THE ROLE OF MICROTUBULES IN REGULATING ONION BULB DEVELOPMENT

MITA Takashi

Contents

General introduction

One of the most prominent features of the plant cell is that it is surrounded by the well developed cell wall. Although the animal cell is also surrounded by the extracellular matrix, it is not so well developed and organised as the plant cell wall. The most of the differences between plants and animals seem to be derived from the presence of the plant cell wall. In fact, it is well recognized that there are no essential differences between plants and animals in fundamental molecular features.

A special plant features which resulted from the presence of the cell wall is that after cell division was completed, the arrangement of cells never changes. Another special feature is irreversible cell expansion. After plant cells competed cell expansion, they never change their cell shapes. This feature can also traced to the presence of rigid plant cell walls.

As the arrangement of cells in plant organs never changes and each cell never changes its shape, we can compare plant organs to brick buildings. The shape of a brick building is determined by the arrangement of bricks and the shape of each brick. Likewise, the shape of plant organ is determined by the arrangement of cells and the shape of each cell. What determine the arrangement of cells is the way of cell division and what determine the shape of cell is the way of cell expansion.

That polarized cell division, which produces two differently characterized doughter cells, plays an important role in plant morphogenesis is well established. The branch formation in a marine alga Callithamnion rosenum provides a good example (Konrad-Hawkins, 1964). A cylindrial tip cell of this alga undergoes oblique mitoses which produce cells with one long side. The branch always protrudes from this side. The formation of stomatal complex in vascular plants includes three polarized cell divisions, one for the formation of a guard mother cell and two for subsidary cells (Stebins and Stah, 1960). The significance of the plane of cell division in determining the shape of plant organs can best be demonstrated in shapeless callus tissues, where the plane of cell division is at random.

Although it is an endless task to cite example which show the importance of cell division in plant morphogenesis, cell division can not determine the shape of plant organs by itself. Without oriented cell expansion, no plant organ can develop properly.

During the development of plant organs, cell division occurs in restricted regions or in limited periods. In stem or root, it occurs only in apical meristems, and in leaf or fruit it occurs only in early period of development. Thus, the final shape and size of stem and root are determined by the direction and the extent of expansion of cells derived from apical meristems. Usually, mature stem or root consist of long cells, but new born stem or root cells are isodiametric in shape. It can be coincided that elongated shape common to stem and root is

attributable to longitudinally oriented expansion of cells.

In fruit development, cell division completes before anthesis. The shape and the size of fruit, therefore, largely depend upon the cell expansion. Thus, we cannot approach the problems of plant morphogenesis without studying the controlling mechanism of cell expansion.

As plant cells are surrounded by cell walls, cell expansion means the expansion of cell wall. Likewise, the oriented cell expansion means the oriented expansion of cell wall. Cell walls expand only when they are subjected to a stress induced by turgor pressure. As the stress induced by turgor pressure pulls the wall in all directions, it cannot be responsible for oriented cell wall expansion. The oriented cell wall expansion must result from the anisotropic structure of the cell wall.

The plant cell wall is composed mainly of polysaccharides which can be divided into three major components; cellulose, hemicellulose and pectin. Cellulose molecules form strong aggregates, cellulose microfibrils, which are embeded in cell wall matrix composed of hemicellulose and pectin. Usually, cellulose microfibrils are running parallel to each other and are thought to give an anisotropic nature to the cell wall (Green, 1962., Probine and Preston, 1962., Roelofsen, 1966).

In internode cells of Nitella, a majority of cellulose microfibrils are oriented transverse or nearly transverse to the cell axis.

These cells expand only in the direction parallel to the cell axis, namely perpendicular to the direction of cellulose microfiblirs. Green (1962) showed that the orientation of cell expansion in Nitella internode cells was controlled by the arrangement of cellulose microfibrils. He treated a shoot of Nittella with colchicine. In colchicine treated Nitella shoots, internode cells expanded isodiametically and cellulose microfibrils in such cells were randomly oriented. As colchicine had been known to disrupt spindle fibers, Green hypothesized that the direction of cellulose microfibrils was controlled by a machnery of spindle-fiber character.

One year later, Green's hypothesis was verified by the findings of Ledbetter and Porter (1963) who showed that slender tubules are present in the periphery of plant cells and that the tubules, which they named microtubules, are identical in morphology to spindle fibers. They also showed that microtubules near the side walls of a root cell are running perpendicular to the long axis of the cell and mirror cellulose microfibrils of adjacent cell walls. Based on these findings, they hypothesized that microtubules in the periphery of plant cells controlled the direction of cellulose microfibrils in the cell walls. Thereafter, Ledbetter and Porter's hypothesis has been supported by many authors who confirmed that microtubule-disrupting agents disturb both normal cellulose microfibril deposition and normal cell morphogenesis, or showed that microtubules near cell walls mirror cellulose microfibrils in the cell walls in many cell types (Newcome, 1969.,

Green, 1969., Hepler and Palevitz, 1974).

As described above, the shape of plant organs depends largely on the shape of cells which construct the organs, and the shape of cells on the direction of cellulose microfibrils in the cell walls, which, in turn, is controlled by microtubules. Thus, microtubules seem to play an important role in plant morphogenesis.

About a half of a century ago Magruder and Allard (1937) found that onion plants form bulbs in response to the stimulus of long-day conditions. Histological observations by Heath (1943) revealed that the bulb is formed by the swelling of leaf sheath cells and cell division is not involved in this phenomenon. Under short-day conditions leaf sheath cells expand longitudinally, but under long-day conditions they grow laterally. This means that leaf sheath cells change the direction of cell expansion in response to the stimulus of long-day conditions. If the direction of cell expansion in onion leaf sheaths also depends on the arrangement of microtubules in the cell periphery, the microtubules must change their arrangement during onion bulb formation. Therefore, I examined the behavior of microtubules near the periphery of the leaf sheath cells of onion plants during bulb development, and revealed that microtubules are oriented transversely to the cell axis when the plants have not yet started bulb formation, but, as the bulb develops and the leaf sheath cells swell, they become disoriented and scattered and finally disappear, suggesting that microtubules are playing an important role in regulating bulb development which occures in

response to the changes in the day-length conditions (Chapter I).

Flower bud formation is a well known phenomenon which also induced by the stimulus of day-length conditions. In this phenomenon , florigen, a hypothetical flowering hormone, has been considered to be produced by leaves which are kept under the inducing day-length conditions and to cause the morphological changes in apical and axillary buds. Flower bud formation is also considered to be controlled by an antagonist of florigen which is supplied from roots. Cytokinin is the best candidate for this antagonist (Miginiac, 1978).

In onion bulb formation, leaf blades are supporsed to produce a "bulbing hormone" in response to the stimulus of long-day conditions. The fact that flower bud formation is controlled by both leaves and roots suggests the possibility that roots may play some role in regulating onion bulb formation. In fact, Kato (1965) reported that onion bulb development was promoted when roots were removed from the plants just before the bulb initiation. In the course of the study, I myself noticed that the accidental root excision, which often occurred when seedlings were transplanted, induced swelling of leaf sheath of young onion seedlings which otherwise showed no swelling. Though this swelling is a kind of artifact, it certainly involves the change in the direction of cell expansion. Thus, I examined the changes in the arrangement of microtubules in the leaf sheath cells of onion seedlings during cell swelling caused by root excision and revealed that root excision caused disorientation of microtubules in leaf sheath cells

before it caused swelling, suggesting that roots are suppressing swelling of leaf sheath by supplying them with some substances which have an ability to prevent microtubules from being disoriented (Chapter II).

Usually, in a phenomenon in which a certain plant organ influences the growth of another organ, plant hormones play the leading part. Thus, it is not ridiculous to suppose that the anti-bulbing root factor is identical to one of the subtances which are widely accepted as a plant hormone. Gibberellin seems to be the best candidate for this factor, because it has been known that roots are the site of gibberellin biosynthesis (Torrey, 1976), and it has also been known that gibberellin has an ability to arrange microtubules perpendicular to the cell axis in azuki bean stem cells (Shibaoka, 1974).

Thus, I checked the possibility that this root factor is gibberellin by two different ways. In one way, I examined whether or not an inhibitor of gibberellin biosynthesis showed the effects similar to those of root excision, and found that such an inhibitor dictated the effects of root excision (Chapter III). In this experiment, I used a newly developed inhibitor of gibberellin biosynthesis, S-3307 (Izumi et al., 1984).

In the other, I examined whether or not gibberellin acted conversely to root excision in onion plants. I also examined whether or not gibberellin acted antagonistic to microtubule-disrupting agents. The examinations revealed that gibberellin acted conversely to root excision in arranging microtubules. It arranged microtubules

perpendicular to the cell axis. It was also revealed that gibberellin stabilizes microtubules (Chapter IV). Probably, gibberellin which is synthesized in roots and transported to leaf sheaths, plays an important role in regulating onion bulb formation by affecting the orientation and the stability of microtubules in leaf sheath cells.

Chapter I

Changes in microtubules in onion leaf sheath cells during bulb development

Summary

Cortical microtubules are oriented transversely to the cell axis in leaf sheath cells of onion plants (Allium cepa L. cv. Osaka-Okute) that have not started bulb formation. As the bulb develops and the leaf sheath cells swell, the microtubules become disoriented and scattered and finally disappear. The microtubule inhibitors colchicine and cremart (O-ethyl O-(3-methl-6-nitrophenyl)N-sec-butylphosphorothioamidate) cause swelling of leaf sheath cells and make the basal part of the plant bulbous. The cortical microtubules may have an important role in regulating bulb development in onion plants.

Introduction

Onion plants form bulbs in response to the stimulus of long-day conditions (Magruder and Allard, 1937). More than 30 years ago, Heath (1945) reported that the bulb is formed by the swelling of leaf sheath cells and that cell division is not involved in this phenomenon. Under short-day conditions, leaf sheath cells expand longitudinally, but under long-day conditions they grow laterally. This means that leaf sheath cells respond to a long-day stimulus by changing the direction of cell expansion. The direction of expansion largely on the orientation of cellulose microfibrils in the cell wall (Green et al. 1970, Preston 1974). This orientation, in turn, is considered to be controlled by cortical microtubules (Newcomb 1969, Hepler and Palevitz 1974, Gunning and Hardham 1982). As the change in the direction of cell expansion is part of bulb development, the cortical microtubules may be involved in the regulation of this development. Therefore, I examined the behavior of cortical microtubules in the leaf sheath cells of onion plants during bulb development.

Materials and methods

A late variety of onion plant (Allium cepa L. cv. Osaka-Okute) grown under natural daylength conditions in a field near Osaka City was used. From April 23, at which time bulb development had not started,

until May 21 when development was visible, swall pieces of tissue (3 / 3 / 3 mm^3) that included the outer epidermis were cut from the leaf sheath of the plants once a week. The daylengths (time from sunrise to sunset) during which tissue was taken are given in Table 1.

The excised tissues were fixed with 2 % paraformaldehyde + 2.5 % glutaraldehyde in 0.025 M pottassium phosphate buffer, pH 7.2, for 2 hours, then with 1 % osmium tetroxide in the same buffer for another 2 hours. The fixed tissue were dehydrated and embedded in Spurr's resin (Spurr 1969). Tangential longitudinal sections were cut and stained with 25 % uranyl acetate in absolute methanol for 35 minutes, then with lead citrate (Raynolds 1963) for 4 minutes, after which they were examined with a JEM-100C electron microscope (Jeol, Tokyo).

As it was not easy to tell which cells in a swollen scale corresponded to the analogous cells in an unswollen sheath, I examined cells from different positions on leaf sheaths of different sizes for each of the plants at various growth stages. The position of the cells examined and the size of the leaf sheath from which they were sampled are listed in Table 1.

Cross sectional areas of the leaf sheath cells were measured by sectioning the resin-embedded tissues transversely. These sections were stained with a mixture of toluidine blue and basic fuchsin then examined under a light microscope equipped with an ocular micrometer.

Onion plants (Allium cepa L. cv. Senshu-Chuko) raised from sets (small bulbs) were used to examine the effects of microtubule

inhibitors. Five onion sets were planted in a small pot containing 30 g of vermiculite, then the plants were grown at 27 C under short-day conditions (12 hour light / 12 hour dark). Light was obtained from 20 W "white light" fluorescent lamps (FL-20W, Toshiba, Tokyo) and the light intensity at plant level was ca. 7.0 W m^{-2}. When the plants reached the 4-leaf stage, 100 ml of 3×10^{-4} M colchicine or 2×10^{-5} M cremart [O-ethyl O-(3-methyl-6-nitrophenyl)N-sec-butylphosphoro-thioamidate] solution was poured into the pot. Nine days after the start of treatment, the swelling of the basal parts of the plants was recorded on photographs. Cremart was obtained from the Sumitomo Chemical Co. (Takarazuka).

Results

Swelling was present in the inner cells of the leaf sheaths, but not in the epidermal cells (Fig. 1). The ratio of the number of epidermal cells to the number of cells just beneath the epidermis was ca. 1.5 : 1 in the unswollen leaf sheath, whereas in the swollen scale it was ca. 3 : 1. This suggests that the number of epidermal cells increased when the inner cells swelled.

I examined the arrangement of cortical microtubules in cells which showed swelling, principally those cells just baneath the epidermis. Transversely oriented microtubules were present near the face of the outer tangential wall of cells sampled from unswollen leaf sheaths.

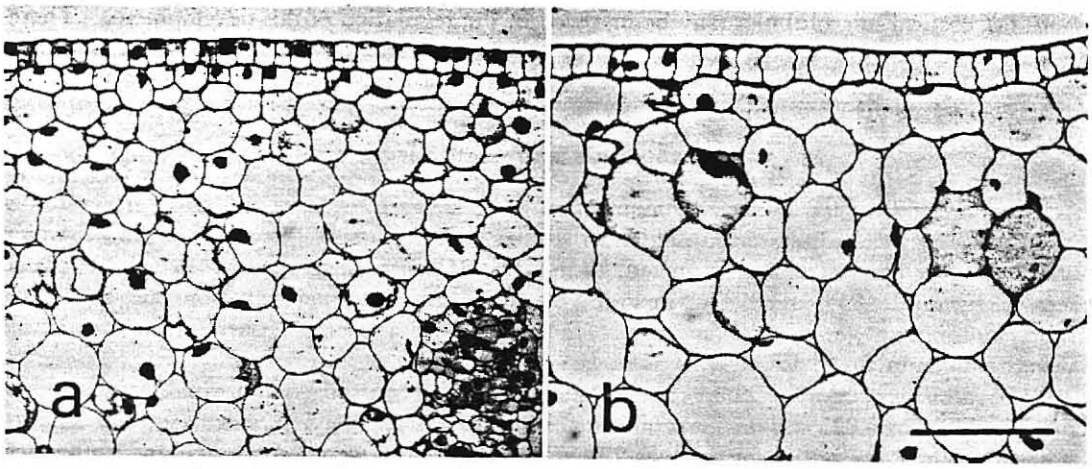


Fig. 1. Transverse sections of onion leaf sheaths.

a. A section cut 5 mm above the basal end of an emerged leaf with a 40-mm-long unswollen leaf sheath, sampled April 23.

b. A section cut 5 mm above the basal end of a swollen scale with a 45-mm-long leaf sheath, sampled May 21.

X 190: bar=100 μm.

They were observed in cells taken both from aged elongated leaves and from young short leaves (Fig. 2a, b). They were found in both cells 5 and 15 mm above the basal end of the leaf sheath and were more dense in the former than in the latter (Fig. 2a, c).

Transverse sections cut from unswollen leaf sheaths showed that the microtubules near the face of the radial and inner tangential walls were also oriented transversely, or nearly transversely, but the microtubules near the edges of cells were oriented longitudinally (data not shown).

As the bulb developed, the microtubules in the leaf sheath cells became disoriented and diffused. Cells sampled on May 14 and 21 showed that transversely oriented microtubules had been replaced by scattered and poorly oriented microtubules (Fig. 2d-g). The number of microtubules decreased gradually; it was less in cells sampled on May 21 that in those sampled on May 14 (Fig. 2d, f) and less in cells cut from 15 mm above the basal end of the leaf sheath than in cells sampled 5 mm above it (Fig. 2e, f).

No microtubules were found in cells 15 mm above the basal end of scales with a 45-mm-long leaf sheath sampled on May 21 (Fig. 2h). Usually, no microtubules were seen near the faces of the walls in the cells taken from swollen scales. Sometimes, however, small number of longitudinally oriented microtubules were present near the edges of the cells (data not shown).

