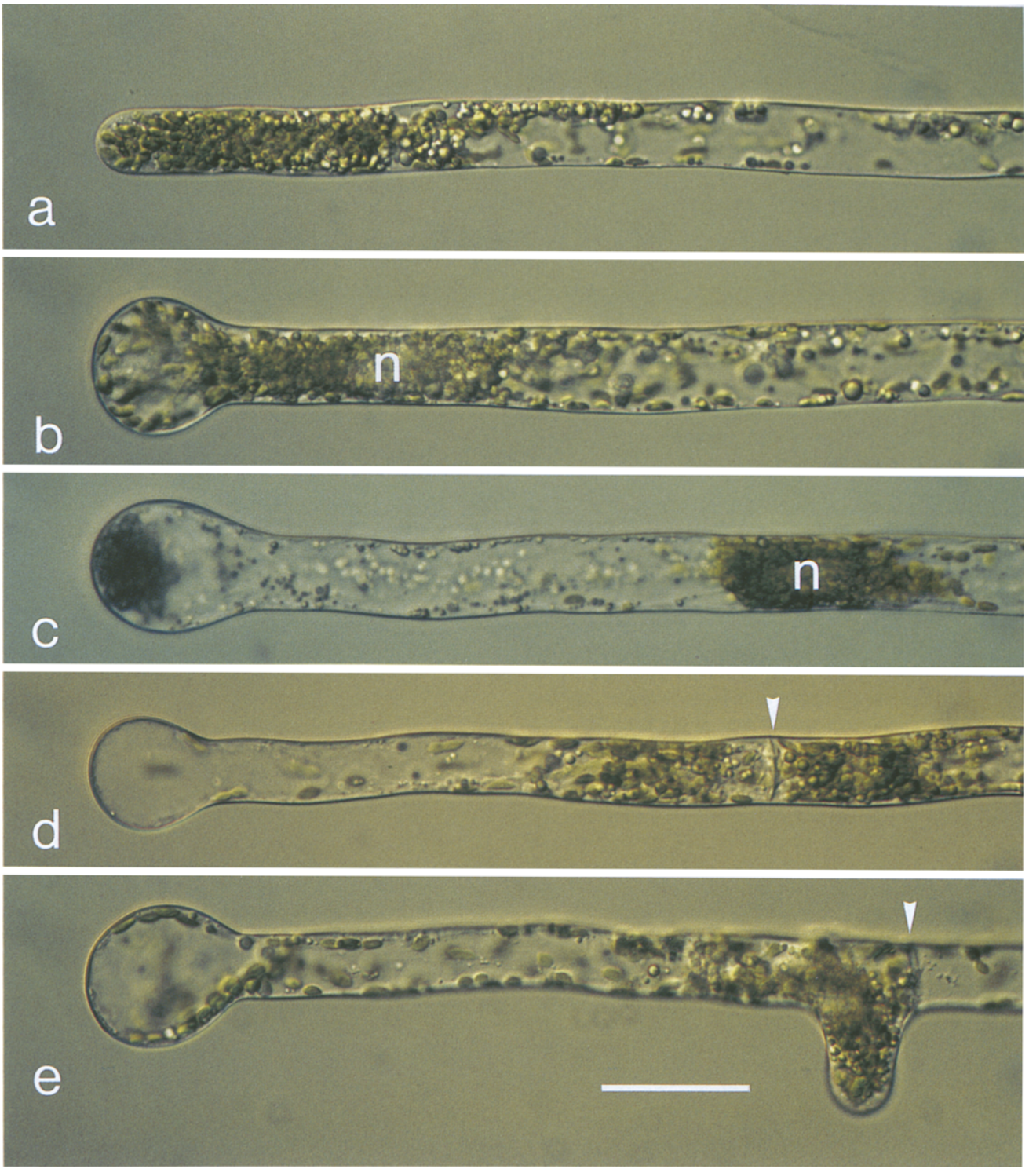

Fig. 1. Photographs of protonemata at various steps of the branching process. A protonema was cultured under continuous red light for 7 days (a), and then under white light for 7 hr (b), and centrifuged for 20 min at 2,000×*g* (c). The protonema transferred to polarized red light divided thereafter (d, arrowhead) and a new branch formed near the septum (e, arrowhead). Bar=50 μm.

