The Roles of the Cytoskeleton and the Cell Wall in Nuclear Positioning in Tobacco BY-2 Cells

Junko Katsuta and Hiroh Shibaoka

Department of Biology, Faculty of Science, Osaka University, Toyonaka 560, Japan

During the course of cell-wall regeneration in protoplasts isolated from tobacco BY-2 cells, the nucleus changed its position from the central region to the cell periphery. This nuclear migration was inhibited by 2,6-dichlorobenzonitrile (DBN), suggesting the involvement of cell walls in nuclear migration in tobacco BY-2 cells.

In spherical cells formed by culturing protoplasts in the presence of DBN or propyzamide, the nucleus was located in the central region of the cells and was tethered by transvacuolar cytoplasmic strands. Nuclei in the spherical cells were displaced by disrupting the actin filaments in the cytoplasmic strands by treating the cells with cytochalasin B (CB), suggesting that the position of the nucleus in the spherical cells is maintained by actin filaments. As the nuclei were located in the central region of the cells even in the presence of propyzamide, microtubules seem not to be involved in nuclear positioning in the spherical cells.

Actin filaments, but not microtubules, also seem to play an important role in nuclear positioning in elongated cells. In these cells, CB greatly enhanced the displacement of the nucleus by centrifugation, while propyzamide showed little effect.

Key words: Actin filaments — Cell wall — 2,6-dichlorobenzonitrile — Microtubules — Nuclear positioning — Tobacco BY-2 cells.

Asymmetric cell division is generally thought to play an important role in cell differentiation in plants. The importance of asymmetric division in plant-cell differentiation is evident in many instances, such as the development of stomatal guard cells, root-hair initial formation and pollen-grain development (Wareing and Phillips 1981). In cells which undergo asymmetric cell division, the nucleus must migrate to the predetermined division site and settle there. Thus, the mechanism that regulates the position of the nucleus is crucially important in cell differentiation.

Nuclei of vacuolated plant cells move to the future division site from their original peripheral position well in advance of the start of cell division and are fixed there by transvacuolar cytoplasmic strands or a thin cytoplasmic layer called a phragmosome (Sinnott and Bloch 1940, 1941). A subcellular structure which tethers the nucleus to the division site is present also in nonvacuolated plant cells (Ôta 1961).

The presence of microtubules in phragmosomes has been reported for various plant species, including Nautilus (Bakhuizen et al. 1985, Venverloo et al. 1980), Fraxinus (Goosen-de Roo et al. 1984) and Pisum (Bakhuizen et al. 1985). Actin filaments also seem to be present in phragmosomes. Goosen-de Roo et al. (1984) showed the presence of microfilament bundles in the phragmosomes of Fraxinus cambial cells, and Kakimoto and Shiibaoka (1987a) showed that actin filaments connect the margin of the protoplast with the cell cortex in highly vacuolated cultured tobacco BY-2 cells.

Although microtubules and/or microfilaments appear to be present in phragmosomes and cytoplasmic strands which tow the nucleus, the presence of these cytoskeletal elements does not necessarily mean that they participate in nuclear migration and/or nuclear positioning. Microtubules do not seem to participate in nuclear migration in Tulipa pollen tetrads undergoing asymmetric cell division.
The nucleus moves to the cell periphery in the presence of colcemid, but cannot remain there if colcemid continues to be present (Tanaka and Ito 1981).

In statocytes of cress roots, the nucleus seems to be positioned by a mechanism which involves actin filaments, since the positioning is disturbed by CB, but not by colchicine (Hensel 1985). In contrast, the maintenance of polarized distribution of other organelles requires microtubules (Hensel 1984). The participation of microfilaments in nuclear positioning is suggested also by the finding that nuclear migration preceding so-called wound-induced cell division is inhibited by CB, but not by colchicine (Schnepph and Traiteur 1973). The use of fluorescence-labeled phalloidins has revealed that actin filaments are present near the nucleus in the cells of various plant species (Clayton and Lloyd 1985, Parthasarathy et al. 1985, Seagull et al. 1987). Few detailed studies have been made, however, of the relationship between the distribution of actin filaments and the position of the nucleus.