Results of the observations on microtubules near the outer

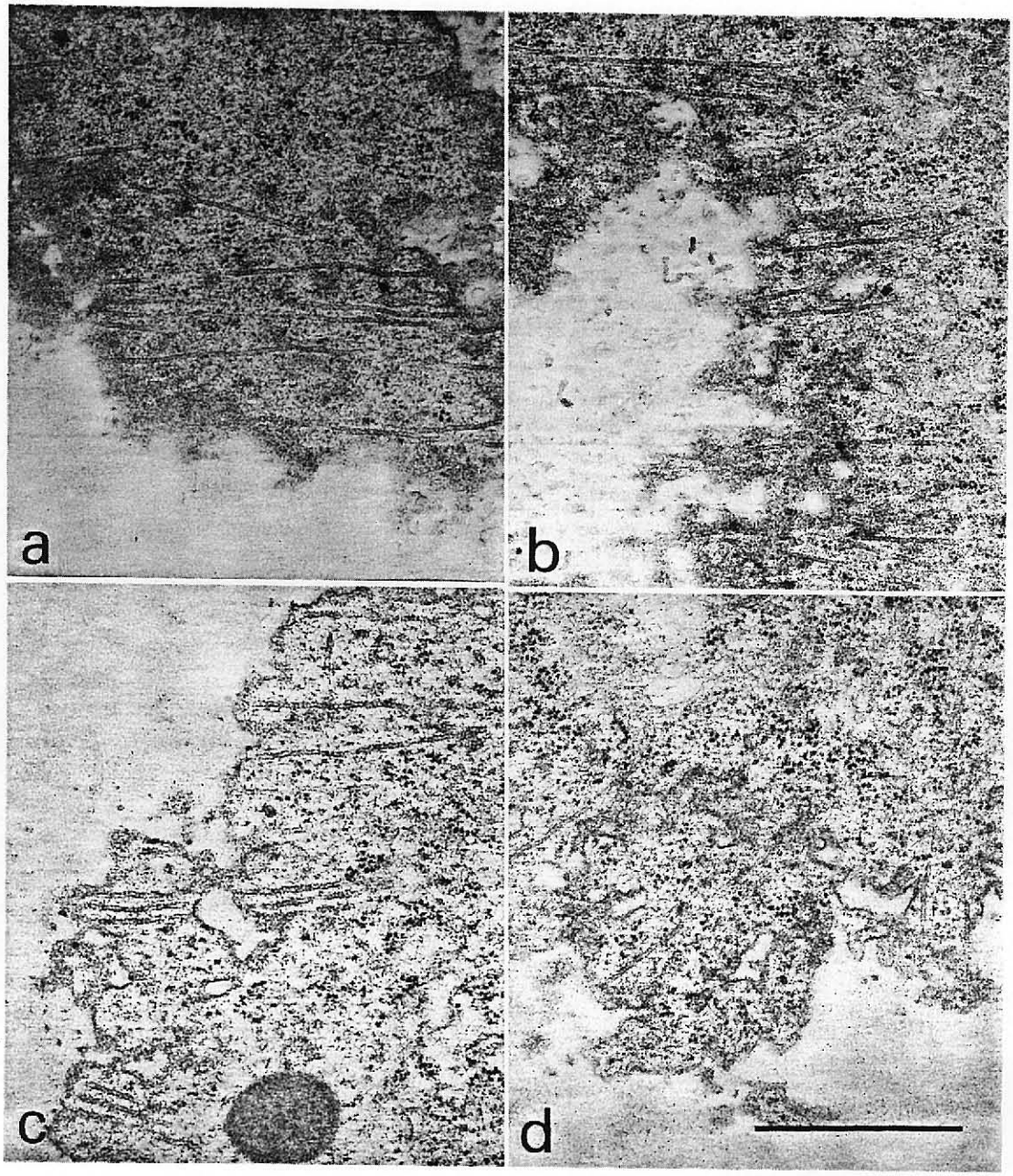
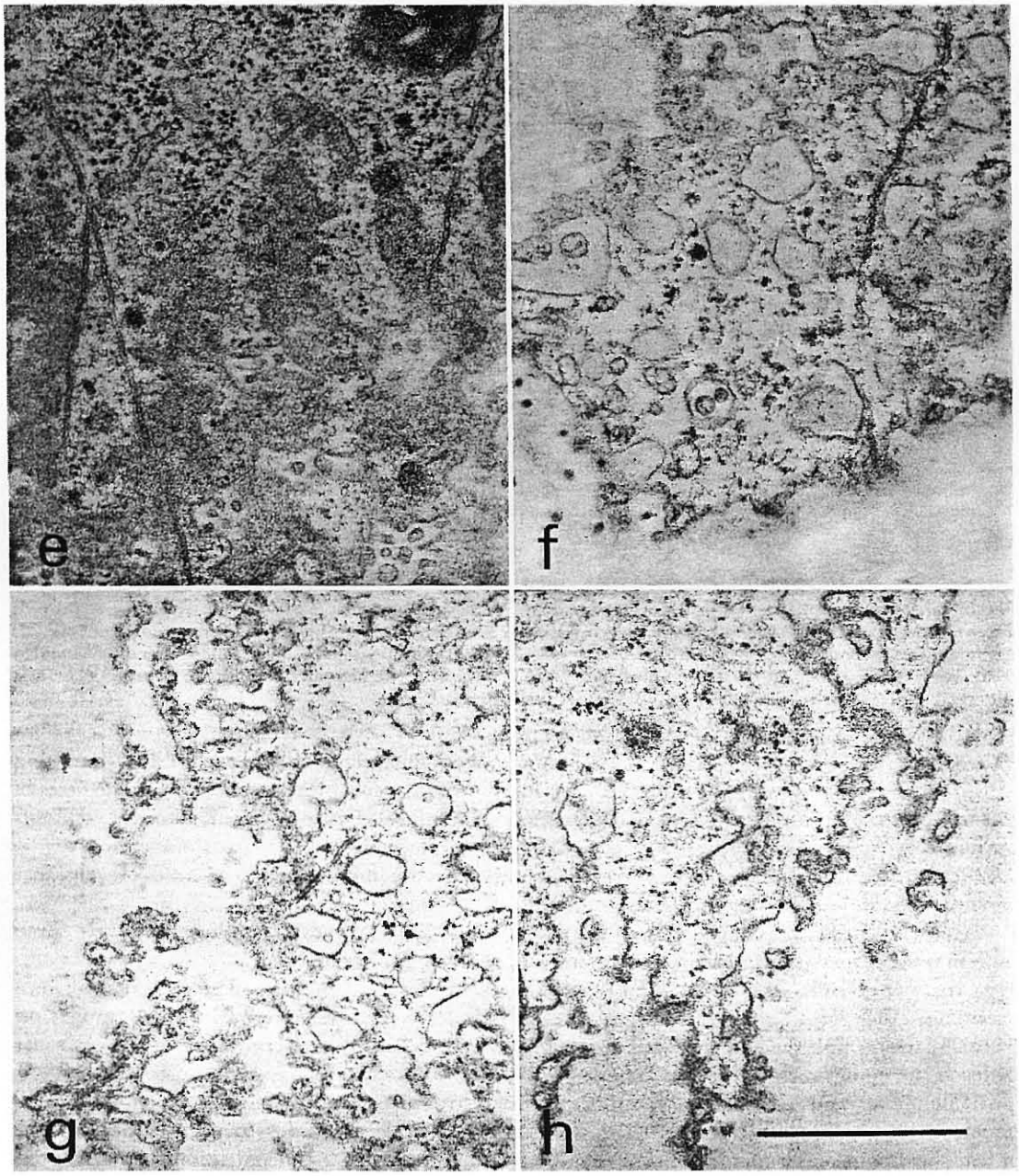


Fig. 2. Microtubules adjacent to the outer tangential wall of a cell just beneath the outer epidermis of an onion leaf sheath.
a. Cell located 5 mm above the basal end of an emerged leaf with a 40-mm-long unswollen leaf sheath, sampled April 23. Abundant, transversely oriented microtubules are present.
b. Cell located 5 mm above the basal end of the innermost emerged leaf with an 8-mm-long unswollen leaf sheath, sampled April 23. Abundant, transversely oriented microtubules are present.
c. Cell located 15 mm above the basal end of an emerged leaf with a 40-mm-long unswollen leaf sheath, sampled April 23. Transvesely oriented microtubules are present.
d. Cell located 15 mm above the basal end of a swollen scale with a 20-mm-long leaf sheath, sampled May 14. Randomly oriented microtubules are present.



e. Cell located 5 mm above the basal end of a swollen scale with a 20-mm-long leaf sheath, sampled May 21. Randomly oriented microtubules are present.

f. Cell located 15 mm above the basal end of a swollen scale with a 20-mm-long leaf sheath,, sampled May 21. A small number of randomly oriented microtubules are present.

g. Cell located 15 mm above the basal end of the innermost emerged leaf with a 70-mm-long swollen leaf sheaths, sampled May 21. A small number of randomly oriented microtubules are present.

h. Cell located 15 mm above the basal end of a swollen scale with a 45-mm-long leaf sheath, sampled May 21. No microtubules are present.

The axis of the cell parallels the length of the page.

X 30000 : bar=1 μm.

tangential wall of cells just beneath the epidermis are summarized in Table 1.

Cross sectional areas of cells just beneath the outer epidermis of the swollen scales were three to four times larger than those of unswollen leaf sheaths (Table 1).

Epidermal cells from unswollen leaf sheaths and those from swollen scales also were examined (Fig. 3). Transversely oriented microtubules were present even in cells sampled on May 21, when bulb development was visible and few microtubules were found in the cells just beneath the epidermis.

Colchicine and cremart [a substance with a structure and function similar to amiprophosmethyl (Sumida and Ueda 1976)] were tested for their abilities to induce the swelling of leaf sheaths in onion plants. Both colchicine and cremart, at a concentration that disrupted microtubules in cells just beneath the outer epidermis of the leaf sheath (data not shown), caused the leaf sheaths of onion plants grown under a short-day regimen to swell: This did not happen in untreated plants. Blades of new leaves emerged from colchicine- and from cremart-treated onion plants whose basal parts had become bulbous, soon after the termination of treatment.

Discussion

The observation that the direction of the microtubules located near

Table 1. Change in arrangement of microtubules adjacent to the outer tangential wall of cells just beneath the outer epidermis of leaf sheaths from onion plants during bulb development

Sampling date[a]	Position and morphology of leaf	Length of leaf sheath (mm)	Position of cells[b] (mm)	Microtubule arrangement[c]		Cross sectional area of cell[e] (μm^2)
				Density[d]	Orientation	
April 23 (13:19)	emerged leaf	40–45	5	++	transverse	704± 25
			15	+	transverse	762± 49
	emerged leaf	20–25	5	++	transverse	526± 49
			15	+	transverse	700± 36
	innermost emerged leaf	8– 9	5	++	transverse	426± 16
May 1 (13:35)	innermost emerged leaf	25–30	5	+	transverse	603± 19
			15	++	transverse	647± 31
	first scale	10	5	+	random	452± 31
May 14 (13:57)	innermost emerged leaf	25–30	5	++	random	1,081± 46
			15	+	random	1,214± 61
	first scale	20–25	5	+	random	727± 38
			15	+	random	634± 27
	second scale	6	5	+	random	382± 22
May 21 (14:07)	innermost emerged leaf	90	5	±	random	2,619±109
			15	±	random	3,544±167
	innermost emerged leaf	70	5	±	random	1,031± 43
			15	±	random	1,174± 63
	first scale	40–50	5	±	random	2,101±118
			15	−	—	3,927±200
	first scale	20–25	5	+	random	569± 26
			15	±	random	861± 41
	second scale	8	5	+	random	445± 19

[a] Bulb development had not started on April 23, but was visible on May 21. In parentheses, hours from sunrise to sunset.

[b] Distance of cells from the basal end of the leaf sheath.

[c] At least 40 cells from 4 different plants were examined.

[d] ++, dense (as in Fig. 2a, b); +, less dense (as in Fig. 2c, d, e); ±, sparse (as in Fig. 2f, g); −, none (as in Fig. 2h).

[e] Cells just beneath the outer epidermis of the leaf sheath were examined. The average of 40 cells with standard error of the mean. Expressed as (radial width) × (tangential width).

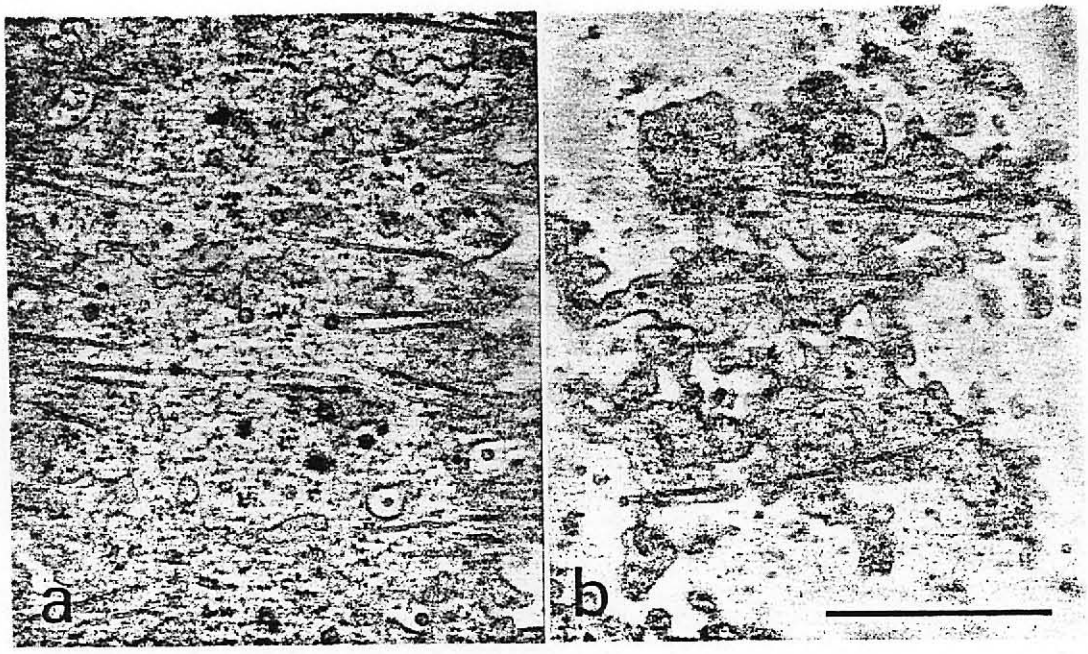


Fig. 3. Microtubules adjacent to the outer tangential wall of an epidermal cell of an onion leaf sheath.

a. Cell located 5 mm above the basal end of an emerged leaf with a 40-mm-long unswollen leaf sheath, sampled April 23. Abundant, transversely oriented microtubules are present.

b. Cell located 5 mm above the basal end of a swollen scale with a 45-mm-long leaf sheath, sampled May 21. Transversely oriented microtubules are present.

The axis of the cell parallels the length of page.
X 30000 : bar=1 μm

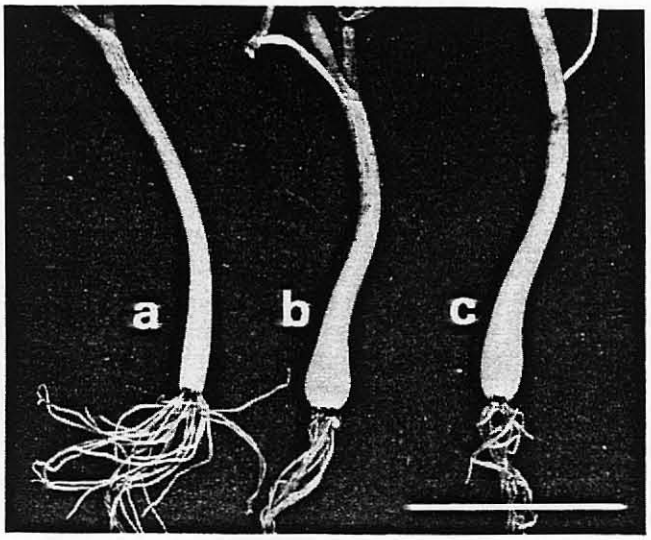


Fig. 4. Colchicine- and cremart-caused swelling of the basal portion of onion plants.

a. Untreated plant.

b. Colchicine (3×10^{-4}M)-treated plant.

c. Cremart (2×10^{-5}M)-treated plant.

The photograph was taken 9 days after the start of treatment.

$\times$ 0.55 : bar=5 cm.

the edges of the cells differed from that of those near the faces of the walls is consistent with the finding of Gunning et al. (1978) that cortical microtubules fan out from electron dense complexes located along the edges of cells. The presence of microtubules in swelling cells only near their edges also is consistent with the hypothesis of Gunning et al. (1978) that these complexes are microtubule-organizing centers (MTOC) because microtubules attached to the MTOC are considered more stable than those that are unattached (Kirschner 1980).

In almost all the cells taken from unswollen leaf sheaths, the microtubules near the faces of the walls were transverse, or nearly transverse, to the cell axis and in none of these cells were obliquely, or longitudinally, arranged microtubules present. This indicates that the walls of these cells do not have a distinct cross polylamellate structure. Probably, the cells reinforce their walls only with transversely oriented cellulose microfibrils that suppress the swelling of the cells.
In contrast, no, or only scattered and poorly oriented, microtubules were present in swollen cells. This swelling of the cells may be caused by the lack of the transversely oriented cortical microtubules necessary for the deposition of transversely oriented cellulose microfibrils (Green 1962, Hogetsu and Shibaoka 1978, Takeda and Shibaoka 1981).

The disruption of cortical microtubules is known to bring about a loss of polarity in cell expansion and to induce swelling (Green 1962, Hogetsu and Shibaoka 1978, Marchant and Hines 1979). That the lack

of microtubules caused the swelling of onion leaf sheaths was shown by the results of the colchicine and cremart experiments; they suggest that the long-day conditions induced onion bulb swelling by disrupting the cortical microtubules of the leaf sheath cells. The presence of transversely oriented microtubules in the unexpanded epidermal cells of swollen scale also indicated the importance of cortical microtubules in the regulation of the direction of cell expansion.

It has been suggested that long-day conditions stimulate the production of a "bulbing hormone" in onion leaf blades, and that the stimulus of long-day conditions slow down cell division in the meristematic region of onion plants (Heath and Holdsworth 1948). The "bulbing hormone" may thus be a microtubule-disrupting agent which induces bulb swelling by disrupting cortical microtubules and which slows down cell division by disrupting spindle microtubules.

Growth of leaves in bulbs formed under long-day conditions has been reported not to start for about 40 to 50 days after the completion of bulb development (Aoba 1964). Onion bulb development seems to include at least two different events, the swelling of leaf sheaths and the onset of dormancy. The fact that colchicine- and cremart-treated onion plants sprouted soon after termination of treatment may mean that the disruption of microtubules alone cannot accomplish bulb development: Some dormancy-inducing factors must be involved in this phenomenon.

It has been reported that microtubules near the outer tangential wall of epidermal cells from gibberellin-treated azuki bean epicotyls

were oriented transversely, whereas those of the control epicotyl were oriented randomly (Shibaoka 1974). Recently, Durnam and Jones (1982) found that gibberellin suppressed a decrease in the number of cortical microtubules in cells of excised lettuce hypocotyl segments. If gibberellin does act in onion leaf sheath cells, as it acts in azuki bean and lettuce cells, changes in the microtubule arrangement during onion bulb development can be explained in terms of changes in the endogenous gibberellin content. Therefore, changes in the amount of endogenous gibberellin during bulb development and the effect of gibberellin on microtubule arrangement in onion leaf sheath cells need to be examined.