Material and Methods

Plant material and aseptic culture

Culture methods of protonemal cell of *Adiantum capillus-veneris* L. were basically the same as Murata and Wada (1989). Briefly, spores of *Adiantum*, which were collected in a greenhouse of Tokyo Metropolitan University in 1990, were sown between two layers of thin gelatin-agar films, and cultured in modified Murashige and Skoog's mineral salt solution for 6 or 7 days under red light (approximately 0.5 Wm^{-2}) irradiated horizontally. The resulting protonemata growing horizontally (Fig. 1a) were cultured under white light (4 Wm^{-2}) for 7 hr to induce cell division (Fig. 1b), centrifuged basipetally (Fig. 1c) and then transferred to vertically irradiated continuous polarized red light which has an electric vector (E-vector) parallel to the growing axis of protonema, or into darkness, where cell division occurred (Fig. 1d). The reason why the protonemata cultured in the dark for 2 days, in the latter case, before transferring to polarized red light were to induce branching synchronously. The temperature was 25C throughout experiments.

Centrifugation

Centrifugation was done as described by Wada *et al.* (1983). Briefly, the protonemata cultured under white light were transferred aseptically into a custom-made centrifuge cuvette under white light conditions, and centrifuged using a swing type rotor at 2,000×g for 20 min basipetally at 25C under darkness, unless otherwise stated.

Light source

Red light was obtained by a fluorescent tube (FL20SD, Toshiba Lighting and Technology Corp., Tokyo) with a red acrylic plate (Shinkolite A 102, Mitsubishi Rayon Co. Ltd., Tokyo). Polarized light was obtained through polarizer (HN38, Nippon Polaroid K.K., Tokyo). White light of about 4 Wm^{-2} for the induction of cell division was provided by a fluorescent lamp (FL40SD, Toshiba Lighting and Technology Corp.). Dim green safe light (Kadota *et al.* 1984) was used if necessary.

Continuous recording of branching processes was done under infrared light (obtained through IR85, Hoya Corp., Akishima, Japan) using a custom-made epi-microbeam irradiator (Wada *et al.* 1983) connected to a video recording system (see below). Monochromatic red light was provided through an interference filter (IF-BPF-660, Vacuum Optics Co. of Japan, Tokyo) which has a peak of 660 nm and half-band width of 34 nm.