In preliminary experiments, we noticed that the nuclei of protoplasts of cultured tobacco cells migrated, as the protoplasts regenered cell walls and then elongated, to settle eventually at the cell periphery of the middle region of the elongated cells. In the present study using cell-wall regenerating tobacco protoplasts, we examined whether or not microtubules and/or actin filaments are involved in nuclear migration and positioning.

Materials and Methods

Plant material—A tobacco suspension culture, cell line BY-2, (Nicotiana tabacum 'Bright Yellow 2') was maintained in Linsmaier and Skoog's medium modified by raising the concentration of KH2PO4 to 370 mg/liter and supplemented with 3% sucrose and 0.2 mg/liter 2,4-D. The medium was adjusted to pH 5.8 prior to autoclaving. The cell suspension was subcultured every 7 days (Nagata et al. 1981).

Protoplasts were isolated from cells in the logarithmic phase of growth according to Nagata et al. (1981). Briefly, cell walls were digested by an enzyme solution containing 1% Cellulase Onozuka RS (Yakult Honsha, Higashi-Shinbashi, Tokyo, Japan), 0.1% Pectolyase Y23 (Seishin Pharmaceutical Co., Nagareyama, Chiba, Japan) and 0.4 mM mannitol, pH 5.5. Protoplasts were washed twice with 0.4 mM mannitol solution and suspended at a cell density of 5–8×10^6/ml in BA-NAA medium. This medium was the same as that used for suspension culture except that it contained 1.0 mg/liter BA and 0.1 mg/liter NAA instead of 0.2 mg/liter 2,4-D (Hasezawa and Syōno 1983). Unless otherwise stated, 0.4 mM mannitol was added to the medium to protect protoplasts from osmotic injury.

Protoplast suspensions of 3 ml each were poured into glass petri dishes (6 cm in diameter) and cultured statically at 27°C in the dark.

Treatments with inhibitors—Propyzamide (Tokaruzu Ka Research Center, Sumitomo Chemical Co., Takarazuka, Japan) and DBN (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) were dissolved in DMSO to make 2 or 20 mM stock solutions. The stock solution was added to BA-NAA medium to yield a final propyzamide or DBN concentration of 10^−3 M.

CB (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in DMSO at 2 mg/ml and used at 20 or 30 μg/ml. The concentration of DMSO in the medium for control experiments was adjusted by adding corresponding amounts of DMSO. DMSO at the concentrations used (below 2.5%) showed no appreciable effects on the cells during the period of the experiments.

Staining—Microtubules were stained by the indirect-immunofluorescence technique according to Wick et al. (1981). Cells were fixed with 3% formaldehyde dissolved in KP buffer (50 mM potassium phosphate buffer, 5 mM EGTA, 0.3 mM PMSF, pH 6.8), extracted with 0.1% Triton X-100, and incubated first with mouse monoclonal anti-chick brain α-tubulin antibody (Amersham, Buckinghamshire, England and then with FITC-conjugated rabbit anti-mouse IgG (Miles Laboratories, Naperville, IL, U.S.A.). Stained cells were mounted with glycerol/PBS-TA (8 mM PBS, 0.05% Tween 20, 0.05% NaN3, pH 8) mixture (1:1, w/v) containing 0.1% p-phenylenediamine, and examined with an Olympus BHS-RFK epifluorescence microscope (Olympus, Tokyo, Japan) using BP 490 and EY 455 excitation filters and an O 515 barrier filter. Photographs were made using Kodak Tri-X Pan film (ASA 400).