As ethylene is known to suppress longitudinal cell expansion and to cause lateral expansion, it is tempting to say that ethylene is a factor in onion bulb swelling. But, the recent finding of Lang et al. (1982) that microtubules run longitudinally in cortical cells of ethylene-treated pea epicotyls makes this supposition unlikely. The microtubules in cells from swelling leaf sheaths of onion plants grown under long-day conditions are not oriented longitudinally; they are scattered and poorly oriented. Naturally, we must determine whether ethylene acts in onion leaf sheath cells in the same way it acts in pea epicotyl cells before we can exclude it from having a part in onion bulb development.

Chapter II

Effects of root-excision on swelling of leaf sheath cells and on the arrangement of cortical microtubules in onion seedlings

Summary

Unlike abundantry-leafed mature plants, 2- to 3-leafed young onion seedlings (Allium cepa L. cv. Senshu-Chuko) showed no swelling of leaf sheaths in response to the stimulus of long-day conditions. But, such young seedling showed swelling when their roots were excised. Before swelling became evident, changes in the arrangement of cortical microtubules occurred in leaf sheath cells of root-excised seedlings. Root excision exerted such effects only in seedlings grown under long-day conditions ; it caused neither swelling of leaf sheaths nor changes in microtubule arrangement.

Introduction

In the preceding chapter, the behavior of cortical microtubules in the leaf sheath cells of onion plants during bulb development was examined and obtained the results that microtubules were oriented transversely to the cell axis in leaf sheaths of onion plants while the daylength was short, but as the daylength became long and bulbs developed they became disoriented and scattered and finally disappear. In the course of the study, I noticed that swellings of leaf sheaths of seedlings in the two or three leaf stage had never occurred even if the seedlings were grown under continuous light condition, but if the roots of such seedlings were excised (it often happened when the seedlings were transplanted), the swelling of leaf sheath occurred. Though this swelling was a kind of artifact, it certainly involved the change in direction of cell expansion. Thus, I examined the behavior of microtubules in the leaf sheath cells of onion plants during cell swelling caused by root excision.

In order to know whether or not root excision-induced swelling of leaf sheaths of seedlings occurs irrespective of daylength, the experiments were carried out under short-day conditions as well as under long-day conditions.

Materials and methods

Seeds of onion plants (Allium cepa L. cv. Senshu-Chuko) were sown in vermiculite and the seedlings were raised at 27°C under long-day conditions (continuous light) or short-day conditions (12 hr light / 12 hr dark). Light came from 20 W "Biolux" fluorescent lamps (FL20SBR, Sylvania, Tokyo) and the light intensity at plant level was ca. 7.0 W/m^2. About 23 days after sowing, when the 2nd leaf of the seedling was 8-10 cm, the roots of the seedling were excised by a razor blade and then the root-excised seedling was laid on wet filter papers (Fig. 1). A set of two circles of filter papers (12.5 cm in diameter) was folded into two to make four half-circles of them. The folded filter papers were placed in a Petri dish, 17 cm in diameter and 2.5 cm in depth. Adjacent to the half-circled filter papers, a silicon rubber plate (2.5 mm in thickness) whose shape is shown in Fig. 1 was placed. Then, water was poured into the dish to become the depth of water 2 mm and the depth was kept constant throughout the experiment by daily water supply. The root-excised seedlings were placed in the Petri dish in two different ways. In one way (Fig. 1b), the basal parts of the seedlings were placed on the surface of water so that they were kept wet. But in the other way (Fig. 1c), the basal parts of the seedlings were placed on the silicon rubber plate so that they were kept dry. As the filter papers on which leaf

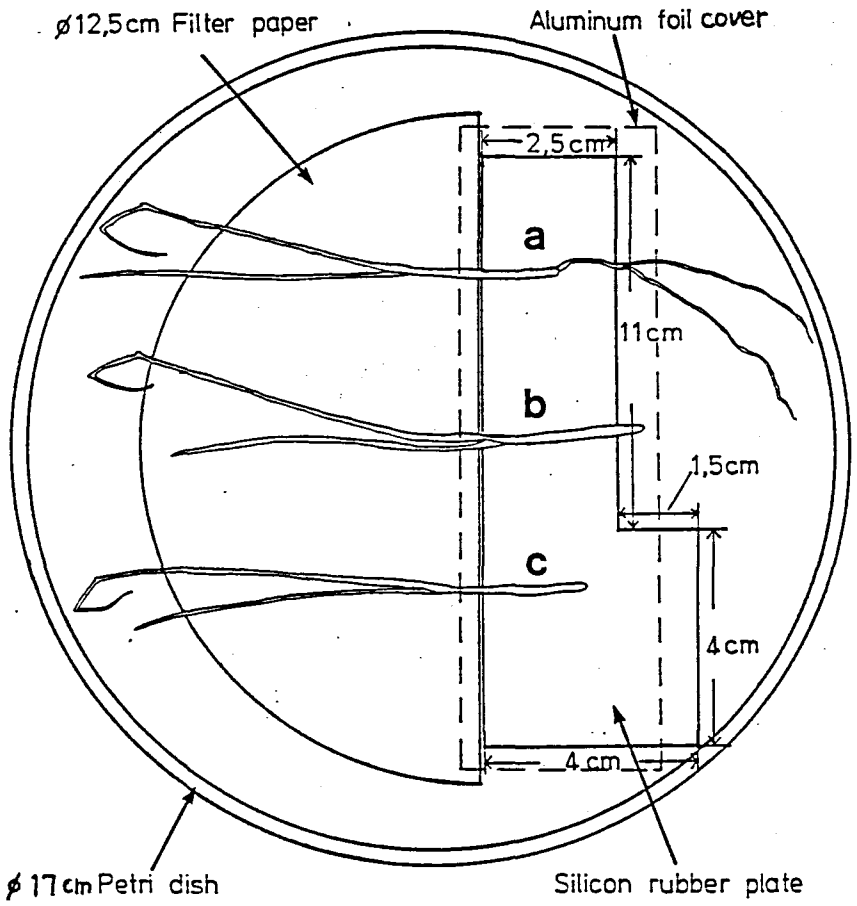


Fig. 1. Schematic illustration of experimental arrangement.

a. Intact seedling.

b. Root-excised seedling whose basal part is kept wet.

c. Root-excised seedling whose basal part is kept dry.

blades of the seedlings were placed were well moistened, the seedlings whose basal parts were kept dry were not suffered from drought. The control seedlings whose roots were not excised were placed in the Petri-dish as shown in Fig. 1a so that their roots were kept wet and the basal parts of their leaf sheaths dry.

In each Petri dish, were placed 8 intact seedlings and 16 root-excised seedlings (basal parts of a half of them were kept wet and those of the others were kept dry). The leaf sheath regions of these seedlings were covered with an aluminum foil to protect these regions from direct light (Fig. 1). The dish was not covered with a lid and was kept under the same conditions with those employed in raising the seedlings.

Leaf sheath segments, 1.5 mm long, were cut from 1 - 2.5 mm above the basal end of the 2nd leaf of seedlings 2, 3, and 5 days after the start of treatment when cell swelling had not yet started, had just started, and had been in progress, respectively.
The sampled tissues were fixed with 0.025 M potassium phosphate buffer pH 7.2 containing 2 % paraformaldehyde, 2.5 % glutaraldehyde, 0.5 % tannic acid and 3 mM EGTA for 2 hours and then with 2 % OsO_4 in the same buffer for another 2 hours at room temperature.
The fixed tissues were dehydrated with ethanol and embedded in Spurr's resin (Spurr, 1969). Tangential longitudinal sections through the outer tangential wall and outer cytoplasm were cut from the epidermal cells. The sections were stained with 25 % uranyl

acetate in absolute methanol for 35 minutes and then lead citrate for 4 minutes. The stained sections were examined with an electron microscope (JEM-100C, Jeol, Tokyo). As can be seen in Fig. 3 or Fig. 6 the thickness of a leaf sheath was not uniform. It was thicker on side of the blade (upper side in Fig. 3 or Fig. 6) and thinner in the side of the axis or the pore (lower side in Fig. 3 or Fig. 6). Throughout the present work, cells on the side of leaf blade were examined.

To measure the cross-sectional area of leaf sheath cells, transverse sections were cut by a razor blade from 2 mm above the basal end of 2nd leaf of seedlings and examined under a light microscope equipped with an ocular micrometer. The cross-sectional area of cells were expressed as (tangential width) X (radial width).

Results

Experiments under long-day conditions

The swelling of the basal parts of leaf sheaths occurred in root-excised seedlings whose basal parts were kept dry, but did not occur in those whose basal parts were kept wet or in seedlings whose roots remained intact (Fig. 2). The root-excised seedlings whose basal parts were kept wet regenerated new roots (Fig. 2b). The conditions favorable for root regeneration seem not to be favorable for swelling. If the Petri dish was covered with a

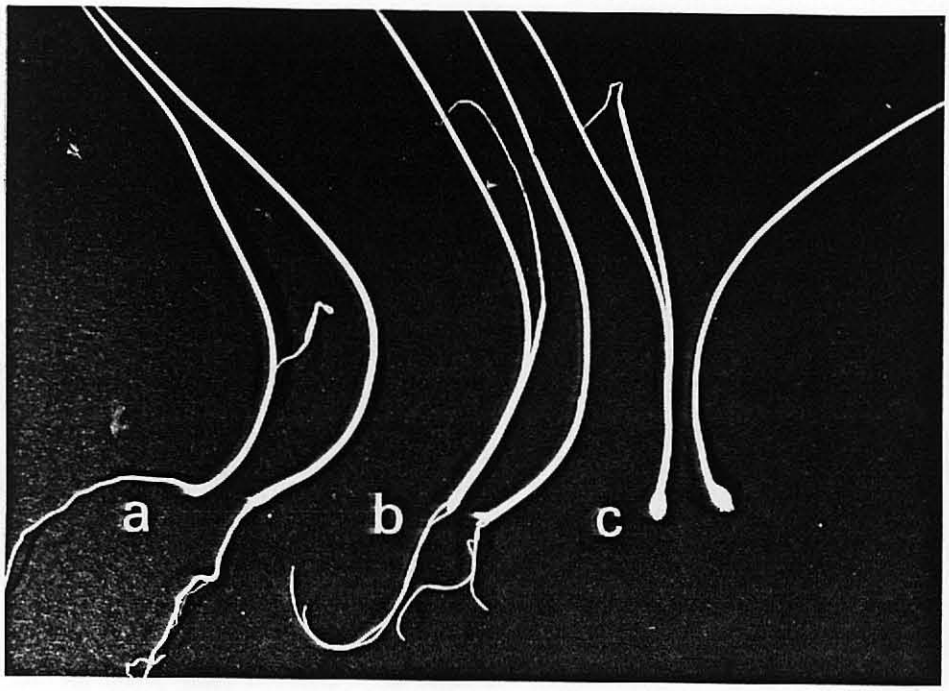


Fig. 2. Effect of root excision on swelling of leaf sheath of young 2- to 3-leafed onion seedlings grown under long-day conditions.

a. Intact seedlings.

b. Root-excised seedlings whose basal parts had been kept wet.

c. Root-excised seedlings whose basal parts had been kept dry.

The 1st leaf was removed from the right side seedling of each pair. The photograph was taken 10 days after the start of treatment.

X 1.15

polyethylene film or was places in a humid incubator so that the humidity of air surrounding the seedlings became high, the seedlings regenerate roots vigorously even when their basal parts were placed on the silicon rubber plate. Under such conditions, swelling did not occur. Seemingly, swelling of basal parts of root-excised seedlings is not induced by the temporal loss of root system, but it requires a prolonged lacking of the system.

Although the water supply from the basal parts of seedling was found to be inhibitory for swelling, the water supply from leaf blades is indispensable. If the half-circled filter papers were not moistened with water and the seedlings could not take up water through their leaf blades, the swelling never occurred. Root-excised seedlings, placed on dry filter papers for 15 days, still could regenerate roots and form new leaves and resume their growth after transplanting in moistened vermiculite, suggesting that the inability of the seedling to cause swelling was not due to the seedling's death from dryness.

Small bulbs formed at the basal parts of the root excised seedlings were planted in wet vermiculite after 11 days from the root excision. New leaf blades and new roots began to emerge from the bulbs soon after the planting. In contrast to that the bulbs formed by the seasonal daylength change show dormancy (Aoba, 1964), the bulbs produced by the root excision seem not to be in a state of dormancy.

Histological observations revealed that the swelling of root excised seedling was due to lateral expansion of leaf sheath cells (Fig. 3). Root excision brought about the increase in the cross-sectional areas both in epidermal cells and in cortical cells (Table 1).

Root excision caused disorientation of microtubules in leaf sheath cells of onion seedlings (Fig. 4). Transversely or nearly transversely oriented microtubules were present near the face of the outer tangential wall of leaf sheath cells sampled from intact seedlings, irrespective of the time of sampling (the cells were sampled 2, 3, and 5 days after the start of the experiment) (Fig. 4a). But, microtubules in the corresponding position of the corresponding cells from root excised seedlings were oriented parallel or obliquely to the cell axis (Fig. 4c and d). Changes in the orientation of microtubules were evident 2 days after the root excision when cell swelling had not yet started. Changes in the orientation of microtubules were not accompanied with the decrease in the number of microtubules. Almost the same number of microtubules as observed in the cells from intact seedlings were present in almost all cells taken 3 days after the root excision, although they were not oriented transversely to the cell axis. The number of microtubules seemed to decrease as cells swell; it was slightly small in the cells taken 5 days after the root excision.

Changes in the orientation of microtubules did not occur in the

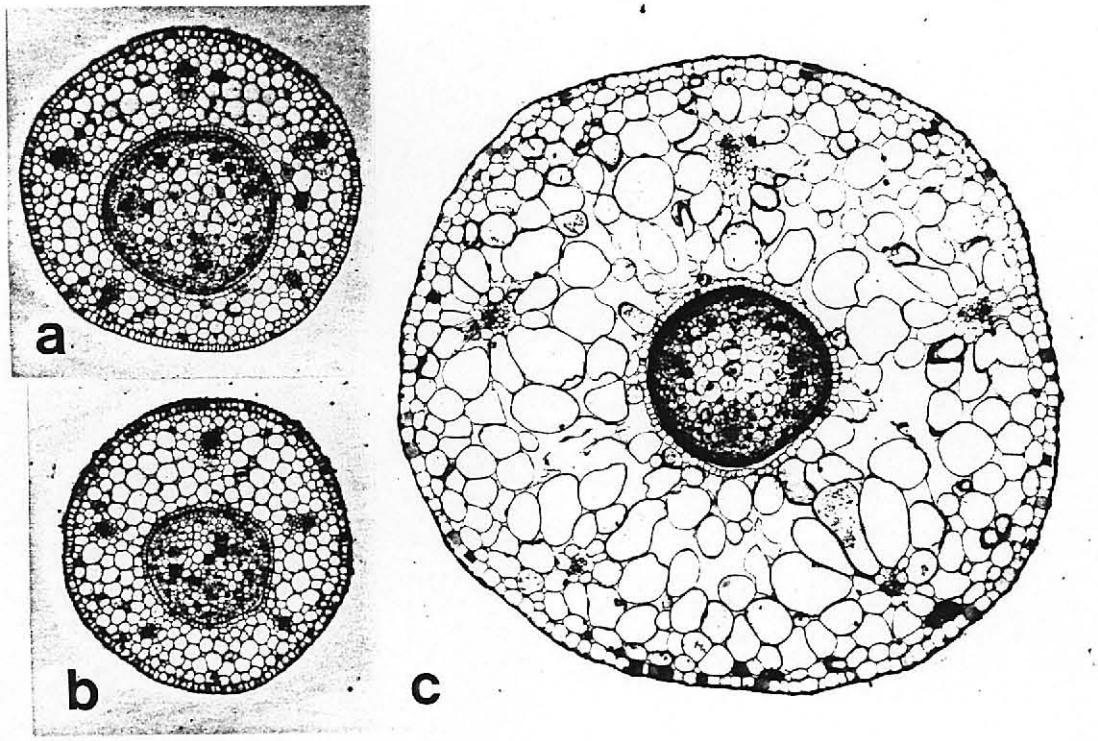


Fig. 3. Effect of root excision on swelling of leaf sheath of young 2- to 3-leafed onion seedlings grown under long-day conditions. Transverse sections of the leaf sheaths of 2nd leaves were cut 2 mm above their basal ends at 10 days after the start of treatment. The blades of 3rd leaves are seen surrounded by the sheaths of 2nd leaves.

a. Intact seedling.

b. Root-excised seedling whose basal part had been kept wet.

c. Root-excised seedling whose basal part had been kept dry.

X 40.

Table 1. Effect of root excision on lateral cell expansion of leaf sheath cells of young 2- to 3-leafed onion seedlings grown under long-day conditions.

Days after treatment	Cross-sectional area of cell (μm^2)*					
	Epidermal cells			2nd layer cells		
	+Root**	-Root (wet)**	-Root (dry)**	+Root**	-Root (wet)**	-Root(dry)**
2	295±6(100)	306±6(104)	290±7(98)	448±19(100)	456±30(102)	483±28(108)
3	303±10(100)	298±4(98)	350±12(116)	427±24(100)	454±30(106)	592±54(139)
5	332±7(100)	348±8(105)	485±19(146)	473±29(100)	484±44(102)	966±65(204)
10	380±11(100)	399±10(105)	1700±169(447)	583±56(100)	674±39(116)	3300±383(566)

* Average of 40 cells with standard error. In parenthesis, percent of that of root-unexcised control.

** +Root, root-unexcised control seedlings; -Root (wet), root-excised seedlings whose basal parts had been kept wet; -Root (dry), root-excised seedlings whose basal parts had been kept dry.