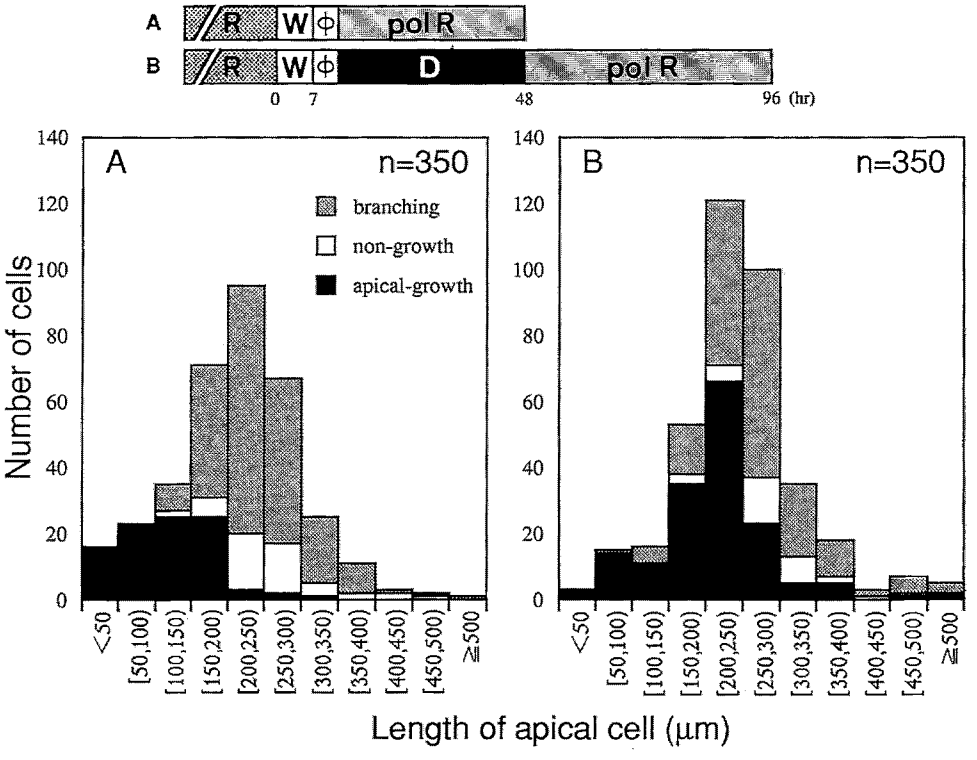

Fig. 2. Relationship between apical cell length and growth pattern of the centrifuged protonemata. Schedules of treatments are shown at the top of figure. Centrifuged protonemata were irradiated with polarized red light with (B) or without (A) insertion of 40 hr dark period after the centrifugation. Protonemata with a branch (or branches) either in the apical and/or basal cells were counted. R; red light, W; white light, o; centrifugation at about 2,000×*g* for 20 min, D; dark, pol R; polarized red light. n=; the number of cells observed. Growth patterns are shown in the figure.

Video recording system

Nuclear behavior and processes of cell division and branching were recorded with a similar video system as described by Kadota and Wada (1995) and recorded images were printed out by video copy processor (SCT-P66, Mitsubishi Electric Corp. Ltd., Tokyo). Positions of two daughter nuclei in a protonema were measured on the printed images. Timing of completion of cytokinesis was decided as the timing of resumption of organelle movement which had ceased just before the cytokinesis (see Wada *et al*. 1982).

Results

Branching under polarized red light

When red light grown protonemata were centrifuged basipetally after irradiation with white light for 7 hr, with which cell division was induced, nuclei surrounded with a mass of cytoplasm of the cells were displaced towards the cell base (Figs. 1c, 2A) at an average of about 200-250 μm. When the centrifuged cells were transferred to polarized red light or to darkness immediately after centrifugation, most of the protonemata divided within 10-20 hr (Fig. 1d). These cells then branched, at a high rate, at the apical and/or basal cell under the subsequent polarized red light (Figs. 1e, 2). Branches developed horizontally at the either side of protonema, probably because higher absorption of the polarized red light may have occurred on both flanks compared to the top and bottom of the protonema. The branches grew perpendicular to the E-vector of the polarized red light, that is perpendicular to the protonemal side wall. This means that even a small cell swelling at a very early stage of branch development could be detected in this system. The relationship between apical cell length and growth or branching patterns was studied (Fig. 2).

When cells were transferred directly to polarized red light immediately after the centrifugation (Fig. 2A), cell growth at the protonemal apex was dominantly induced if the apical cell was shorter than 200 μm. In contrast, if apical cells were longer than 250 μm, a new branch dominantly developed. The cell apices of new branches developed in most cases at the side wall near the septum either in the apical or basal cells (Figs. 1e, 3A) or in both cells as rare cases (Fig. 3). However, if the centrifuged cells were cultured in the dark for 2 days before transferring to the polarized red light, the percentage of branch formation decreased, and percentage of growth from the former apex increased, even in long apical cells (Fig. 2B). Moreover, branch formation had a tendency to occur away from the septum (Fig. 3B). In total darkness, neither branch fomation nor cell growth was observed.