Cell walls were made visible by staining with Calcofluor White-II (Dojin Chemical Laboratory, Kumamoto, Japan). Freshly prepared protoplasts or wall-regenerating protoplasts were collected by centrifugation and suspended in 0.4 mM mannitol solution containing 0.1% Calcofluor White-II. After 5 min at room temperature, the cells were washed twice with 0.4 mM mannitol solution. Stained cells were examined with an Olympus BH-RFL epifluorescence microscope using UG 1 excitation filter and an L 410 barrier filter, and photographed using Kodak Tri-X Pan film. Photographs of stained cells were taken with a fixed aperture of the diaphragm and a fixed exposure time so that the extent of cell-wall regeneration could be determined from the brightness of the cell walls in the photographs.

Actin filaments in the cells were stained with Rh-ph according to the method described by Kakimoto and Shibaoka (1987a). Cells were collected by centrifugation and incubated in a 1:1 mixture of solution A (0.66 μM Rh-ph, 10 mM PBS, 1% BSA, pH 7.3) and solution B (0.05% [w/v] Triton X-100, 100 mM PIPES, 1 mM MgCl2, 5 mM EGTA, 0.3 mM PMSF, 50 μg/ml leupeptin, 3 mM DTT, pH 6.9) for 10 min at room temperature. Stained cells
Table 1  Effects of cytochalasin B (CB) and propyzamide on nuclear displacement by centrifugation

<table>
<thead>
<tr>
<th>CB treatment</th>
<th>Propyzamide treatment</th>
<th>Centrifugal force (×g)</th>
<th>Nuclei in centrifugal third of cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>0</td>
<td>5.6</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>100</td>
<td>12.9</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>100</td>
<td>15.0</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>100</td>
<td>38.4</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>100</td>
<td>56.4</td>
</tr>
</tbody>
</table>

After 66 h of culture in BA-NAA medium, BY-2 protoplasts were incubated with $10^{-4}$ M propyzamide, 30 μg/ml CB or propyzamide plus CB for 5 h and then centrifuged at 100 x g for 30 min. All media contained 2.5% DMSO.

were examined with an Olympus BH-RFL epifluorescence microscope using IF 545 and BG 36 excitation filters and an O 590 barrier filter, and photographed using Kodak Tri-X Pan film. As has been discussed by Kakimoto and Shibaoka (1987a), the patterns of staining with Rh-ph should represent the arrangements of actin filaments in situ.

DNA was made visible by staining with DAPI. Cells were fixed with 3% formaldehyde in KP buffer for 30 min, incubated in 0.2 μg/ml DAPI solution at room temperature for 10 min, then rinsed with distilled water and mounted with glycerol/PBS-TA (1:1, v/v). Stained cells were examined with an Olympus BHS-RFK epifluorescence microscope using a UG I excitation filter, an L 420 barrier filter and a phase-contrast apparatus.

Measurements—The position of the nucleus in the protoplasts cultured in petri dishes was examined with an inverted microscope at designated time intervals. The results of the examination were analyzed for the percentage of cells in which the nucleus was suspended in the inner region of the cells (or the percentage of cells in which the nucleus was located near the cell periphery) to the total cells examined. The position of the nucleus was examined at various levels of focus, so that we could differentiate the nuclei which were in fact suspended in the central region from those which were located near the upper or lower plasma membrane. Since the position of the nucleus was difficult to determine in dividing cells, only nondividing cells were counted. For each determination, more than 200 cells were examined.

In experiments in which the relation between cell size and the position of the nucleus was examined, protoplasts were cultured in the presence of 0.3, 0.4, 0.6 or 0.95 μM mannitol, and photographs were taken at appropriate time intervals. The diameter of spherical cells or the width of elongated cells was measured on the enlarged photographs (×500).