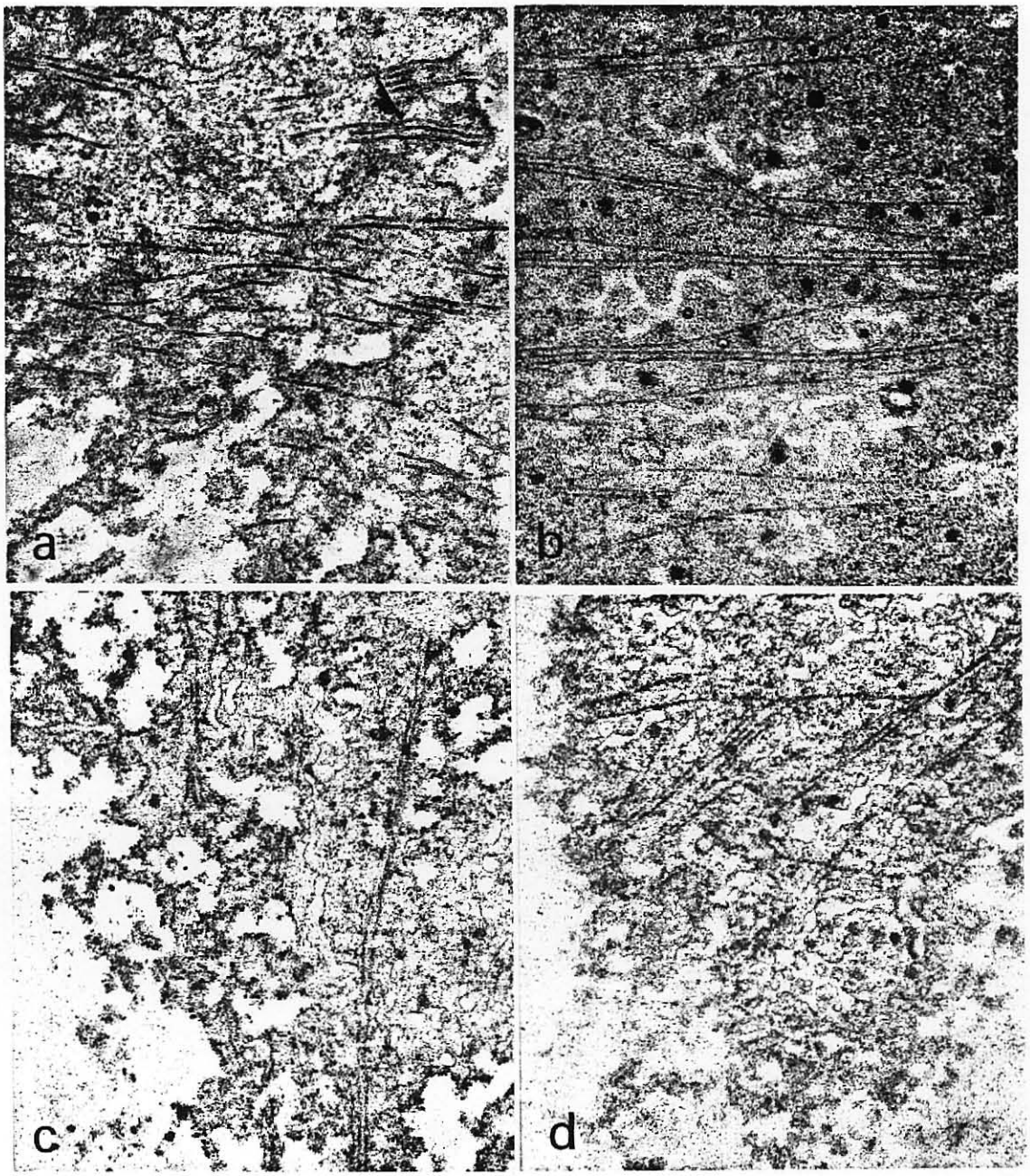


Fig. 4. Effect of root excision on arrangement of microtubules against the face of the outer tangential wall of a leaf sheath epidermal cells of onion seedling grown under long-day conditions.

a. A cell located 1.5 mm above the basal end of the 2nd leaf of intact seedling. They were sampled 3 days after the start of treatment. Microtubules are oriented transversely to the cell axis.

b. A cell located 1.5 mm above the basal endt of the 2nd leaf of root-excised seedling whose basal part had been kept wet. They were sampled 3 days after the start of treatment. Microtubules are oriented transversely to the cell axis.

c. and d. Cells located 1.5 mm above the basal end of the 2nd leaf of root-excised seedlings whose basal parts had been kept dry. They were sampled 3 days (c) and 5 days (d) after the start of treatment. Microtubules are oriented parallel or obliquely to the cell axis.

X 20000

leaf sheath epidermal cells of root-excised seedlings whose basal parts had been kept wet (Fig. 4b). Transversely or nearly transversely oriented microtubules were present in the cells from the seedlings at all 3 different stages (2, 3, and 5 days after the root excision).

Experiment under short-day conditions

Under short-day conditions, root excision caused neither swelling of basal parts of onion leaf sheath, (Fig. 5 and 6) nor lateral expansion of leaf sheath cells (Table 2). It caused no disorientation of microtubules; transversely or nearly transversely oriented microtubules were present both in leaf sheath epidermal cells of intact seedlings and in those of root excised seedlings (Fig. 6). They were observed in the cells of root-excised seedlings at 3 different stages, i.e. 2, 3, and 5 days after the root excision, whether whose basal parts had been kept dry or wet (Fig. 6b).

Discussion

In onion seedling grown under long-day conditions, root excision caused change in arrangement of cortical microtubules in leaf sheath cells, and accompany with this change lateral expansion of these cells occurred, suggesting that the presence of roots kept the microtubules to orient transversely to the cell axis and the cells

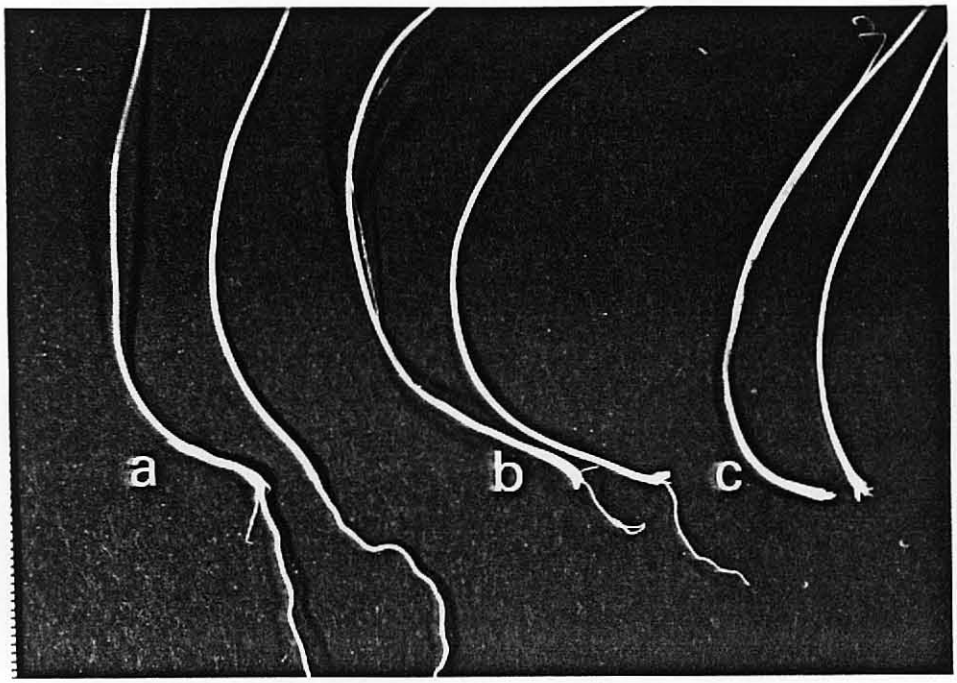


Fig. 5. Ineffectiveness of root excision in swelling of leaf sheath of young 2- to 3-leafed onion seedlings grown under short-day conditions.

a. Intact seedlings.

b. Root-excised seedlings whose basal parts had been kept wet.

c. Root-excised seedlings whose basal parts had been kept dry.

The 1st leaf was removed from the right side seedling of each pair. The photograph was taken 10 days after the start of treatment.

X 1.15

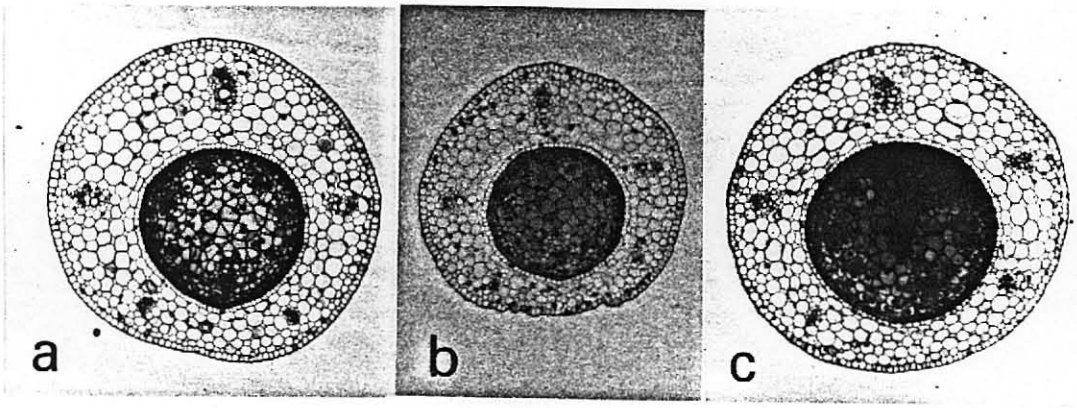


Fig. 6. Ineffectiveness of root excision in swelling of leaf sheath of young 2- to 3-leafed onion seedlings grown under short-day conditions. Transverse sections of the leaf sheaths of 2nd leaves were cut 2 mm above their basal ends at 10 days after the start of treatment. The blades of 3rd leaves are seen surrounded by the sheaths of 2nd leaves.

a. Intact seedling.

b. Root-excised seedling whose basal part had been kept wet.

c. Root-excised seedling whose basal part had been kept dry.

X 40.

Table 2. Effect of root excision on rateral cell expansion of leaf sheath cells of young 2- to 3-leafed onion seedlings grown under short-day conditions.

Days after treatment	Cross-sectional area of cell (um^2)*					
	Epidermal cells			2nd layer cells		
	+Root**	-Root (wet)**	-Root (dry)**	+Root**	-Root (wet)**	-Root (dry)**
2	239±7(100)	256±5(107)	238±4(100)	343±19(100)	375±33(109)	363±20(106)
3	243±8(100)	262±7(108)	279±5(115)	353±20(100)	373±21(106)	360±27(102)
5	308±11(100)	333±8(108)	339±10(110)	378±35(100)	381±31(101)	390±27(103)
10	383±10(100)	378±7(99)	439±6(115)	563±37(100)	456±27(81)	555±39(99)

* Average of 40 cells with standard error. In parenthesis, percent of that of root-unexcised control.

** +Root, root-unexcised control seedlings; -Root (wet), root-excised seedlings whose basal parts had been kept wet; -Root (dry), root-excised seedlings whose basal parts had been kept dry.

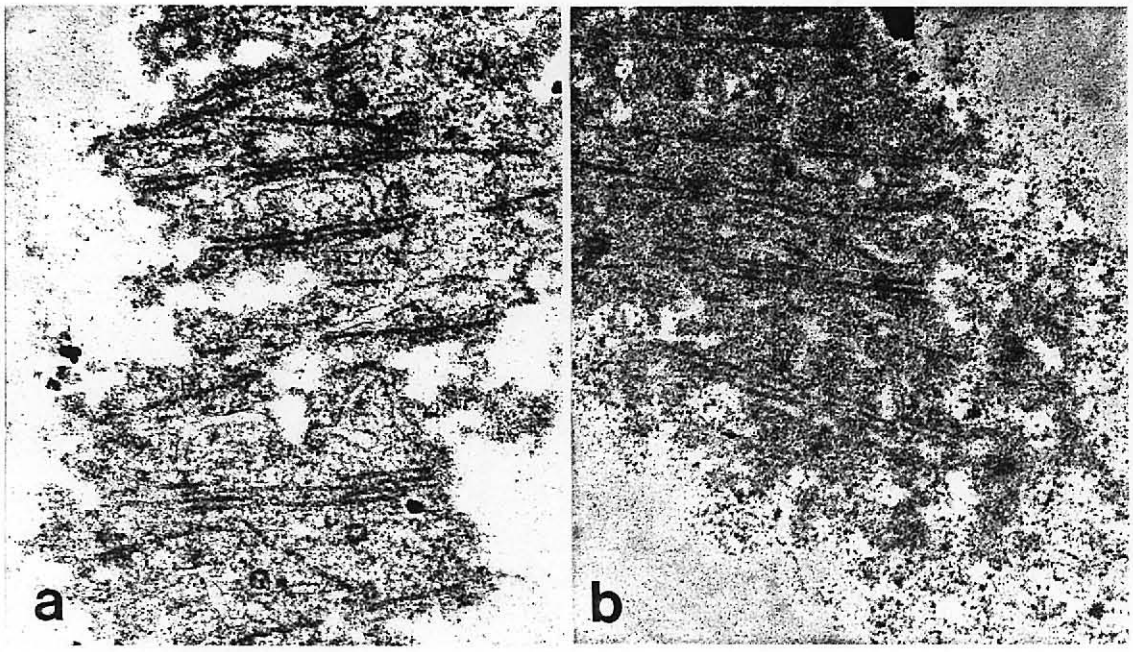


Fig. 7. Ineffectiveness of root excision in changing arrangement of microtubules against the face of the outer tangential wall of a leaf sheath epidermal cells of onion seedlings grown under short-day conditions.

a. A cell located 1.5 mm above the basal end of the 2nd leaf of intact seedling. They were sampled 5 days after the start of treatment. Microtubules are oriented transversely to the cell axis.

b. A cell located 1.5 mm above the basal end of the 2nd leaf of root-excised seedling whose basal part had been kept dry. They were sampled 5 days after the start of treatment. Microtubules are oriented transversely to the cell axis.

X 20000

not to expand laterally. These results suggest that roots are producing some substance which has an ablity to keep microtubules to orient transversely and supplying it to leaf sheath cells. If this is the case, root excision should cause the lack of this substance in leaf sheath cells and, consequently, the change in the orientation of microtubules. The cell having no transverse microtubules may not deposit transverse cellulose microfibrils on the wall (Green, 1969; Hepler and Palevitz, 1974; Gunning and Hardham, 1982), and the cell having the wall not reinforced by transverse cellulose microfibril should expand laterally (Green, 1962; Hogetsu and Shibaoka, 1978).

Probably, gibberellin is one of the candidates for this substance. Because, roots have been considered to produce gibberellins (Torrey, 1976) and gibberellin has been reported to have an ability to arrange cortical microtubules transversely to cell axis (Shibaoka, 1974). This ability of gibberellin has been demonstrated also in onion seedling (Mita and Shibaoka, 1984). Although, I have to examine whether or not gibberellins are synthesized in roots also in onion plants before I reach final conclusions, I had obtained the results which support the idea that the lack of gibberellin supply causes disorientation of cortical microtubules and cell swelling from the experiments in which I examined the effects of (E)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol, an inhibitor of gibberellin biosynthesis. The results

will be reported in the next chapter.

Root excision did not cause swelling of leaf sheath in seedlings grown under short-day conditions, making a contrast with the results that microtubule inhibitors caused the swelling even in the seedlings grown under short-day conditions (Mita and Shibaoka, 1983,1984). This result indicates that the swelling is not caused solely by removal of a factor from roots which supresses swelling, but it requires a "bulbing hormone" which has been considered to be synthesized in leaf blades in response to the stimulus of long-day conditions (Heath and Holdsworth, 1948). Seemingly, the direction of cell expansion of basal regions of onion leaf sheaths is controlled both by the bulbing hormone from leaf blades and by "anti-bulbing hormone" from root system. The increase in amount of bulbing hormone seems to be indispensable for bulb development induced by the seasonal changes in daylength.

The decrease in the amount of an anti-bulbing hormone from roots seems not to be involved in the bulb development induced by the seasonal change in daylength conditions. Because, cortical microtubules in leaf sheath epidermal cells of seedlings whose bulb formation was caused by root excision behaved differently from those in seedlings whose bulb formation was caused by the seasonal change in daylength. When the swelling was induced by root excision, microtubules in leaf sheath epidermal cells were oriented parallel or obliquely to the cell axis (Fig. 4c and d in this

chapter), while those in leaf sheath epidermal cells (not those in cortical cells) remained transversely oriented when the swelling occurred in intact plants grown under natural daylength conditions (Fig. 3b in the preceding chapter), suggesting that the decrease in the amount of anti-bulbing hormone from roots was not involved in the latter case. Thus, I suppose that the direction of cell expansion of basal region of onion leaf sheaths is determined by the proportion of "bulbing hormone" from leaf blades and "anti-bulbing hormone" from root system. When the proportion of the former to the latter is relatively high the cell may swell, and when it is low the cells may continue to elongate. This idea coincides with the facts that seedlings having small number of leaves do not develop bulbs even under long-day conditions and only seedlings having more than a certain number of leaves can develop bulbs. The proportion of "bulbing hormone" from blades to "anti-bulbing hormone" from roots may remain relatively low in seedlings having a small number of leaves even under long-day conditions, because a limited number of leaves may produce only a limited amount of bulbing hormone. Root excision may elevate this proportion by decreasing the amounts of factor from roots, and thus induce cell swelling. The amounts of bulbing hormone will increase as the number of leaves increases, and when it exceded some extent so that bulbing hormone can overcome anti-bulbing hormone from roots, bulb development will begin.

I have reported in the preceding chapter that epidermal cells did not show lateral expansion while the inner cells did in swelling leaf sheaths of onion plants grown under natural daylength conditions, but have not shown the reason of why these 2 types of cells differently responded to the same stimulus. The result that both epidermal cells and inner cells showed lateral expansion in root-excised seedlings suggests that anti-bulbing hormone from roots play an important role in producing this difference. Further studies on this anti-bulbing hormone will give us useful information to elucidate this difference.

Chapter III

Effects of S-3307, an inhibitor of gibberellin biosynthesis, on swelling of leaf sheath cells and on the arrangement of cortical microtubules in onion seedlings.

Summary

Treatment with S-3307, a newly developed growth retardant, caused swelling of leaf sheaths of 2- to 3- leafed young onion seedlings, which otherwise showed no swelling even under long-day conditions. Before swelling became evident changes in the arrangement of cortical microtubules occurred in leaf sheath cells of S-3307-treated seedlings. S-3307 exerted their effects only in seedlings grown under long-day conditions; it caused neither swelling of leaf sheaths nor changes in microtubule arrangement.