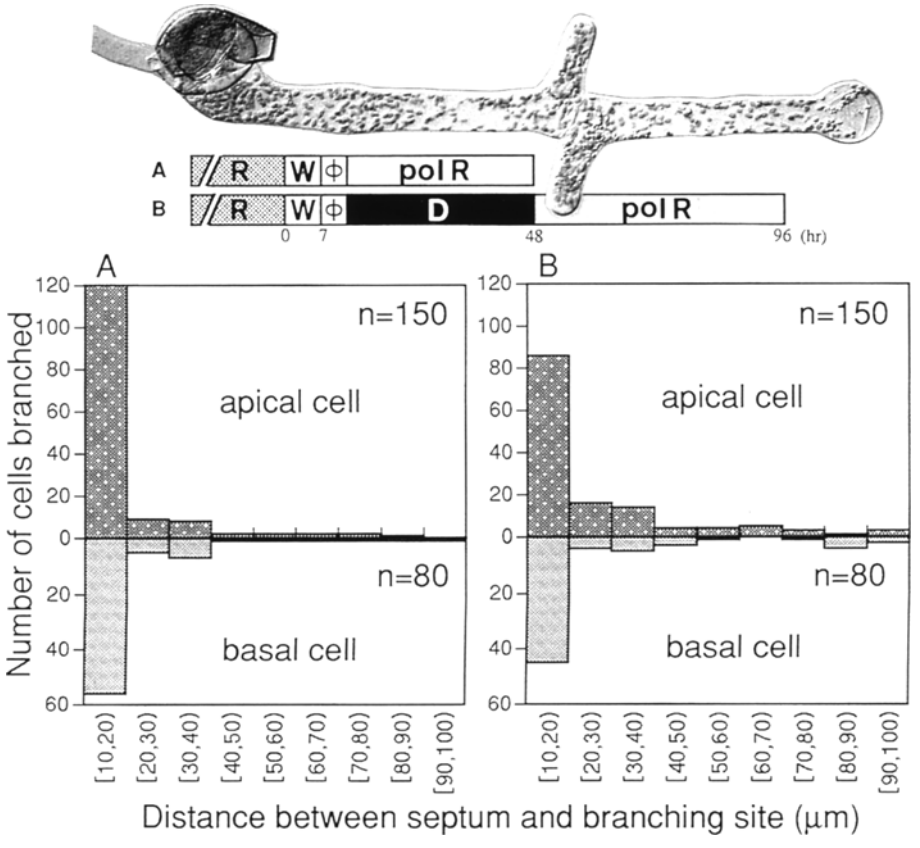

Fig. 3. Frequency of new branches both in the apical and basal cells in relation to apical cell length. Other details are the same as Fig. 2.