Centrifugation experiments—Protoplasts were cultured in BA-NAA medium. After 66 h of culture, propyzamide and/or CB stock solution was added to obtain the final concentration of $10^{-4}$ M or 30 μg/ml, respectively, and culture was continued for 4-7 h. After treatment with propyzamide and/or CB, cells were made to adhere to cover slips coated with polylysine (average mol wt 75,000, Sigma Chemical Co., St. Louis, MO, U.S.A.). A 1-mm thick silicon-rubber plate with a square hole (14 × 16 mm$^2$) was placed on a glass slide. The hole was filled with a small amount (224 μl) of medium containing one or both of the inhibitors and then covered with a cover slip with adhering cells, so that the cells on the cover slip came in contact with the medium. Another glass slide was placed on the cover slip. The cover slip and the silicon-rubber plate sandwiched between the two glass slides were set in a centrifuge tube and spun at 100 x g for 30 min, so that the direction of the centrifugal force was parallel to the surface of the cover slip. Immediately after centrifugation, the cells were fixed with 3% formaldehyde in KP buffer and stained with DAPI. The position of the nucleus (centripetal, middle or centrifugal third) was recorded for elongated cells which lay with their long axes lying in the direction within 45° to the direction of centrifugal force. The percentage of cells in which the nucleus was in the centrifugal third of the cell was determined. For each treatment, more than 300 cells were examined.

Results

Changes in cell shape and microtubular arrangement during cell culture—Immediately after isolation, protoplasts were more or less spherical in shape, but some of them showed irregular contours. The surface of such protoplasts had caved in at the points where transvacuolar cytoplasmic strands met the plasma membrane (Fig. 1). Within 2 h after isolation, however, the surfaces of all protoplasts became smooth.

Protoplasts cultured in BA-NAA medium regenerated cell walls (see below) and then elongated. As reported by Hasezawa and Syono (1983), the rate of cell division was reduced in BA-NAA medium. Elongation was apparent in some cells within 1 day and in most of the cells within
3 days of culture (Fig. 2a). They continued to elongate throughout the experiment for 7 days. When protoplasts were cultured in BA-NAA medium containing $10^{-3}$ M propyzamide, they did not elongate, but expanded isotropically, and highly developed cytoplasmic strands were observed (Fig. 2b). Protoplasts cultured in BA-NAA medium containing $10^{-3}$ M DBN showed isotropic expansion during the first 4 days (Fig. 2c). However, after 5 or more days, i.e., shortly after the cells had become Calcofluor White-positive (see below), they expanded un-isotropically and assumed an ellipsoidal or dumbbell shape.

As reported by van der Valk et al. (1980), somewhat disordered parallel microtubules were present in freshly prepared tobacco protoplasts. In cells which had elongated during culture for 3 days or more in BA-NAA medium, cortical microtubules ran parallel to each other transversely (Fig. 2d), but hardly any microtubules were observed around the nucleus or in the transvacuolar cytoplasmic strands (Fig. 2d'). This network of cortical microtubules was not present in cells cultured in the presence of $10^{-3}$ M propyzamide, only fragmented cortical microtubules being observed (Fig. 2e). In cells cultured in the presence of $10^{-3}$ M DBN, a well-developed network of cortical microtubules was observed, although they were randomly oriented (Fig. 2f).

Cell-wall regeneration during cell culture—Protoplasts immediately after isolation were not stained with Calcofluor White, indicating that no cellulose was present on the cell surface. Within 1 day of culture in BA-NAA medium, however, the cells became Calcofluor White-positive (Fig. 3a), indicating that they had started to regenerate cellulose cell walls within 24 h. The cells cultured in medium containing propyzamide were stained as intensely as those cultured without propyzamide (Fig. 3b).

Cells cultured in the presence of $10^{-3}$ M DBN were only faintly stained by Calcofluor White in the early stage of protoplast culture (Fig. 3c, d), suggesting that cellulose synthesis was greatly reduced by DBN. The cells became Calcofluor White-positive after 4 or more days (Fig. 3e). Probably, cellulose synthesis was not inhibited completely by DBN at $10^{-3}$ M and the cells accumulated cellulose synthesized at a reduced rate.