Simultaneously applied gibberellin reversed the effects of S-3307, indicating that S-3307 acted as an inhibitor of gibberellin biosynthesis in onion seedlings as had been reported by Izumi et al. in rice plants (1984).

Introduction

Gibberellin has been found to have an ability to arrange cortical microtubules transversely to the cell axis in azuki bean stem cells (Shibaoka, 1974) and onion leaf sheath cells (Mita and Shibaoka, 1984). In the preceding chapter, I reported that root excision caused swelling of basal parts of young 2- to 3-leafed onion seedlings, which otherwise showed no swelling even under long-day conditions, and that before swelling become evident changes in the arrangement of cortical microtubules occurred in leaf sheath cells of root-excised seedlings.

Based on these findings and a well known fact that roots are the sites of gibberellin biosynthesis (Torrey, 1976), I hypothesized that roots were suppressing lateral cell expansion of leaf sheath cells by supplying them with gibberellin.

To check this hypothesis I had undertaken the experiments in which an inhibitor of gibberellin biosynthesis was examined for its ability to cause swelling of onion leaf sheath cells. I also examined whether or not the inhibitor causes changes in the arrangement of cortical microtubules in the leaf sheath cells before they begin to swell.

Recently, S-3307, a newly developed growth retardant, has been revealed to inhibit gibberellin biosynthesis in rice plants (Izumi et al., 1983). I used this substance in this experiment.

Materials and methods

Seeds of onion plants (Allium cepa L. cv. Senshu-Chuko) were sown in vermiculite and seedlings were raised at 27°C under long-day (continuous light) or under short-day (12 hr light / 12 hr dark) conditions. Light came from 20 W "Biolux" fluorescent lamps (FL 20SBR Sylvania, Tokyo) and the light intensity at plant level was about 7.0 W m^{-2}. About 23 days after sowing, when the 2nd leaves of the seedlings were 8-10 cm long, seven seedlings were transplanted in a small pot containing 30 g vermiculite moistened with 100 ml of 1×10^{-4} M S-3307 emulsion or control emulsion. They were kept under the same conditions with those employed in raising the seedlings. After 2, 3, 5, and 10 days, the cross sectional area of leaf sheath cells of 2nd leaves were measured.

Leaf sheath segments, 1.5 mm long, were cut from 1.0-2.5 mm above the basal ends of the 2nd leaves of the S-3307 untreated and treated seedlings 2 days after the start of the treatment when cell swelling had not yet started, after 3 days when cell swelling had just started, and after 5 days when cell swelling had become evident (Table 1). The sampled tissues were fixed, dehydrated and embedded in Spurr's resin (Spurr 1964) as described in the preceding chapter. Tangential longitudinal sections through the outer tangential wall and outer cytoplasm were cut from the outer epidermal cells. The sections were stained with uranyl acetate in absolute methanol for 35 minutes and then with lead citrate for 4 minutes. The stained sections were examined with an

electron microscope (JEM-100C, Jeol, Tokyo). As in the experiments in the preceding chapter, leaf sheath cells on the side of the leaf blade were examined throughout the experiments in this chapter.

S-3307, (E)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol, which we obtained from Sumitomo Chemical Co. Ltd. (Takatsukasa, Takarazuka, Hyogo, Japan) was dissolved in cyclohexane and to S-3307 cyclohexane solution was added tween 20 to give 5 % S-3307 stock solution (S-3307 : cyclohexane : tween 20 = 5 : 85 : 10, W/V/V). To make 1×10^{-4}M S-3307 test emulsion, 60 ul of S-3307 stock solution was mixed with 100 ml distilled water. To make control emulsion, 60 ul of cyclohexane-tween 20 mixture (85 : 10, V/V) was mixed with 100 ml distilled water.

In experiments in which the antagonistic effects of gibberellin A_3 (GA_3) to the effects of S-3307 were examined, seven seedlings having the 2nd leaves of 7-9 cm long were planted in a pot containing 30 g vermiculite moistened with 100 ml of distilled water or 1×10^{-4}M GA_3 solution, and after 2 days both water-pretreated and GA_3-pretreated seedlings were transplanted in a pot containing 30 g vermiculite moistened with 100 ml of control emulsion, 1×10^{-4}M S-3307 emulsion, control emulsion containing 1×10^{-4}M GA_3 or 1×10^{-4}M S-3307 emulsion containing 1×10^{-4}M GA_3. The cross sectional area of leaf sheath cells of the seedlings were measured 10 days after the start of the 2nd treatment, i.e. 12 days after the start of the experiments.

To measure the cross sectional areas of the leaf sheath cells,

transverse sections were cut with a razor blade from 2 mm above the basal end of the 2nd leaves of the seedlings and examined under a light microscope equipped with an ocular micrometer. The cross sectional areas of cells were expressed as (tangential width) X (radial width).

Results

Experiments under long-day conditions

S-3307 at 1×10^{-4}M suppressed leaf growth and caused swelling of leaf sheaths in onion seedlings grown under long-day conditions (Fig. 1). At lower concentrations, such as 1.7×10^{-5} and 3.3×10^{-5}M S-3307 was active both in suppressing leaf growth and in causing swelling.

As shown in Fig. 2a and b, S-3307-induced swelling was brought about by lateral expansion of leaf sheath cells and the increase in the number of cells was not involved in this phenomenon. S-3307 caused lateral expansion in both epidermal cells and cells just beneath the epidermis, but the S-3307-induced increment of cross-sectional areas of the former was smaller than those of the latter. S-3307 at 1×10^{-4}M increased the cross sectional area of epidermal cells by 300 to 500 %, and those of cells just benerth the epidermis by about 600 % (Table 1 and 2).

Results shown in Table 1, and Fig. 2 were obtained with sections cut from 2 mm above the basal ends of the 2nd leaf sheaths. The examination of sections cut from near basal and upper ends of leaf sheaths revealed that S-3307 caused lateral cell expansion along entire

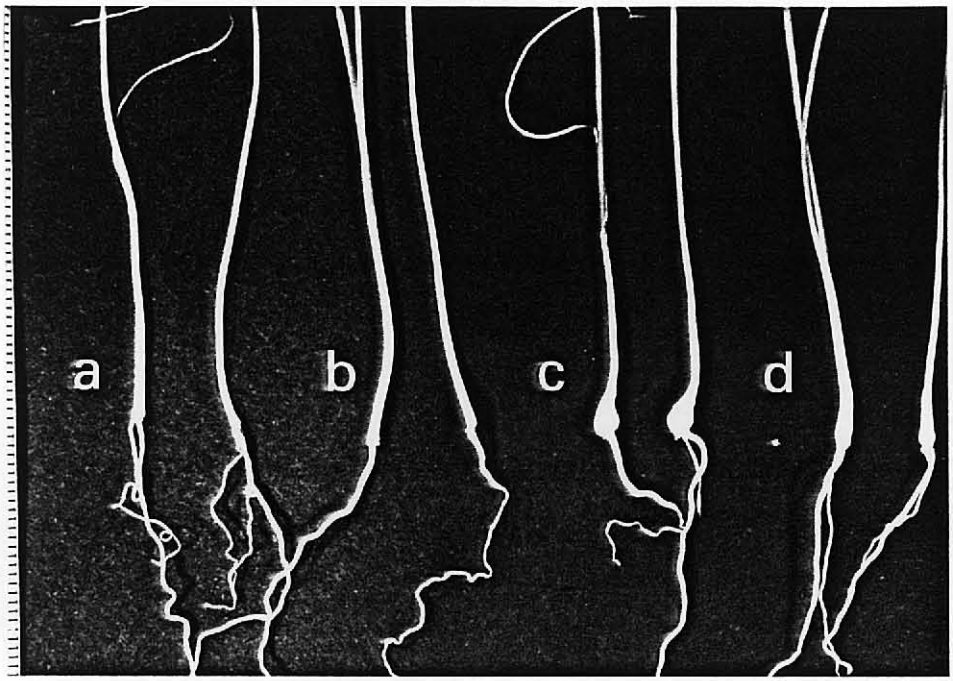


Fig. 1. Antagonism between GA_3 and S-3307 in swelling of the basal parts of onion seedlings grown under long-day conditions.

a. Pretreated with water for 2 days and again with water.

b. Pretreated with GA_3 (1×10^{-4}M) for 2 days and again with GA_3 (1×10^{-4}M).

c. Pretreated with water for 2 days and then with S-3307 (1×10^{-4}M).

d. Pretreated with GA_3 (1×10^{-4}M) for 2 days and then with GA_3 (1×10^{-4}M) + S-3307 (1×10^{-4}M).

The 1st leaf was removed from the right side seedling of each pair. The photograph was taken 12 days after the start of the experiment (10 days after the start of the 2nd treatment).

× 1.2

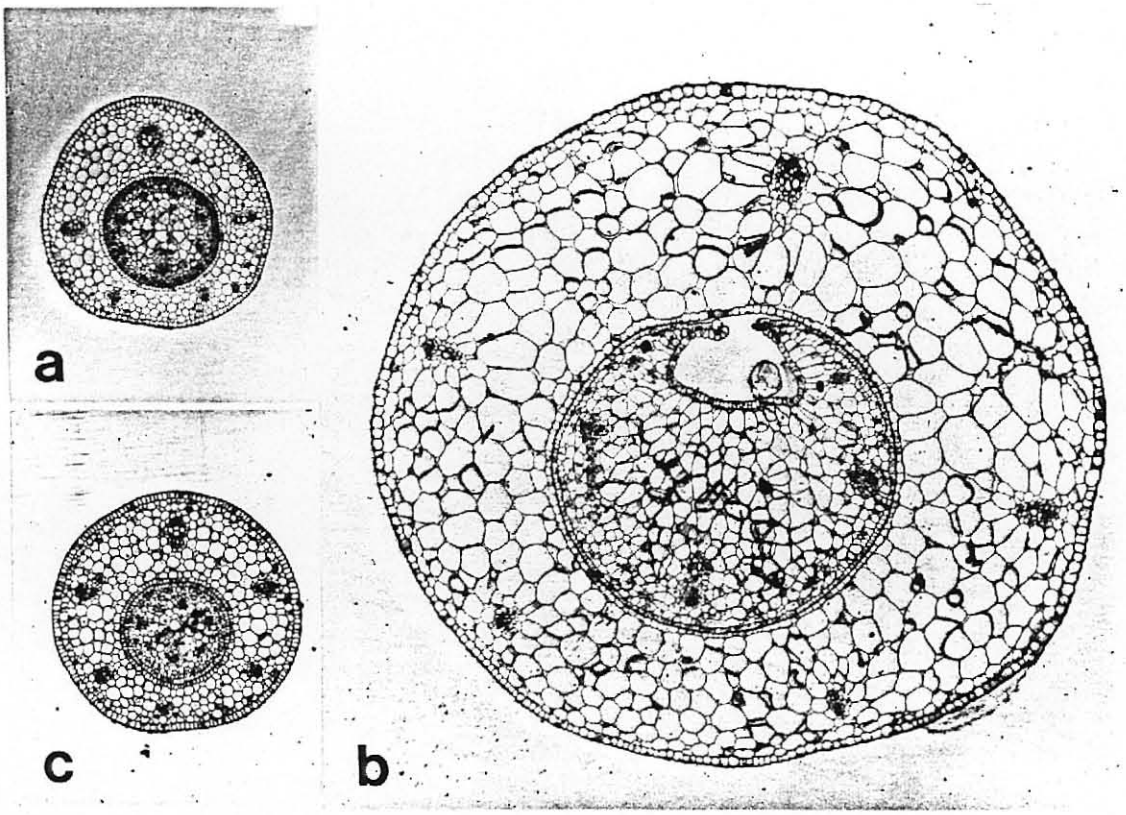


Fig. 2. Antagonism between GA_3 and S-3307 in swelling of the basal parts of onion seedlings grown under long-day conditions. Transverse sections of the 2nd leaves were cut 2 mm above their basal ends 12 days after the start of the experiment. The blades of 3rd leaves are seen surrounded by the sheaths of 2nd leaves.

a. Pretreated with water for 2 days and again with water.

b. Pretreated with water for 2 days and then with S-3307 (1×10^{-4}M).

c. Pretreated with GA_3 (1×10^{-4}M) for 2 days and then with GA_3 (1×10^{-4}M) + S-3307 (1×10^{-4}M).

× 35

Table 1. Effect of S-3307 on lateral expansion of leaf sheath cells of onion seedlings grown under long-day conditions.

Days after treatment	Cross-sectional area of cell (μm^2) *			
	Epidermal cells		2nd layer cells	
	Control	S-3307 ($1 / 10^{-4}$ M)	Control	S-3307 ($1 / 10^{-4}$ M)
2	284±6 (100)	268±6 (94)	436±22 (100)	426±16 (98)
3	279±6 (100)	312±7 (112)	410±19 (100)	456±31 (111)
5	317±10 (100)	746±72 (235)	474±29 (100)	1524±203(322)
10	410±9 (100)	2464±159(601)	655±58 (100)	4562±346(696)

* Average of 40 cells with standard error. In parenthesis, percent of that of control.

Table 2. Interaction of S-3307 with GA_3 in lateral expansion of onion leaf sheath cells

Treatment		Cross-sectional area of cell (μm^2) *	
0 - 2 days	2 - 12 days	Epidermal cell	2nd layer cell
H_2O	H_2O	406 ± 9.6 (100)	523 ± 39 (100)
H_2O	S-3307 ($1X10^{-4}M$)	1582 ± 77 (390)	3727 ± 294 (713)
H_2O	GA_3 ($1X10^{-4}M$)	433 ± 10 (107)	579 ± 36 (111)
H_2O	S-3307 ($1X10^{-4}M$)+GA_3 ($1X10^{-4}M$)	515 ± 26 (127)	719 ± 47 (137)
GA_3 ($1X10^{-4}M$)	S-3307 ($1X10^{-4}M$)	575 ± 17 (142)	848 ± 69 (162)
GA_3 ($1X10^{-4}M$)	GA_3 ($1X10^{-4}M$)	410 ± 7 (101)	547 ± 34 (105)
GA_3 ($1X10^{-4}M$)	S-3307 ($1X10^{-4}M$)+GA_3 ($1X10^{-4}M$)	622 ± 13 (153)	743 ± 43 (142)

* Average of 40 cells with standard error. In parenthesis, percent of that of control.

lengths of leaf sheaths.

Transversely or nearly transversely oriented microtubules were present near the face of the outer tangential walls of leaf sheath epidermal cells sampled from S-3307-untreated seedlings (Fig. 3a). They were observed irrespective of the time of sampling.

S-3307 at 1×10^{-4}M caused changes in the arrangement of microtubules. Longitudinally or obliquely oriented microtubules were present in cells sampled from S-3307-treated seedlings. They were observed in cells sampled 2 days after the start of treatment when the swelling had not yet started. The changes in the direction of microtubules was not accompanied with the decrease in the number of microtubules (Fig. 3c). No sign of the decrease in the number of microtubules was shown in cells sampled 3 days after the start of treatment, although microtubules were oriented longitudinally or obliquely in almost all cells examined. The decrease in the number of microtubules was not noticed untill cell swelling become evident; it was noticed after 5 days.

GA_3 acted antagonistic to S-3307. The lateral cell expansion caused by 1×10^{-4}M S-3307 was greatly reduced either by simultaneous GA_3 (1×10^{-4}M) application or by prior GA_3 (1×10^{-4}M) application (Table 2). In some experiments, simultaneous GA_3 application or prior GA_3 application did not antagonize S-3307 sufficiently, but the combination of these two treatment always gave satisfactory results. Seedlings pretreated with GA_3 (1×10^{-4}M) for 2 days and then with

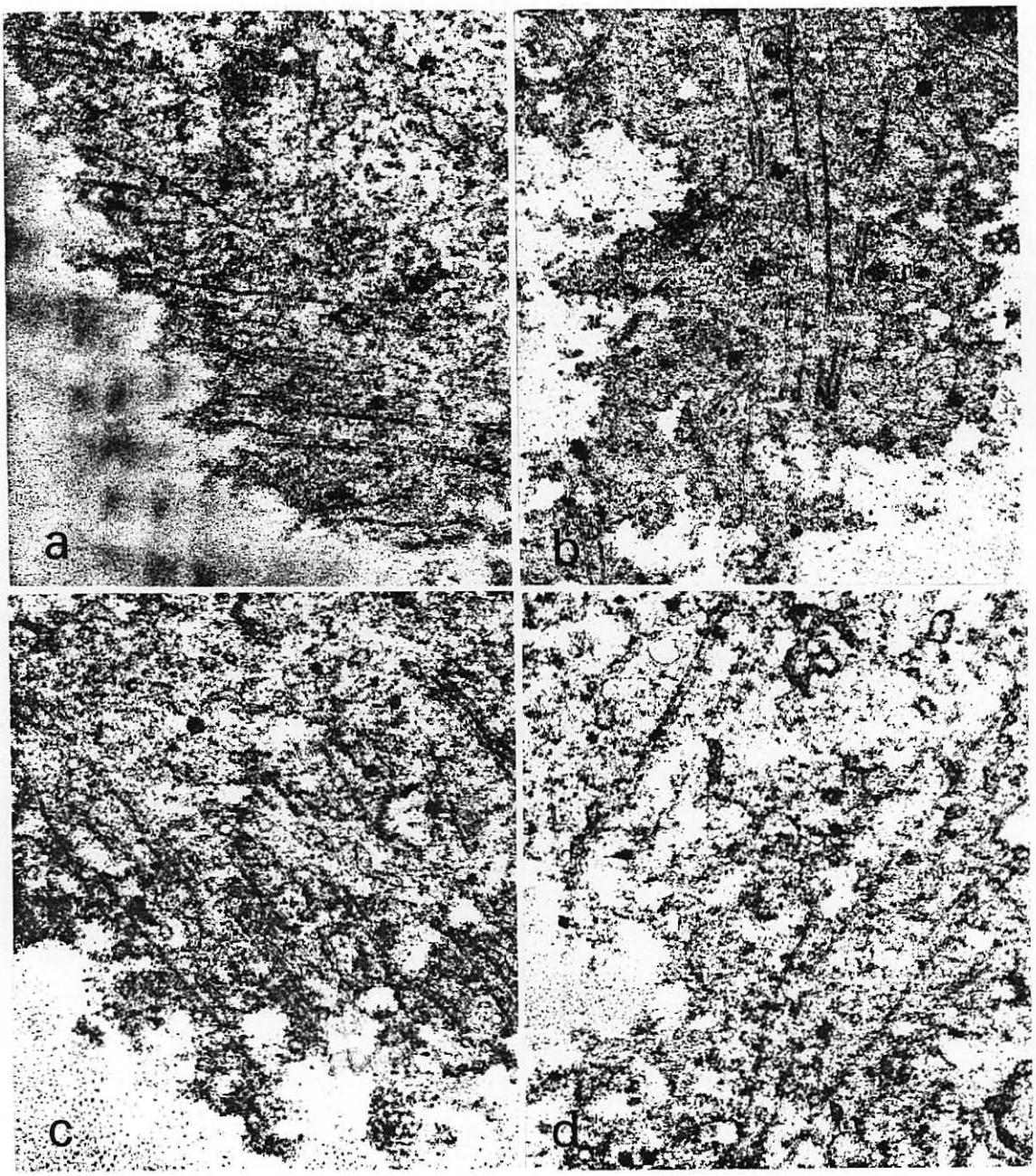


Fig. 3. Effect of S-3307 on the arrangement of microtubules against the face of the outer tangential wall of leaf sheath epidermal cells of onion seedlings grown under long-day conditions.

a. A cell located 1.5 mm above the basal end of the 2nd leaf of water-treated seedling sampled 5 days after the start of treatment. Microtubules are oriented transversely to the cell axis.

b., c., and d. Cells located 1.5 mm above the basal end of 2nd leaves of S-3307 (1×10^{-4} M) treated seedlings sampled 2 days (b), 3 days (c), and 5 days (d) after the start of treatment. Microtubules are oriented parallel or obliquely to the cell axis.