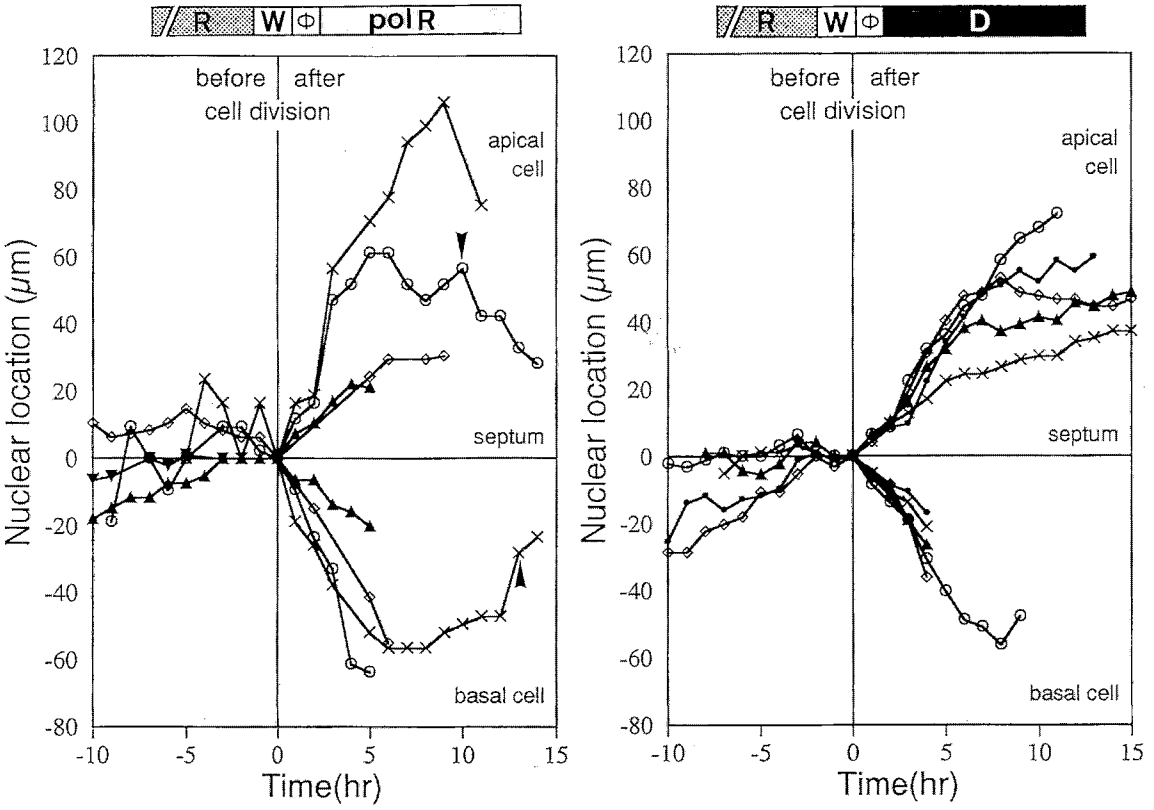

Fig. 4. Position of nuclei in a centrifuged protonema before and after cell division induced by white light. Note that both daughter nuclei moved away from the newly made septum as soon as cell division completed, but returned towards the septum under polarized red light. Arrowheads show the timing when apical growth of the new branch started. Different symbols show nuclei of different cells. Other details are the same as Fig. 2.

Based on these results, protonemata with apical cells longer than 250 μm were used in the following experiments.

Nuclear movement after cell division

The centrifuged cells were cultured under polarized red light or in the dark, and nuclear movement before and after cell division was recorded continuously by a video system (Fig. 4). Although the nuclei were surrounded with other organelles, the nuclear location was easily detected. As soon as cell division was completed, the daughter nuclei moved away from the septum towards the apex in the apical cell and towards the base in the basal cell in both light conditions. Under polarized red light, nuclei in the apical and basal cells changed their direction of movement some time after cell division, and returned towards the septum. Branch formation had already commenced during this nuclear returning process (arrowheads in Fig. 4). In the dark, nuclei stayed around the middle part of the long apical cells, as shown in the case of apical cells kept for 2 days in the dark after centrifugation (Fig. 5).

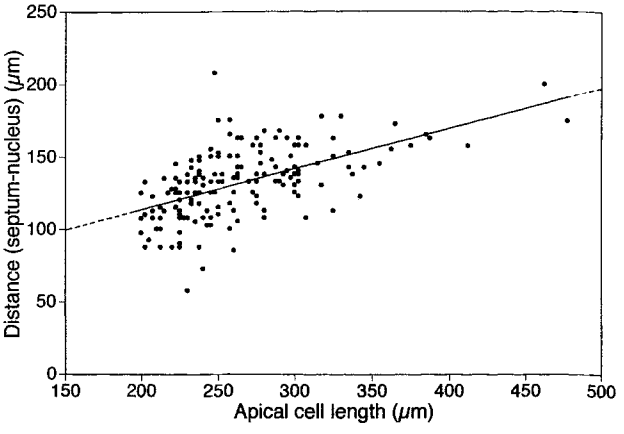

Fig. 5. Nuclear position in a protonema in relation to apical cell length. The distance between septum and nucleus was measured in protonemata cultured in the dark for two days after centrifugation (2,000×g for 20 min) following 7 days red and 7 hr white light irradiation. Solid line indicates a regression line obtained from the all data points.

Nuclear movement during branching processes

When a new branch began to develop at a site close to

the septum, nuclei were still located far from the branching site under polarized red light (Figs. 6, 7) as shown in Fig. 4. Data in Fig. 7 were obtained from fixed and DAPI stained cells showing branch formation close to the septum in apical cells. Considering the protonemal cell width is less than 20 μm, most of the nuclei were not at the branching site, although many of them were within 60 μm range of the branching site. The timing of nuclear migration into the branches is shown in Fig. 7 with black circles. Nuclei moved into branches when the latter became more than 20 μm in length. Nuclei stained with DAPI showed that the nuclei moved into branches from their basal end in the branch of apical cell and from the apical end in the branch of basal cell (Fig. 6).