Nuclear migration during cell culture—In freshly prepared protoplasts, the nuclei were distributed rather randomly. As shown in Fig. 4, the nucleus was located at the cell periphery in about 40% of total protoplasts, and in the inner region in about 60% of total protoplasts. During the first 24 h of incubation in BA-NAA medium, the percentage of cells with the nucleus in the inner region increased, and then decreased. With prolonged incubation, the cells with peripheral nuclei increased to over 80% after 7 days (Fig. 4). The nucleus migrated toward the cell periphery from its central position mainly in the direction perpendicular to the cell long axis. After 3 days, when nearly all cells had begun to elongate, the nucleus was present in the middle third portion in about 90% of the cells (Fig. 2a).

During the first 2 days of culture in the presence of $10^{-3}$ M propyzamide, the cells showed the same time course of change in the position of the nucleus as those cultured without propyzamide. That is, the cells with the nucleus in the inner region of the cell increased during the first day, and then decreased during the second day. Unlike in the control culture, however, cells of this type increased again gradually after 3 or more days (Fig. 4).

The change in nuclear position in the cells cultured in the presence of $10^{-3}$ M DBN was quite different from that in control or propyzamide-treated cells (Fig. 4). During the first 3 days, the percentage of cells with the nucleus in the inner region increased to 90% and remained at this level for the next 2 to 3 days. The nucleus was at the center of the cell, supported by highly-developed transvacuolar cytoplasmic strands (Fig. 2c). After 5 or more days, however, the percentage of cells with peripheral nuclei increased as the cells began to assume an ellipsoidal or dumbbell shape.

Relation between cell size and nuclear position—Both propyzamide and DBN increased the percentage of cells with a central nucleus. At the same time, they caused swelling of the cells. During 3 days of culture in medium containing 0.4 M mannitol, the average diameter of the cells became 40.1 and 67.7 $\mu$m in the presence of propyzamide and DBN, respectively, while it was only 32.3 $\mu$m in their absence (in this case, the width of the cells was measured). These results strongly suggested that propyzamide and DBN caused centripetal nuclear migration by inducing cell swelling. To check this possibility, the effects of propyzamide and DBN on the change of nuclear position were examined in the presence of different concentrations of mannitol. The results of the experiments with 0.3 M and 0.95 M mannitol are shown in Fig. 5.
Nuclear positioning in tobacco BY-2 cells

The diameters of cells cultured in the presence of propyzamide did not increase when the medium contained 0.95 M mannitol (Fig. 5c), but did increase when mannitol was added to the medium at 0.3 M (Fig. 5a). Propyzamide increased the percentage of cells with a central nucleus (Fig. 5b) in the presence of 0.3 M mannitol, but not in the presence of 0.95 M mannitol (Fig. 5d). Thus centripetal nuclear migration observed in the presence of propyzamide seems to occur as a result of cell swelling caused by propyzamide.

The presence of 0.95 M mannitol in the medium greatly reduced DBN-induced cell swelling, but did not suppress the effect of DBN on nuclear positioning. After 3 days of culture, the nucleus was located in the central region in
about 75% of DBN-treated cells, whereas it was located near the cell periphery in about 60% of cells not treated with DBN (Fig. 5f), although the diameter of the former was almost the same as that of the latter, i.e. the width of the cell (Fig. 5e). Thus, centripetal nuclear migration which occurs in the presence of DBN does not seem to depend on cell swelling.

In the presence of 0.95 M mannitol, elongation of the cells cultured in the absence of propyzamide or DBN was greatly suppressed and the cells remained spherical in shape during at least first 3 days of culture. In the meantime, the percentage of cells with a peripheral nucleus continued to increase (Fig. 5d).

Actin filament distribution—The nucleus was located in the central region of the cell when microtubules were fragmented by treatment with propyzamide. This result strongly suggested that the nucleus in such cells was held in place by cytoskeletal elements other than microtubules. Thus the possible involvement of actin filaments in nuclear positioning was examined.