X 20000

GA_3 (1×10^{-4}M) + S-3307 (1×10^{-4}M) showed no or only small swelling (Fig. 1 and 2). GA_3 reversed inhibition of leaf growth by S-3307 when S-3307 was used at 1.7×10^{-5}M, but did not when S-3307 was used at 1×10^{-4}M.).

Experiments under short-day conditions.

S-3307 at 1×10^{-4}M did not cause noticeable swelling of basal parts of leaf sheaths of seedlings grown under short-day conditions (Fig. 4, 5, and Table 3), while it suppressed leaf growth of the same seedlings (Fig. 4). Inhibiting effect of S-3307 on leaf growth was also observed at 1.7×10^{-5}M and 3.3×10^{-5}M where S-3307 showed no effect on lateral cell expansion. At 1.7×10^{-4}M S-3307 was toxic to seedlings.

Changes in the arrangement of cortical microtubules by S-3307 which was clearly obserbed in seedlings grown under long-day conditions did not occur in seedlings grown under short-day conditions (Fig. 6).

Discussion

S-3307 which recently had been found to inhibit gibberellin biosynthesis (Izumi et al., 1984) caused changes in the arrangement of microtubules in onion leaf sheath cells and made the basal parts of onion seedlings bulbous. As the effect of S-3307 on cell swelling was reversed by gibberellin, we may consider that the effect of

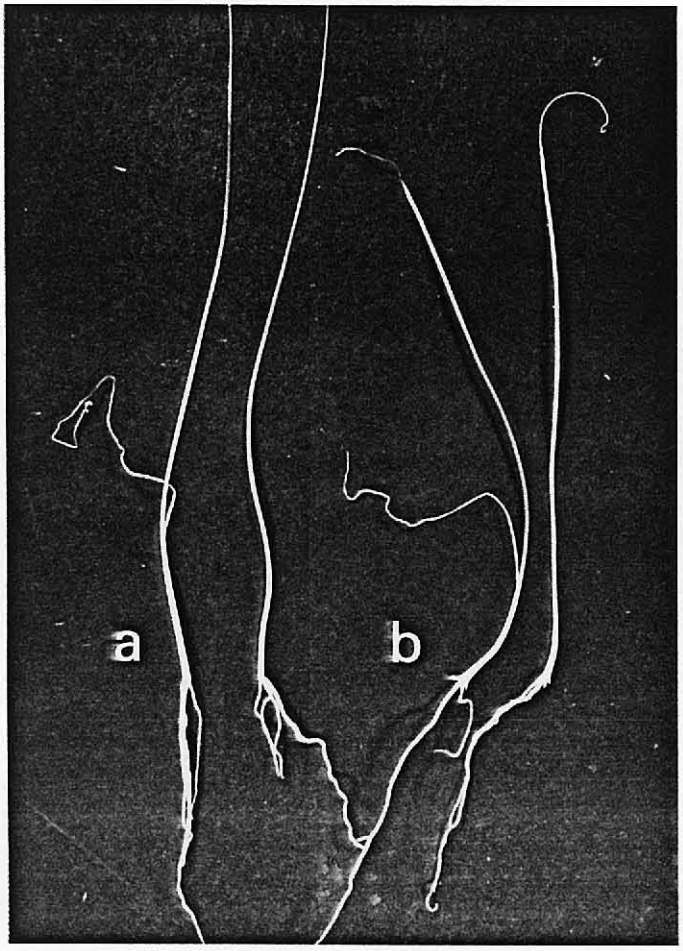


Fig. 4. Inconspicuous effect of S-3307 on swelling of the basal parts of onion seedlings grown under short-day conditions.

a. Control.

b. S-3307 (1×10^{-4}M).

The 1st leaf was removed from the right side seedling of each pair. The photograph was taken 10 days after the start of treatment.

× 0.83

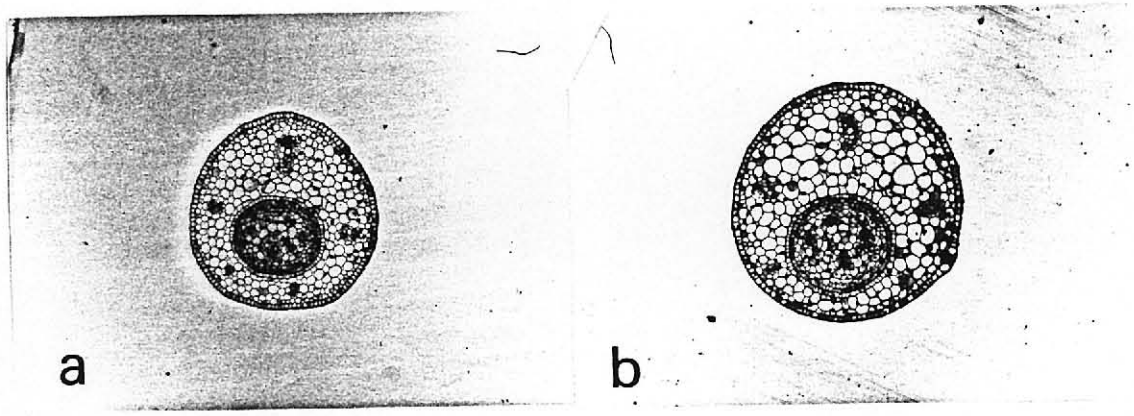


Fig. 5. Inconspicuous effect of S-3307 on swelling of the basal part of a seedling grown under short-day conditions. Transverse sections of the 2nd leaves were cut 2 mm above their basal ends at 10 days after the start of treatment. The blades of the 3rd leaves are seen surrounded by the sheaths of the 2nd leaves.

a. Control.

b. S-3307 (1×10^{-4} M).

$\times 35$

Table 3. Inconspicuous effect of S-3307 on lateral expansion of leaf sheath cells of onion seedlings grown under short-day conditions.

Days after treatment	Cross-sectional area of cell (μm^2)*			
	Epidermal cells		2nd layer cells	
	Control	S-3307($1X10^{-4}M$)	Control	S-3307($1X10^{-4}M$)
2	254±5 (100)	240±4 (94)	372±22 (100)	363±21 (98)
3	252±8 (100)	260±8 (103)	366±26 (100)	368±30 (100)
5	290±4 (100)	320±8 (110)	358±22 (100)	422±43 (118)
10	406±10(100)	484±18(119)	592±38 (100)	682±50 (115)

* Average of 40 cells with standard error. In parenthesis, percent of that of control.

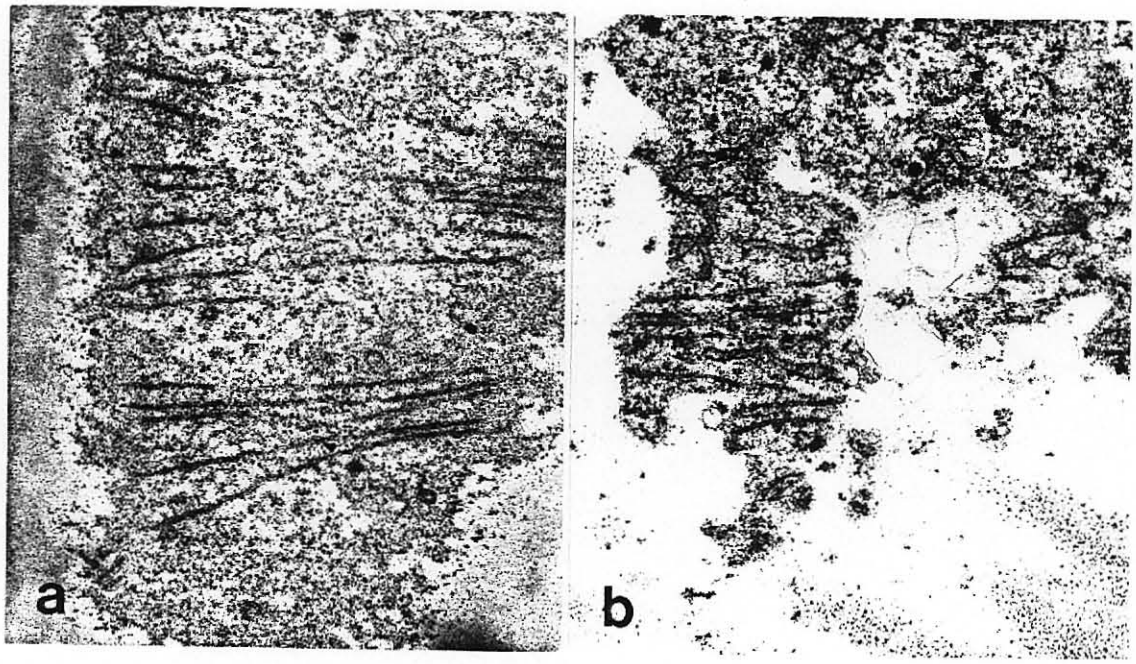


Fig. 6. Ineffectiveness of S-3307 in changing arrangement of microtubules against the face of the outer tangential wall of a leaf sheath epidermal cell of an onion seedling grown under short-day conditions.

a. A cell located 1.5 mm above the basal end of 2nd leaf of water-treated seedling sampled 5 days after the start of treatment. Microtubules are oriented transversely to the cell axis.

b. A cell located 1.5 mm above the basal end of 2nd leaf of S-3307-treated seedling sampled 5 days after the start of treatment. Microtubules are oriented transversely to the cell axis.

X 20000

S-3307 on cell swelling was brought about by its action to inhibit gibberellin biosynthesis. The results that S-3307 acted conversely to gibberellin in arranging cortical microtubules, i.e. the former arranged microtubules parallel or obliquely to the cell axis while the latter caused a predominance of transversely oriented microtubules (Mita and Shibaoka, 1984), may indicate that endgenous gibberellin are playing an important role in arranging cortical microtubules transversely to the cell axis.

What onion seedlings showed in response to S-3307 bore many resemblances to what they did in response to root excision (chapter II) as shown below.

1) Both S-3307 and root excision showed their effects on microtubule arrangement and cell swelling only in seedlings grown under long-day conditions; neither changes in microtubule arrangement nor cell swelling was observed in S-3307-treated or root-excised seedlings grown under short-day conditions.

2) Epidermal cells as well as cortical cells showed swelling both in leaf sheaths of S-3307-treated seedlings and in those of root-excised seedlings, while only cortical cells showed swelling in leaf sheaths of plant whose bulb formation was induced by seasonal changes in daylength (Mita and Shibaoka, 1983).

3) Both S-3307 and root excision caused changes in the arrangement of cortical microtubules in leaf sheath cells without decreasing the number of microtubules.

4) Changes in the arrangement of cortical microtubules was observed in leaf sheath epidermal cells both in S-3307-treated seedlings and in root excised-seedlings, while it was not observed in epidermal cells of leaf sheaths of plants whose bulb formation was induced by seasonal changes in daylength (Mita and Shibaoka, 1983).

Above mentioned resemblances between S-3307's action and root-excision's would lend support to the hypothesis which I proposed in the preceding chapter that a substance which is produced by roots and has an ability to supress swelling of leaf sheaths is gibberellin and root excision causes swelling of leaf sheaths by depleting gibberellin in leaf sheaths. Young onion seedlings having a small number of leaves do not form bulbs even when they were kept under long-day conditions. Probably, leaf blades of such young seedlings produce "bulbing hormone" in response to the stimulus of long-day conditions, but the amount of bulbing hormone in leaf sheaths is not sufficient to overcome anti-bulbing action of gibberellin supplied from root system.

Chapter IV

Gibberellin stabilizes microtubules in onion leaf sheath cells

Summary

Colchicine and cremart (O-ethyl O-(3-methyl-6-nitrophenyl)N-sec-butylphosphorothioamidate) disrupt microtubules in leaf sheath cells of onion plants (Allium cepa L. cv. Senshu-Chuko) and cause cell swelling to make the basal parts of the plants bulbous. Gibberellin A_3 (GA_3) protects microtubules from disruption by colchicine and cremart and suppresses the swelling caused by them. GA_3 also protects microtubules from disruption by low temperature.

Introduction

Leaf blades of onion plants have been considered to form a "bulbing hormone" in response to the stimulus of long-day conditions (Heath and Holdsworth 1948). In Chapter I, I showed that microtubules are oriented transversely to the cell axis in leaf sheath cells of onion plants kept under short-day conditions, but as bulbs develop these microtubules become disoriented and scattered and finally disappear. As these results suggested the involvement of microtubules in regulating bulb development in onion plants, I examined the effects of microtubule inhibitors, such as colchicine and cremart (a substance with a structure and function similar to amiprophos-methyl [Sumida and Ueda 1976]), and found that they cause swelling and make the basal parts of the plants bulbous. Based on these results I assumed that the Heath and Holdworth's bulbing hormone was a microtubule-disrupting agent. As the results in Chapter II and III suggested that gibberellin supplied from roots antagonized the bulbing hormone from leaf blades, it is probable that gibberellin antagonizes microtubule disrupting agents.

In fact, colchicine is known as an antagonist of gibberellin. Gibberellin promotes stem elongation and colchicine reverses its effects in azuki bean epicotyls (Shibaoka 1972, 1974), lettuce hypocotyls (Sawhney and Srivastava 1974) and other plant materials (Atsmon and Lang 1968). However, in Cucumis hypocotyls (Das et al. 1966) and

wheat coleoptiles (Fragata 1970), gibberellin reverses the inhibitory effect of colchicine on growth.

As previous results showed that colchicine causes swelling of onion leaf shesths, I examined whether or not gibberellin acts as an antagonist of colchicine both in swelling of onion leaf sheaths and in destruction of microtubules. The interaction between gibberellin and cremart was also studied.

Materials and methods

Seeds of onion plants (Allium cepa L. cv. Senshu-Chuko) were sown in vermiculite and the seedlings were raised at 27°C under short-day conditions (12 hours light / 12 hours dark). Light came from 20-W "Biolux" fluorescent lamps (FL20SBR Sylvania, Tokyo) and the light intensity at plant level was ca. 7.0 W/m^2. About 20 days after sowing, when the 2nd leaf of the seedling was 6-8 cm long, six seedlings were transplanted in a small pot containing 30 g vermiculite moistened with 100 ml distilled water, 1×10^{-4}M gibberellin A_3 (GA_3), 3×10^{-4}M colchicine, 3×10^{-4}M colchicine + 1×10^{-4}M GA_3, 2×10^{-5}M cremart, or 2×10^{-5}M cremart + 1×10^{-4}M GA_3. Cremart (O-ethyl O-(3-methyl-6-nitrophenyl) N-sec-butylphosphorothioamidate) was obtained from Sumitomo Chemical Co. Ltd. (Takatsukasa, Takarazuka, Hyogo, Japan).

Leaf sheath segments, 2.0 mm long, were cut from 1-3 mm

above the basal end of the 2nd leaf of seedlings 2 days after the start of treatment at which time cell swelling had not yet started (Table 1). The sampled tissues were fixed with 2 % paraformaldehyde + 2.5 % glutaraldehyde in 0.025 M potassium phosphate buffer, pH 7.2, for 2 hours and then with 2 % OsO_4 in the same buffer for another 2 hours at room temperature. The fixed tissues were dehydrated with ethanol and embedded in Spurr's resin. Tangential longitudinal sections through the outer tangential wall and outer cytoplasm and longitudinal sections through the junction of the outer tangential wall and radial wall were cut from the outer epidermal cells as shown by lines a and b in Fig. 1 , respectively. The sections were stained with 25 % uranyl acetate in absolute methanol for 35 minutes and then with lead citrate for 4 minutes. The stained sections were examined with an electron microscope (JEM-100, Jeol, Tokyo). As can be seen in Fig. 3 , the thickness of a leaf sheath was not uniform. It was thicker on the side of the blade (upper side in Fig. 3) and thinner on the side of the axis, or the side of the pore (lower side in Fig. 3). Throughout the present work, cells on the side of the leaf blades were examined.

To measure the cross-sectional areas of the leaf sheath cells, resin-embedded tissue was sectioned transversely. The sections were stained with a mixture of toluidine blue, basic fuchsin and sodium borate and examined under a light microscope equipped with an ocular micrometer. The cross-sectional areas of cells were expressed as (tangential width) × (radial width).