Effect of nuclear dislocation on branch formation

To know whether nuclear location is important for a new apex development, protonemata kept in the dark for 2 days after centrifugation were centrifuged acropetally or basipetally again at about 2,000×g for 20 min and then irradiated with polarized red light for 2 days. The rates of apical growth and branch formation were counted. As shown in Table 1, when a nucleus was dislocated up to the apex, the rate of apical growth increased and branch formation was inhibited. In contrast, when a nucleus was dislocated to the basal end of the apical cell, branch formation was promoted and apical growth at the apex was inhibited. These results show that apical growth as well as branch formation can be induced artificially by changing nuclear location. Spindle-shaped nuclei were always seen in parallel with the protonemal axis in these experiments and travelled up and down without changing their axis.

Figure 8 shows a typical example of change in a growing apex. Nuclear location was changed back and forth by centrifugation. After a branch became about 80 μm in length, the nucleus in the cell was dislocated by centrifugation up to the protonemal apex to induce apical growth. When apical growth started the nucleus was dislocated again to the branching site to let the branch

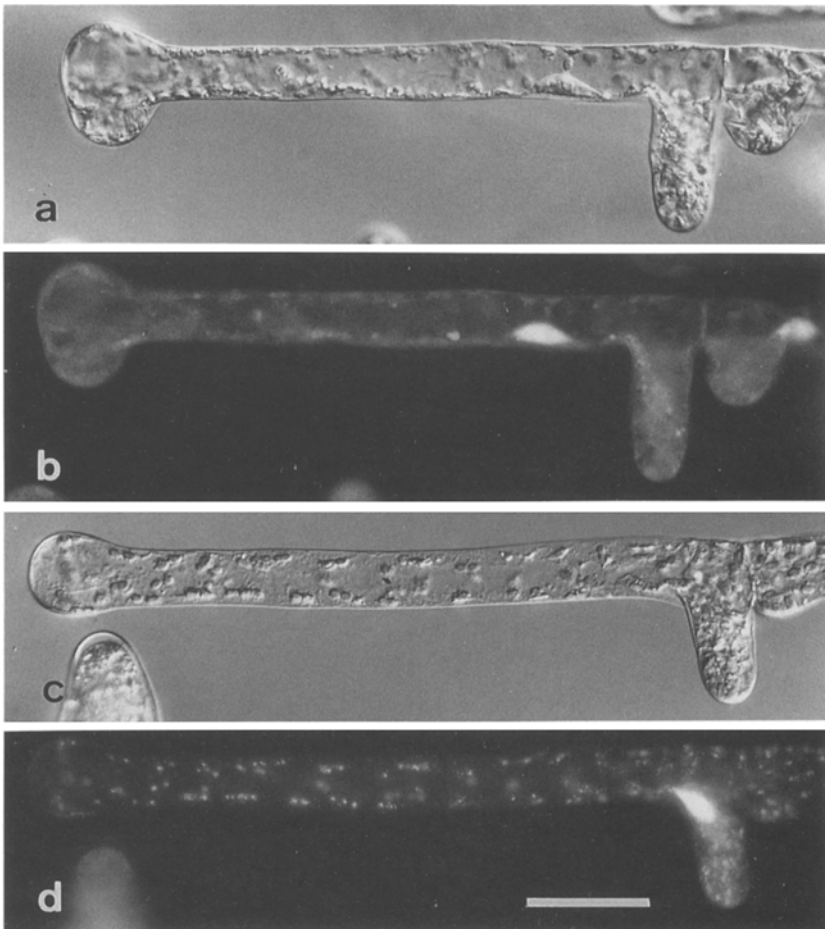

Fig. 6. A pair of photographs of differential interference contrast (a, c) and DAPI staining (b, d) showing nuclear position in relation to branch development. Centrifuged protonemata were irradiated with polarized red light for 1 day after 2 days incubation in the dark. Note that the nucleus is localized far from the branching site even when the branch developed (a, b). A nucleus moved in a branch (c, d). Bar=50 μm.