First, cells were stained for actin filaments with Rh-ph. In cells cultured in BA-NAA medium for 3 days, bundles of actin filaments running parallel to the cell long axis were observed. Some of them were distributed close to the nucleus. An actin-filament network was also present in the cortical cytoplasmic layer (Fig. 6a). Cortical networks of actin filaments were observed also in cells cultured in the presence of $10^{-3}$ M propyzamide or $10^{-2}$ M DBN for 3 days. Actin filaments were distributed in the cytoplasm around the nucleus and in the transvacuolar cytoplasmic strands between the cell periphery and the nucleus (Fig. 6b, c).

Second, the effect of an actin-filament poison on nuclear position was examined. Cells were cultured for 66 h in BA-NAA medium in the presence and absence of propyzamide or DBN and then treated with 20 $\mu$g/ml CB. In almost all CB-treated cells, cytoplasmic strands disappeared. The nucleus was displaced from its original central position to the cell periphery in both propyzamide- and DBN-treated cells (Figs. 7b, c, 8b, c). These changes started within 10 min of CB treatment. The percentage of cells with a central nucleus was decreased within 3 h from 38% to 13% in control cells (Fig. 7a), from 65% to 25% in propyzamide-treated cells (Fig. 7b), and from 87% to 12% in DBN-treated cells (Fig. 7c). Cytoplasmic strands reappeared and the nucleus returned to its original central position within 3 h after the removal of CB (data not shown), suggesting that the effect of CB is reversible.

In elongated control cells, the disappearance of cytoplasmic strands occurred within 30 min of CB treatment, but the displacement of the nucleus occurred much later. After 10-h treatment with CB, the nucleus was located outside the central region of the cell only in a small fraction of the treated cells (Fig. 8a). DMSO alone showed no effect on nuclear positioning at the concentration of 1.5% used for CB treatment.

The effect of CB on actin-filament distribution was also examined. Cells were cultured for 66 h as described above and treated with 20 $\mu$g/ml CB for 2 h. As shown in Fig. 8d-f, the number of actin filaments was reduced by the CB treatment and the remaining filaments were fragmented both in propyzamide- or DBN-treated spherical cells and in untreated elongated cells.

Nuclear displacement by centrifugation—As mention-
Nuclear positioning in tobacco BY-2 cells

Fig. 4  Time course of the change in the position of nucleus in tobacco BY-2 cells. ○: Control cells cultured in BA-NAA medium. ▼: Cells cultured in BA-NAA medium containing 10^{-5} M propyzamide. □: Cells cultured in BA-NAA medium containing 10^{-3} M DBN. All media containing 0.5% DMSO.

ed before, the nucleus was located in elongated cells at the cell periphery. However, based on the position in the direction of the cell long axis, the nucleus was present in the middle region of the cells, suggesting the existence of some mechanism to locate the nucleus there. In order to find out what cytoskeletal elements were involved in this mechanism, centrifugal force was applied to the elongated cells in the direction of their long axis in the presence and absence of propyzamide or CB.

As shown in Table 1, before centrifugation the nucleus

Fig. 5  Time course of the change in cell size and the position of nucleus in tobacco BY-2 cells. a, b: Protoplasts were cultured in BA-NAA medium (○) or BA-NAA medium containing 10^{-5} M propyzamide (▼). Both media contained 0.3 M mannitol. c, d: Protoplasts were cultured in BA-NAA medium (●) or BA-NAA medium containing 10^{-3} M propyzamide (★). Both media contained 0.95 M mannitol. e, f: Protoplasts were cultured in BA-NAA medium containing 0.3 M mannitol (○), or 10^{-3} M DBN and 0.95 M mannitol (●). a, c, e: Cell size. b, d, f: Position of the nucleus. All media contained 0.5% DMSO.
was located in the middle region in about 90% of the cells, and only 5.6% had the nucleus in the prospective centrifugal third of the cell. Centrifugation at 100 × g for 30 min increased the percentage of cells with the nucleus in this region from 5.6% to 12.9% in the absence of propyzamide or CB.