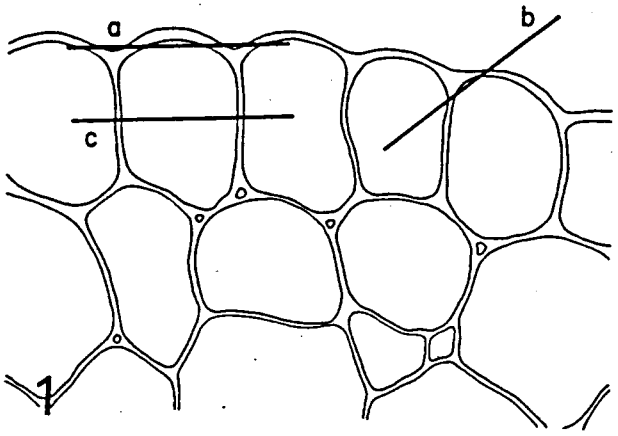


Fig. 1. A sketch of part of a cross-section of onion leaf sheath showing the positions of (a) the longitudinal section through the outer tangential wall and outer cytoplasm, (b) the longitudinal section through the edge between the outer tangential wall and radial wall and (c) the tangential longituginal section through the radial walls.

To measure the orientation of microtubules adjacent to the outer tangential wall of the outer epidermal cells, photographs of glancing tangential sections through the outer tangential wall and outer cytoplasm were taken. The angle of each microtubule to the cell axis was measured on the photograph with a protractor. The results are displayed in the form of a histogram showing the percentage of microtubules running in the direction within the angular ranges 0-10, 10-20 to 80-90 to the cell axis. To make one histogram about 400 microtubules in 20 cells from four different seedlings were examined.

Six onion seedlings having 2nd leaves 6-8 cm long were planted in a pot containing 30 g vermiculite moistened with 100 ml of distilled water or 1×10^{-4} M GA_3. After 2 days of treatment, the pot with the seedlings was transferred to a cold room (1°C) and kept there for 2.5 hours. Leaf sheath segments, 2.0 mm long, were then cut from 1-3 mm above the basal end of the 2nd leaf of seedlings. Microtubules in the outer epidermal cells of the sampled leaf sheaths were examined according to the procedure described above, except that fixation was carried out at 1°C.

Results

Antagonism between GA_3 and microtubule inhibitors in swelling of onion leaf sheath cells

GA_3 antagonized colchicine and cremart in causing the swelling.

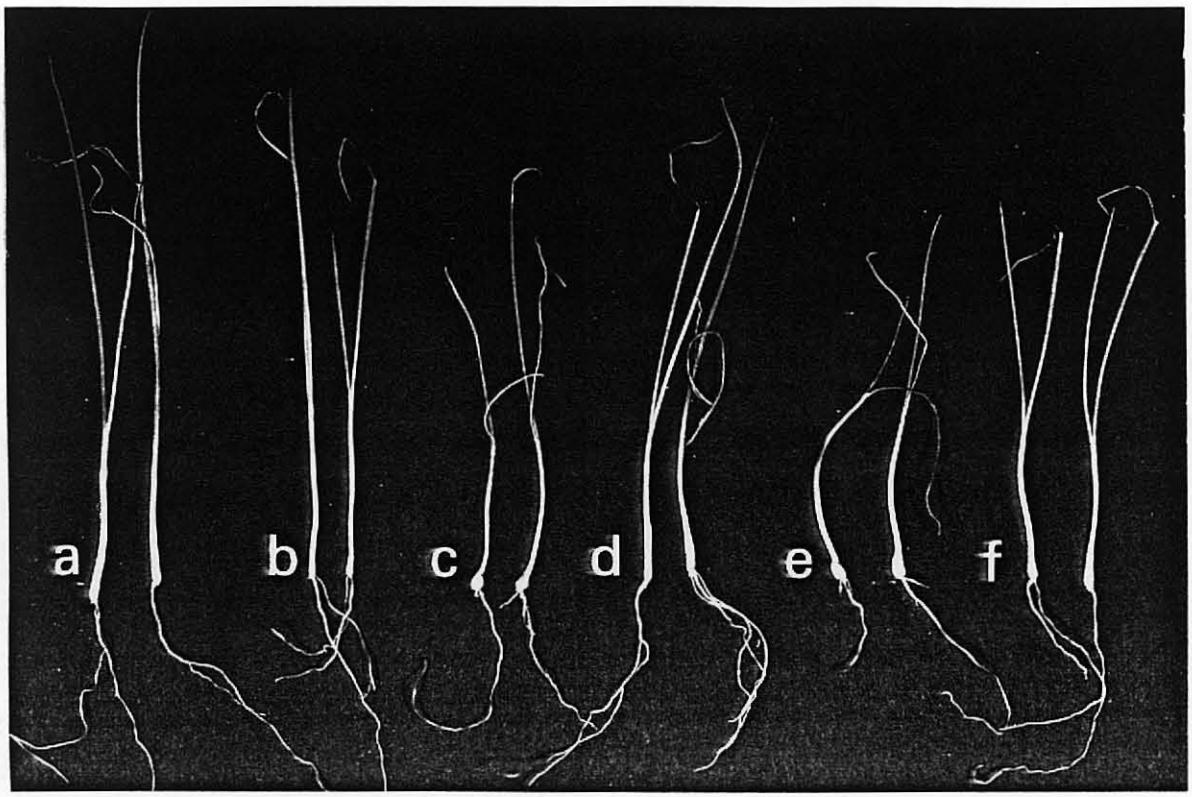


Fig. 2. Antagonism between GA_3 and microtubule inhibitors in swelling of the basal parts of onion seedlings.

a. Control

b. GA_3 (1×10^{-4}M)

c. Colchicine (3×10^{-4}M)

d. Colchicine (3×10^{-4}M) + GA_3 (1×10^{-4}M)

e. Cremart (2×10^{-5}M)

f. Cremart (2×10^{-5}M) + GA_3 (1×10^{-4}M)

The photograph was taken 9 days after the start of treatment.

× 0.5 Bar = 5 cm

The basal parts of the onion seedlings treated with colchicine or cremart became bulbous (Figs. 2c and e), but not those of seedlings treated with colchicine + GA_3 or cremart + GA_3 (Figs. 2d and f). GA_3 did not promote leaf elongation in onion seedlings.

Figs. 3a and c show that colchicine-induced swelling was brought about by lateral expansion of leaf sheath cells. Colchicine increased the cross-sectional areas of epidermal cells and those of cells just beneath the epidermis at almost the same rate. The cross-sectional areas of both epidermal cells and cells just beneath the epidermis of colchicine-treated seedlings were about 8 times as large as those of the corresponding cells of water-treated seedlings (Table 1). GA_3 alone at 1×10^{-4}M slightly promoted lateral cell expansion, but it almost completely reversed the effect of 3×10^{-4}M colchicine (Figs. 3c and d, and Table 1).

Cremart also brought about lateral expansion of leaf sheath cells. Cremart at 2×10^{-5}M showed a greater effect than 3×10^{-4}M colchicine on lateral cell expansion. The cross-sectional areas of cells from cremart-treated seedlings were about 12 times those of the corresponding cells from water-treated seedlings (Table 1). GA_3 at 1×10^{-4}M partially reversed the effect of 2×10^{-5}M cremart on lateral cell expansion.

The results in Table 1 and Fig. 3 were obtained with cells about 2 mm above the basal end of the 2nd leaf sheath. I also examined cells near the basal and the upper ends of the leaf sheath. Promoting effects

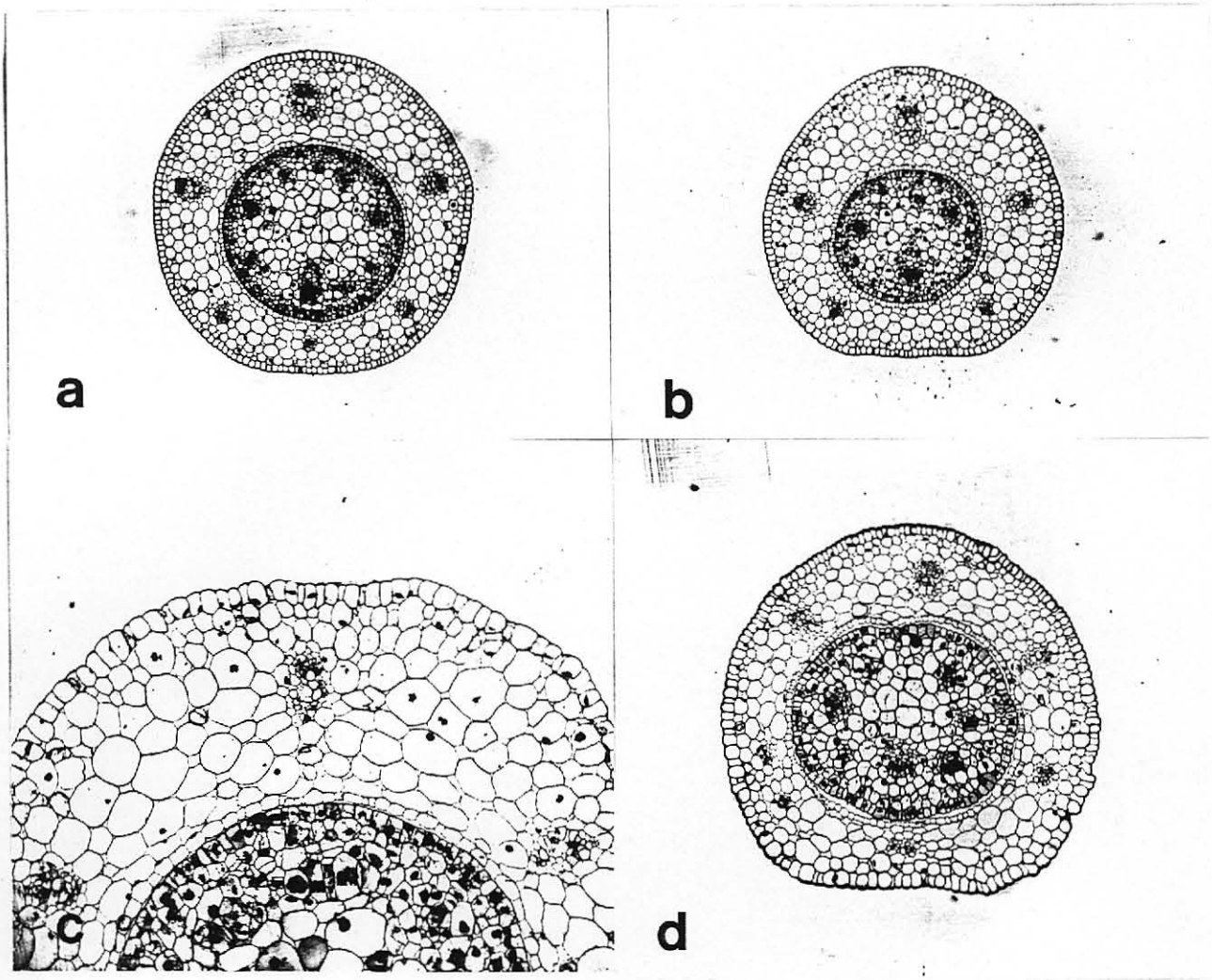


Fig. 3. Antagonism between GA_3 and colchicine in lateral expansion of onion leaf sheath cells. Transverse sections of the leaf sheaths of 2nd leaves were cut 2 mm above their basal ends 9 days after the start of treatment. The blades of the 3rd leaves are seen surrounded by the sheaths of the 2nd leaves.

a. Control

b. GA_3 (1×10^{-4} M).

c. Colchicine (3×10^{-4} M).

d. Colchicine (3×10^{-4} M) + GA_3 (1×10^{-4} M).

× 52 Bar = 500 um.

Table 1. Antagonism between microtubule inhibitors and GA_3 in lateral cell expansion of onion leaf sheath cells

Days after the start of treatment	Treatments	Cross sectional area of cell (μm^2)*	
		Epidermal cell	2nd layer cell**
2	water	224 ± 5	300 ± 21
	colchicine (3×10^{-4}M)	222 ± 5	335 ± 27
	cremart (2×10^{-5}M)	229 ± 5	359 ± 23
9	water	263 ± 6	385 ± 25
	GA_3 (10^{-4}M)	310 ± 7	489 ± 29
	colchicine (3×10^{-4}M)	2,343 ± 128	3,343 ± 270
	" + GA_3 (10^{-4}M)	360 ± 6	457 ± 29
	cremart (2×10^{-5}M)	3,116 ± 202	4,629 ± 475
	" + GA_3 (10^{-4}M)	1,078 ± 66	1,035 ± 66

* Cells 2 mm above the leaf sheath of the 2nd leaf were examined. Average of 45 cells with standard error of the mean. Cells on the side of the leaf blade were examined. Expressed as (tangential width) X (radial width).

** Cell just beneath the epidermis.

of colchicine and cremart on lateral cell expansion and the antagonistic effect of GA_3 were observed also in these cells.

Arrangement of microtubules in onion leaf sheath epidermal cells

Microtubules against the face of the outer tangential wall of onion leaf sheath cell ran transverse or nearly transverse to the cell axis (Fig. 10 a). They were traced from eight adjacent serial sections through the outer tangential wall and outer cytoplasm (Fig. 4). As it is difficult to follow microtubules from the longitudinal view from one section to another (cf., Hardham and Gunning 1978), Fig. 4 only shows the orientations of microtubules and no significance is to be attached to their lengths. This also applies to Figs. 5,6, and 7. Although the transversely oriented microtubules were predominant, some obliquely oriented microtubules were also present. The frequency distribution of microtubule orientation is shown in Fig. 11.

Transversely oriented microtubules were predominant also near the faces of the radial walls of epidermal cells. Near the inner tangential wall, however, obliquely oriented microtubules were frequently observed. Seemingly, this arises from the fact that most of the inner tangential walls have edges at the middle of their faces. (Fig. 3).

Microtubules near the edges of cells were randomly oriented. Fig. 5, which was constructed from eight serial sections through the edge between the outer tangential wall and the radial wall of an

epidermal cell (three of them are shown in Fig. 8), shows longitudinally oriented microtubules as well as obliquely and transversely oriented microtubules. Complexes from which microtubules fanned out, like those reported by Gunning et al. (1978) in Azolla root cells fixed shortly after completion of cytokinesis, were not observed along the edges of onion leaf sheath epidermal cells undergoing cell expansion.

Effect of GA_3 on microtubule arrangement

As shown in Figs. 6 and 10b, microtubules running transverse to the cell axis were predominantly observed in cells from GA_3-treated seedlings as in cells from water-treated seedlings. But detailed examination of the directions of the microtubules revealed that their angular frequency distribution in cells from GA_3-treated seedlings was different from that of microtubules in cells from water-treated seedlings. In cells from GA_3-treated seedlings, almost all microtubules (80 %) were running strictly transverse (80-90°) to the cell axis (Fig. 11). But in cells from water-treated seedlings, they ran in a relatively wide range of directions, though a majority (65 %) ran transverse or nearly transverse (60-90°) to the cell axis. An array of microtubules near the edge of a leaf sheath epidermal cell from a GA_3-treated onion seedling (Fig. 7) was reconstructed from five adjacent serial sections through the edge between the outer tangential wall and the radial wall of the cell (three are shown in Fig. 9). Microtubules

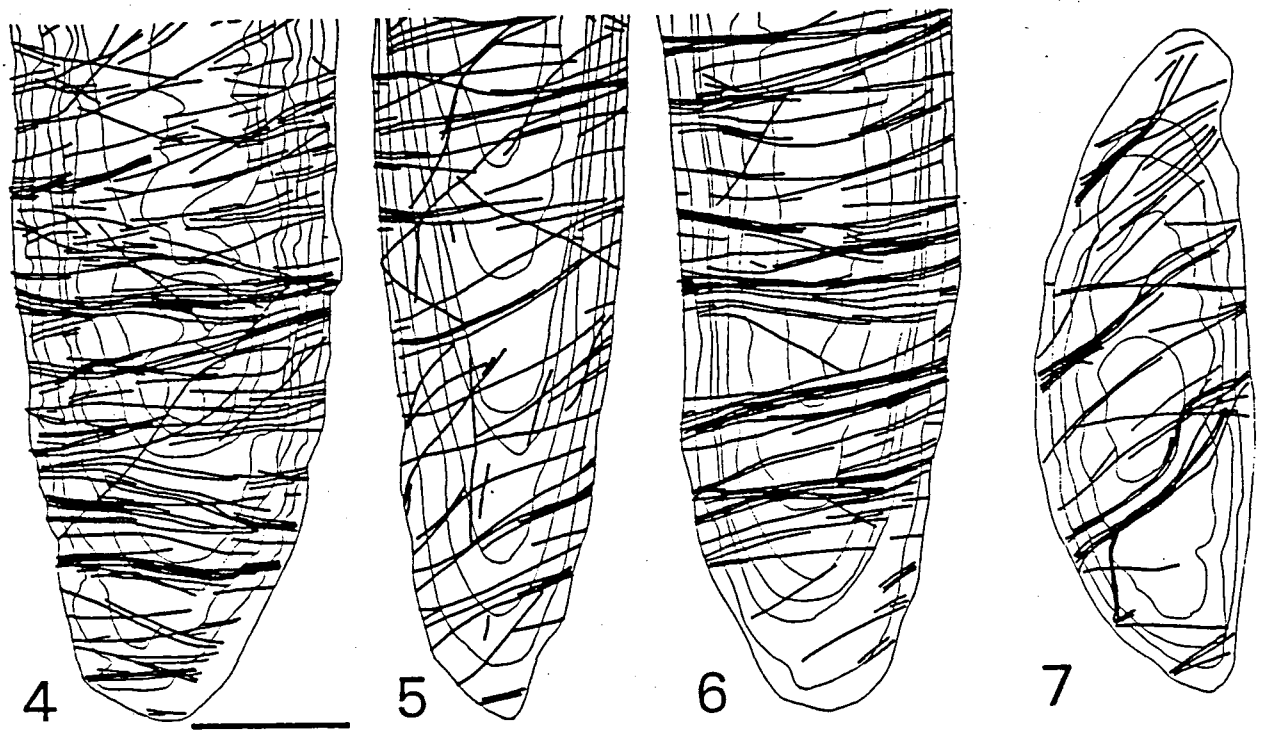


Fig. 4. Arrangement of microtubules against the face of the outer tangential wall of a leaf sheath epidermal cell from a water-treated onion seedling. Microtubules were traced from 8 adjacent serial sections cut from a cell 2 mm above the basal end of the 2nd leaf sampled 2 days after the start of treatment.