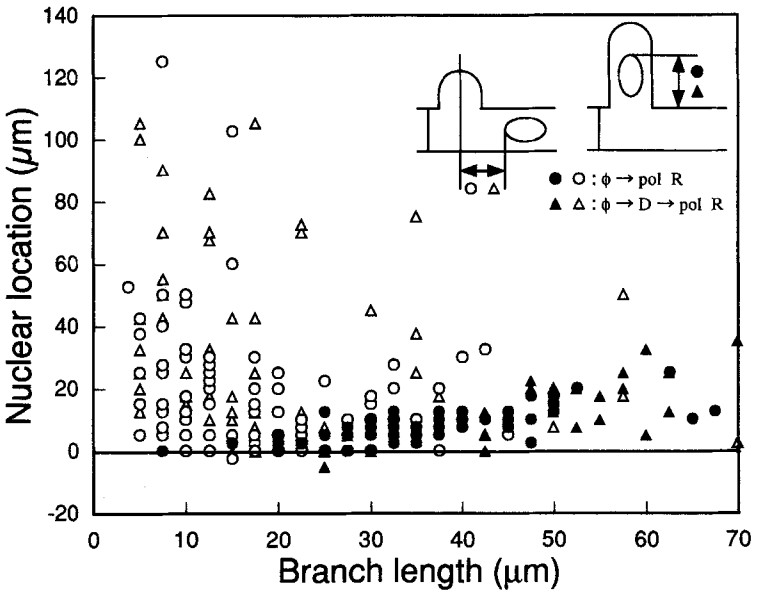

Fig. 7. Nuclear location in protonemata in relation to branch length. Nuclear position was measured as 1) distance between the center of branch at the branch base and nuclear tip when the nucleus was still in the main part of protonema (open symbols), and 2) distance between the branch base and nuclear tip when at least a part of the nucleus was already in the branch (closed symbols). Round and triangular symbols show the data obtained from the protonemata transferred directly to polarized red light or after insertion of 40 hr dark period after centrifugation, respectively.

Table 1. Effect of 2nd centrifugation on branch development and apical growth in apical cells of *Adiantum* protonemata

Centrifugation	Type of protonemal growth (%)		
	branch	apical growth	non-growth
non-centrif.	17.4±2.9	32.7±6.9	49.9±4.5
acropetal	3.6±1.9	41.6±7.5	49.9±8.7
basipetal	42.1±4.1	1.9±1.9	56.0±5.2

Cells were centrifuged at 2,000×*g* for 20 min after dark incubation for 2 days following the 1st centrifugation, and then irradiated with polarized red light for 2 days.
Each datum is the average±standard error obtained from triplicates in which more than 30 protonemal cells of different dishes were counted. Apical cells longer than 250 μm were used.

grow at its apex again. To distinguish between the first and the second growth induction of the branch apex, the direction of the electric vector of the first and the third polarized red light was changed by 45°, so that the change of growth direction was clear.

Discussion

Nuclear head and tail

In many plant and animal cells, since nuclei are usually spherical, the idea of nuclear polarity is not obvious. However, in apically growing cells, nuclei are often spindle-shaped and have a clear axis. Moreover, these nuclei migrate forward, maintaining their axes without turning around during cell growth, suggesting that they have polarity, at least physiologically. Electron microscope studies on *Adiantum* red-light-grown protonemata revealed that the front end of the spindle-shaped nuclei had an invagination, where a fine fibrous structure could be seen (Wada and O'Brien 1975). A cytoskeletal strand of both microtubules and microfilaments, emanating from the front and rear ends of the nucleus could be seen by fluorescence microscopy. The role of the cytoskeleton was recently suggested to be different at the front and the rear part of the nucleus after work using inhibitors (Schmiedel and Schnepf 1980, Kadota and Wada 1995). These results suggest that the *Adiantum* nucleus has polarity, i.e. a head and a tail, in a growing protonema morphologically, and probably physiologically as well. In *Drosophila* salivary glands, the nucleus also has polarity such that the chromocenters are fixed on the nuclear membrane at opposite sides of telomers, which are also in contact with the membrane (Zakian 1984).

In the study presented here, it has been shown that after cell division, if an apical cell was long enough, a new branch developed close to the septum, and the nucleus which had migrated towards the apex returned and entered the branch from its basal end. This means that the basal end (tail) of the nucleus in the main part of protonema became an apical end (head) in the branch. But if the apical cell was short, the cell resumed to grow at the former apex and the nucleus did not change its polarity and kept its apical end as before.