Treatment with propyzamide alone had little effect on the displacement of nucleus by centrifugation. On the contrary, treatment with CB greatly enhanced the displacement of nucleus by centrifugation: it increased the percentage of cells in which the nucleus was displaced by centrifugation by 25.5%. Thus, actin filaments seem to play a more important role than microtubules in nuclear positioning also in elongated cells.

Although propyzamide alone showed little effect on nuclear displacement by centrifugation, it exerted a distinct effect when it was applied together with CB. Treatment with propyzamide plus CB increased the percentage of cells in which the nucleus was displaced by centrifugation by 43.5%. Thus, the role of microtubules in nuclear positioning cannot be ignored.

**Discussion**

*Migration of nucleus toward cell periphery*—During the course of cell-wall regeneration, the nuclei of protoplasts isolated from tobacco BY-2 cells changed their position from the central region to the cell periphery. This nuclear migration was inhibited by propyzamide, a microtubule inhibitor (Bartels and Hilton 1973), and by DBN, a cellulose-synthesis inhibitor (Hogetsu et al. 1974). However, when cell swelling was prevented under hypertonic conditions, the nuclear migration was not inhibited by propyzamide, suggesting that microtubules are not involved in the migration of the nucleus. In contrast, DBN inhibited the migration of the nucleus toward the cell periphery even under conditions where cell swelling was greatly suppressed. This strongly suggests that cellulose synthesis or regeneration of the cell walls is prerequisite for nuclear migration. The percentage of cells with a peripheral nucleus was high in the elongated control cells and low in the DBN-treated spherical cells. These results suggest that cell elongation may cause nuclear migration toward the cell periphery. Although we cannot exclude this possibility, it is unlikely that the former is prerequisite for the latter, because the nucleus migrated toward the cell periphery in the control cells which were cultured under hypertonic conditions and showed no cell elongation.

Hahne and Hoffmann (1984) found that *Hibiscus* protoplasts showed somewhat irregular contours. Based on the finding that this irregularity was diminished by treatment with CB, they suggested that it was caused by tension generated by actin filaments. In the present experiments, freshly prepared tobacco BY-2 protoplasts often showed irregular contours. As in the case of *Hibiscus* protoplasts, indentations on the surface of the BY-2 protoplasts were always associated with underlying transvacuolar cytoplasmic strands extending from the nucleus. This fact and the presence of actin filaments in these cytoplasmic strands strongly suggest that actin filaments are associated with both the nucleus and the plasma membrane and generate tension between them.

The cell walls probably affect how the actin filaments are associated with the plasma membrane and thereby influences the position of the nucleus. Actin filaments in

---

**Fig. 6** Actin-filament distribution in tobacco BY-2 cells. Protoplasts cultured for 66 h in BA-NAA medium were stained for actin filaments. a: Control cell cultured in BA-NAA medium for 66 h. b: Cells cultured in BA-NAA medium containing 10^{-5} M propyzamide for 66 h. c: Cells cultured in BA-NAA medium containing 10^{-5} M DBN for 66 h. Bar=50 μm.
cultured mammalian cells are associated with an extracellular matrix through some plasma membrane components (Beckerle 1986, Heath and Dunn 1978). It seems probable that actin filaments in plant cells are associated with cell walls by an analogous mechanism. Further studies are necessary to show how actin filaments are associated with the plasma membrane and how this association is affected by the cell walls.

*Role of cytoskeleton in nuclear positioning*—In freshly prepared protoplasts and in cells cultured in the presence of propyzamide or DBN, the nucleus was positioned in the central region of cell and tethered with transvacuolar cytoplasmic strands containing actin filaments, suggesting the involvement of actin filaments in nuclear positioning in spherical cells. The finding that the nucleus was easily displaced from its original central position to the cell periphery by disrupting the actin filaments with CB also supports this idea.

Microtubules seem not to be involved in nuclear positioning in spherical cells, because none were observed in the cytoplasmic strands which tethered the nucleus to the cell periphery and the nucleus was positioned in the central region even in the presence of propyzamide, a microtubule-disrupting agent.

The involvement of actin filaments in nuclear positioning has been reported in several plant species. In *Tradescantia* leaf epidermal cells, Schnepf and Traiteur (1973) found that the traumatotactic movement of nuclei was inhibited by treatment with CB. The displacement of the nucleus by treatment with CB was reported in *Funaria* caulonema cells (Schmiedel and Schnepf 1980) and in *Lepidium* statocytes (Hensel 1985).

For actin filaments to maintain the position of a nucleus, they must be closely associated with the nucleus. The presence of actin filaments near nuclei has been demonstrated in cells of various plant species both by fluorescence microscopy using fluorescence-labeled phalloidins (Clayton and Lloyd 1985, Parthasarathy et al. 1985, Seagull et al. 1987) and by electron microscopy using skeletal muscle heavy meromyosin (Kakimoto and Shibaoka 1987b). However, details of the association between actin filaments and the nucleus are not known.

Centrifugation of elongated cells in the absence and presence of CB revealed that actin filaments also play an important role in maintaining the position of the nucleus in elongated cells. The nucleus was displaced by centrifugation far more readily in the presence of CB than in its absence. Microtubules, however, do not seem to be primarily important in nuclear positioning in elongated cells. Treatment with propyzamide alone did not facilitate displacement of the nucleus by centrifugation. The observation that propyzamide amplified the effect of CB on nuclear displacement, however, indicates that microtubules are also involved in maintaining the position of the nucleus. Thomas et al. (1977) reported that both colchicine and CB promoted the displacement of nuclei by centrifugation in *Avena* coleoptile cells. In *Avena* cells, however, treatment with colchicine alone promoted the displacement, suggesting that microtubules are more important for nuclear positioning in *Avena* cells than in tobacco BY-2 cells.

The involvement of microtubules in premitotic nuclear
Fig. 8 Effect of CB on the position of nucleus and actin-filament distribution in tobacco BY-2 cells. Protoplasts were cultured in the absence of CB for 66 h and then treated with 20 μg/ml CB for 10 h (a–c) or 2 h (d–f). a: Control cells. b: Propyzamide-treated cells. c: DBN-treated cells. d: Actin filaments in a control cell. e: Actin filaments in a propyzamide-treated cell. f: Actin filaments in a DBN-treated cell. During the period of CB treatment, all medium contained 1.5% DMSO. Bar=50 μm.

positioning has been reported in pollen tetrads of Liliaceae plants. Tanaka and Ito (1981) showed that the mechanism which maintained the polarized position of the premitotic nucleus in *Tulipa* pollen tetrads was colcemid-sensitive. The presence in *Lilium* pollen tetrads of microtubules which radiate from the envelope of the nucleus in premitotic position and extend toward the plasma membrane has been reported (Dickson and Sheldon 1984). Microtubules seem to be involved in premitotic nuclear positioning also in *Adiantum* protonema tip cells (Mineyuki and Furuya 1986).

In the present study, we used tobacco BY-2 cells cultured in a medium favorable for cell elongation but not for cell division (Hasezawa and Syōno 1983), and found that actin filaments, not microtubules, were primarily important in nuclear positioning. It may be hypothesized, therefore, that microtubules play a principal part in premitotic nuclear positioning, and actin filaments in nuclear positioning in interphase cells. Studies on tobacco BY-2 cells cultured in a medium favorable for cell division are under way to check this hypothesis.

This work was supported in part by Grants-in-Aid for Special Project Research (No. 61129005) and for Scientific Research (No. 62480009) from the Ministry of Education, Science and Culture, Japan.
Nuclear positioning in tobacco BY-2 cells

References


(Received July 22, 1987; Accepted January 15, 1988)