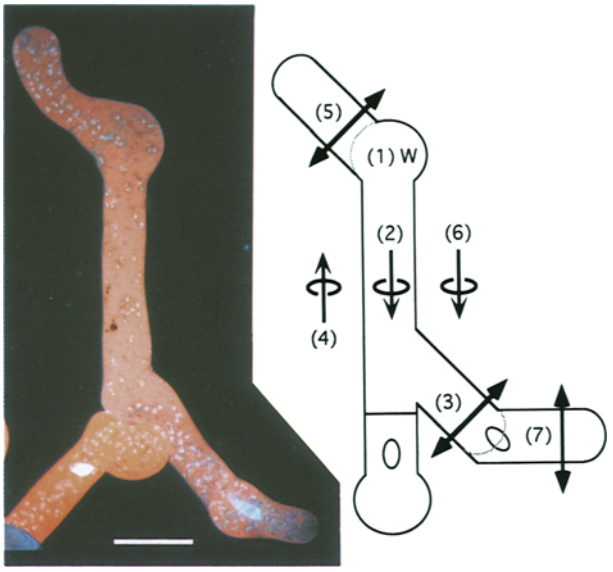

Fig. 8. A fluorescence micrograph of a double-headed protonema stained with DAPI and a scheme of how the protonema was induced. (1) Red light grown protonema was irradiated with white light (W) for 7 hr to induce cell division, (2) centrifuged basipetally by 2,000×*g* for 20 min to move nucleus downward, (3) irradiated with polarized red light with an E-vector of 45° to cell axis for 2 days to induce a branch, (4) centrifuged acropetally by 2,000×*g* for 20 min to move the nucleus upward, (5) irradiated again with the polarized red light for one day to induce apical growth, (6) centrifuged basipetally again at 2,000×*g* for 20 min to bring back the nucleus into the branch, and finally, (7) irradiated with polarized red light with an E-vector of 90° to cell axis for one day to induce branch growth again. Direction of E-vector of the first and the third polarized red light were changed by 45° each other to distinguish the growth occurred at both red light treatments. Bar=50 μm.

When a nucleus was dislocated to the apical or basal part of the apical cell by centrifugation just before branch induction, apical growth or branch formation was induced, respectively. Moreover, the relocation of the nucleus in the branch to the former apical part by acropetal centrifugation changed the growing apex from the branch apex to the main cell (Table 1, Fig. 8). Given these facts, both ends of the nucleus may be able to become a leading head (front head) depending on which cell apex (branch apex or main cell apex) was the closest. The nucleus may thus not have a real head and tail physiologically in strict sense. Alternatively, the nucleus may be able to instantaneously reverse its morphological and physiological polarity when necessary. In *Pteris vittata*, although a side branch was induced under similar conditions by Ootaki (1963), he did not describe nuclear migration in the protonema nor nuclear polarity, probably because the nuclei of *Pteris* in his experimental system are small and spherical, at least judging from his figures, so that he might not have been able to observe nuclear behaviour precisely in living cells of *Pteris*.

Nucleus may remotely control apex differentiation

The site of cell division is predicted by the location of a preprophase band of microtubules (PPB) (Gunning 1982). In *Adiantum* protonemata, a PPB develops at the cortical ectoplasm closest to the nucleus, even in a cell with its nucleus displaced artificially by cell centrifugation (Murata and Wada 1989), indicating that the position of the nucleus is important for the site of cell division. For the differentiation of a new cell apex, however, the results presented here show that nuclear position is not as important as in PPB development in *Adiantum*. As Figs. 4, 6 and 7 show, when a new cell apex (branch) starts to develop, the nucleus is still located far away from the branching site in most cases. Nuclear position is random in relation to the branching site in *Adiantum* (as shown Fig. 7), compared to the following cases in which the relationship is clear. In *Pteris*, a side branch develops where cytoplasm (including the nucleus) gathers near the basal end of the apical cell (Ootaki 1963). In the moss *Funaria hygrometrica*, caulonema filaments, which show rhythmic cell division every 120 μm, a side branch occurs 40-50 μm apical to the nucleus, on the cell flank opposite the nucleus (Schmiedel and Schnepf 1979a). The reason why the nuclear position in the apical cell has no relation to the branching site in *Adiantum* is so far unclear. Nucleus may remotely control the apex differentiation, or alternatively an unknown factor in cytoplasm may exist at the branching site.

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