Fig. 5. Arrangement of microtubules near the edge between the outer tangential wall and the radial wall of a leaf sheath epidermal cell from a water-treated onion seedling. Microtubules were traced from 8 adjacent serial sections cut from a cell 2 mm above the basal end of the 2nd leaf sampled 2 days after the start of treatment.

Fig. 6. Arrangement of microtubules against the face of the outer tangential wall of a leaf sheath epidermal cell from a GA_3 ($1X10^{-4}M$)-treated onion seedling. Microtubules were traced from 7 adjacent serial sections cut from a cell 2 mm above the basal end of the 2nd leaf sampled 2 days after the start of treatment.

Fig. 7. Arrangement of microtubules near the edge between the outer tangential wall and radial wall of a leaf sheath epidermal cell from a GA_3 ($1X10^{-4}M$)- treated seedling. Microtubules were traced from 5 adjacent serial sections cut from a cell 2 mm above the basal end of the 2nd leaf sampled 2 days after the start of treatment.

Fig. 4. to 7., cell axis is parallel to the length of the page.
X 6900 Bar = 3 μm

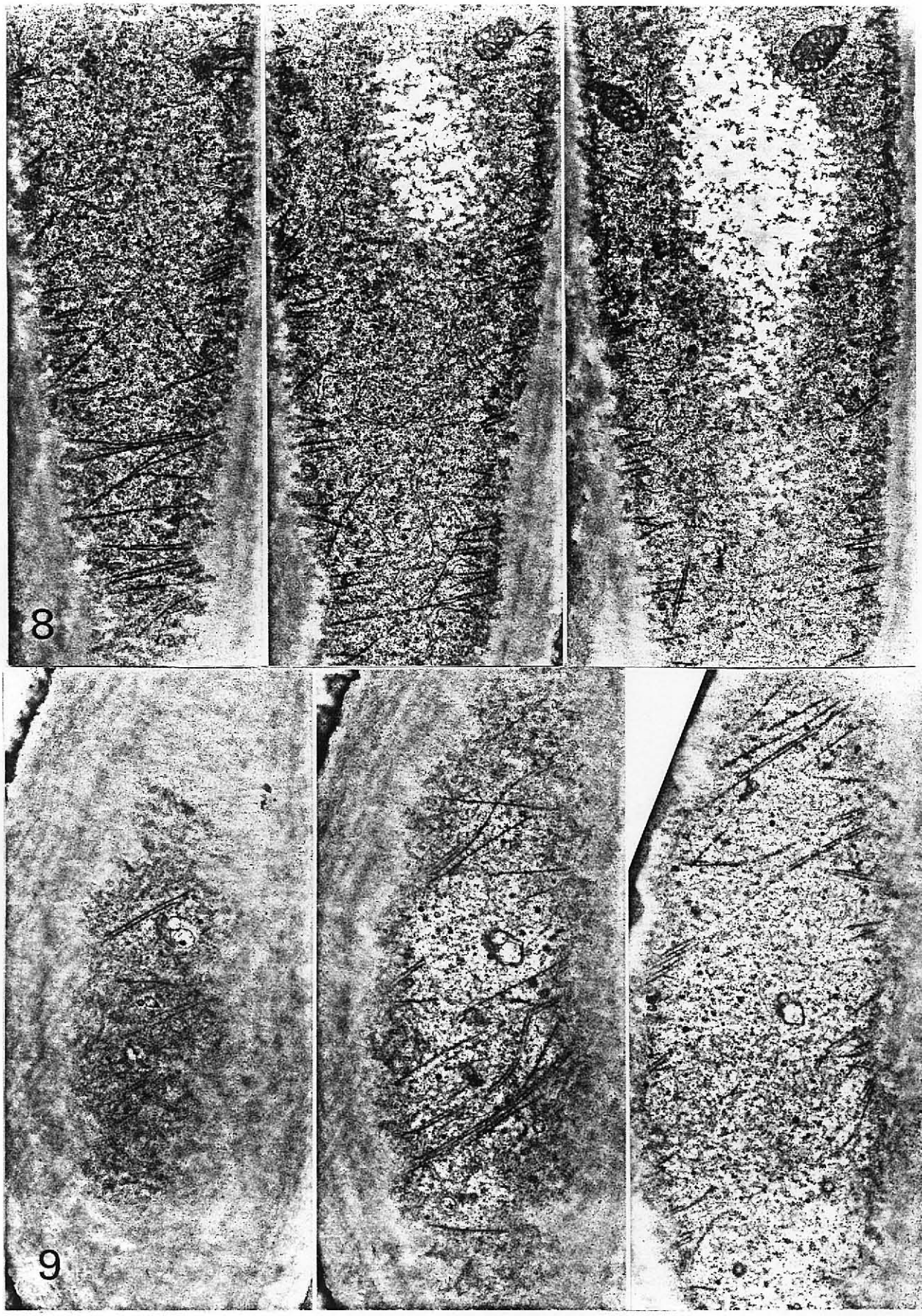


Fig. 8. Three adjacent serial sections through the edge between the outer tangential wall and the radial wall of a leaf sheath epidermal cell from a water-treated onion seedling. Sections were cut from a cell 2 mm above the basal end of the 2nd leaf sampled 2 days after the start of treatment.

Fig. 9. Three adjacent serial sections through the edge between the outer tangential wall and the radial wall of a leaf sheath epidermal cell from a GA_3 (1×10^{-4} M)-treated onion seedling. Sections were cut from a cell 2 mm above the basal end of the 2nd leaf sampled 2 days after the start of treatment.

Cell axis is parallel to the length of the page.
X 12100 : bar = 2 μm.

running continuously from one face to another were present. In most cases, their orientation near the cell edge was different from their orientation near the faces. They were longitudinally oriented near the edge. No complexes from which microtubules were fanning out were observed.

Antagonism between GA_3 and microtubule inhibitors in microtubule disruption

Both colchicine and cremart disrupted microtubules. Only a small number of microtubules were observed cells from colchicine- or cremart-treated seedlings (Fig. 10c and e). The number of microtubules in cells from seedlings treated with 2×10^{-5} M cremart was much smaller than that in cells from seedlings treated with 3×10^{-4} M colchicine, suggesting that cremart more effectively disrupted microtubules. Interestingly, GA_3 protected microtubules from disruption by colchicine and cremart. Transversely oriented microtubules were abundant in cells from seedlings treated with colchicine + GA_3 or cremart + GA_3 (Fig. 10 d and f). As I examined cells which had not yet started to swell, the changes in microtubule arrangement (Fig. 10) might not be results of cell expansion.

Microtubule-disrupting effects of colchicine and cremart and the antagonistic effect of GA_3 were observed also in cells just beneath the epidermis (data not shown).

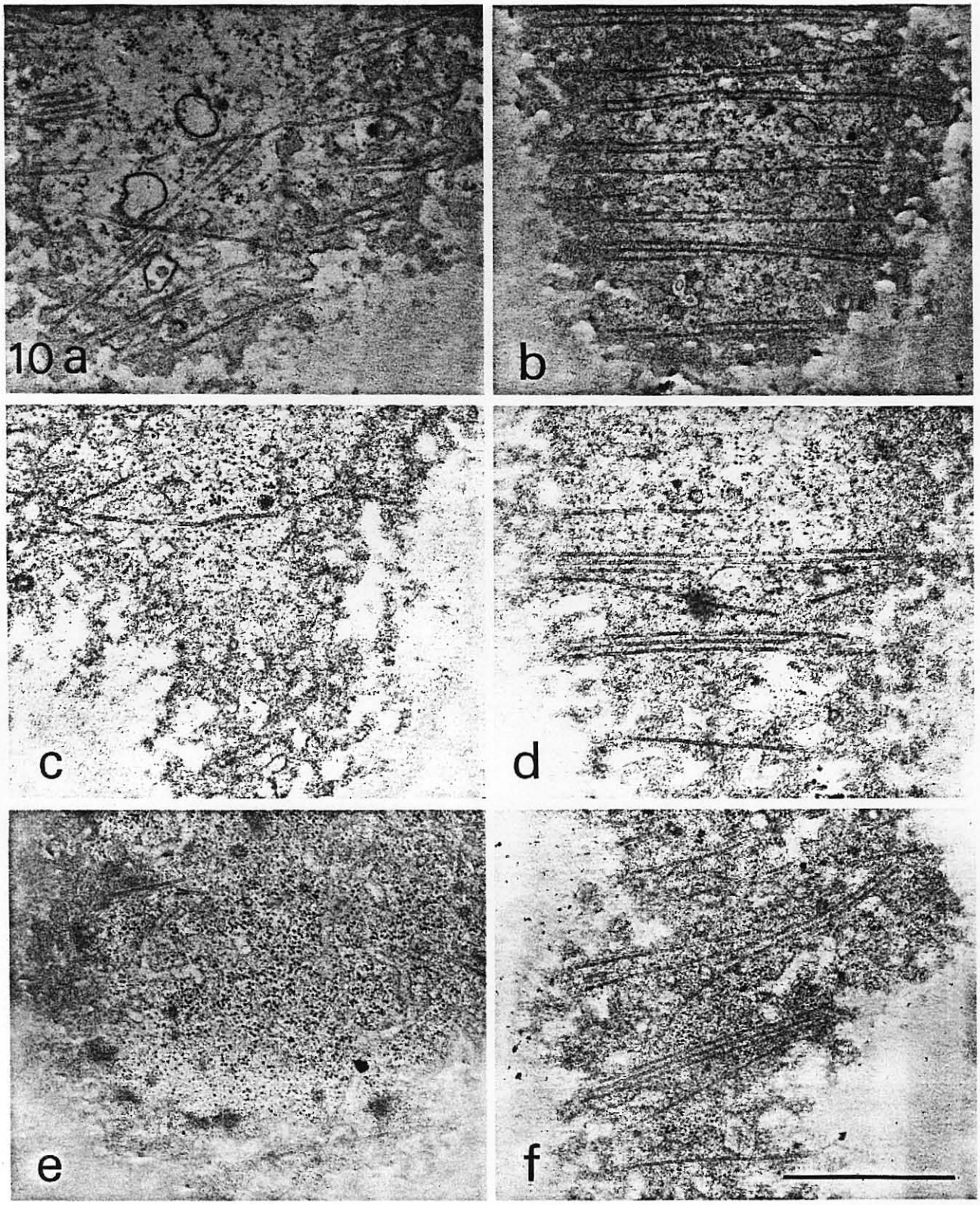


Fig. 10. Colchicine- and cremart-induced microtubule disruption and its prevention by GA_3 in onion leaf sheath cells. Tangential longitudinal sections through the outer tangential wall and outer cytoplasm were cut from epidermal cells 2 mm above the basal end of the 2nd leaf sampled 2 days after the start of treatment. a. control; b. GA_3 (1×10^{-4} M); c. colchicine (3×10^{-4} M); d. colchicine (3×10^{-4} M) + GA_3 (1×10^{-4} M); e. cremart (2×10^{-5} M); f. cremart (2×10^{-5} M) + GA_3 (1×10^{-4} M).
Cell axis is parallel to the length of the page.
X 30000 : bar = 1 um

Microtubule-stabilizing effect of GA_3

As the results in Fig. 10 indicated that GA_3 protected microtubules from disruption by colchicine and cremart, I examined whether it would also protect them from disruption by low temperature.

No microtubules or only a small number of them were observed in cells from about 40 % of the seedlings pretreated with water for 2 days and kept at 1°C for 2.5 hours (Fig. 12 a). However, cells from about 60 % of the low-temperature-treated seedlings had a considerable number of microtubules, although they were not clearly outlined (Fig. 12 b). Tangential longitudinal sections through the radial walls of cells with microtubules near the outer tangential wall that were not clearly outlined were cut as shown by line c in Fig. 1. The sections revealed that microtubules in low-temperature-treated cells were C-shaped from the cross-sectional view (Fig. 12 c).

GA_3 protected microtubules from disruption by low temperature. Abundant microtubules were observed in cells from the seedlings which were pretreated with GA_3 for 2 days and then kept at 1°C for 2.5 hours. They were clearly outlined from the longitudinal view (Fig. 12 d) and showed normal profiles from the cross-sectional view (Fig. 12 e).

Discussion

Gibberellin protected microtubules in onion leaf sheath cells from disruption by colchicine and cremart and, probably by this action,

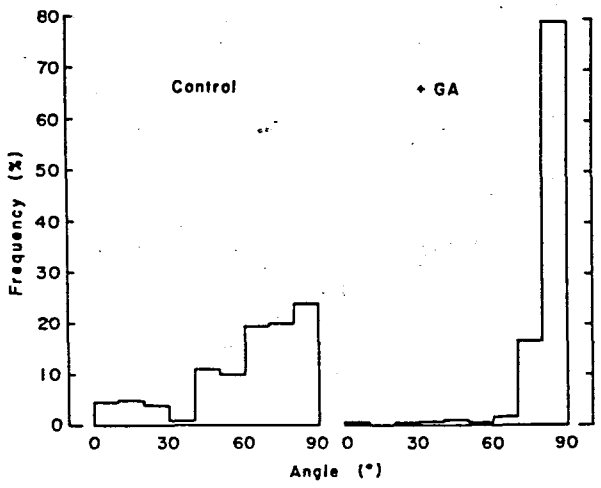


Fig. 11. Effect of GA_3 on the arrangement of microtubules adjacent to the outer tangential walls of onion leaf sheath epidermal cells. Cells 2 mm above the basal end of the 2nd leaf sampled 2 days after the start of treatment were examed. The histogram shows the percentage of microtubules running in the direction within the angular ranges of 0-10, 10-20 to 80-90° to the cell axis.

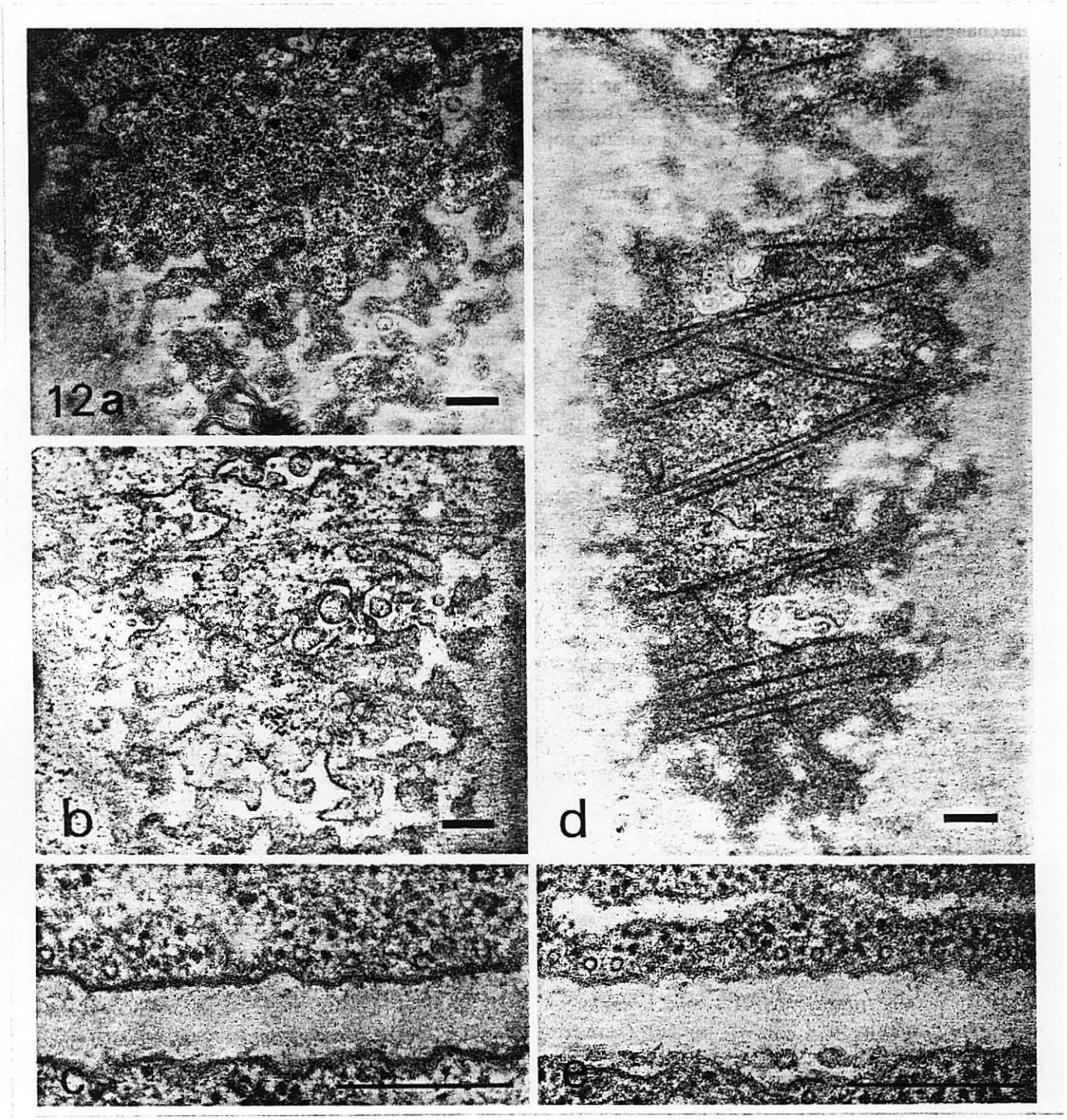


Fig. 12. Low-temperature-induced microtubule disruption and its prevention by GA_3 in onion leaf sheath cells. Seedlings were pretreated with water or GA_3 ($1X10^{-4}$ M) for 2 days and then kept at low temperature (1°C) for 2.5 hours. Outer epidermal cells 2 mm above the basal ends of the 2nd leaves were examed.
a. and b. Tangential longitudinal sections through the outer tangential walls and outer cytoplasm of cells from water-pretreated seedlings. X 30000.
c. Tangential longitudinal sections through the radial wall of a water-treated cell with microtubules near the outer tangential wall that are not clearly outlined. X 100000. d. Tangential longitudnal section through the outer tangential wall and outer cytoplasm of a cell from a GA_3-pretreated seedlings. X 30000.
e. Tangential longitudinal section through the radial wall of a cell from a GA_3-pretreated seedling. X 100000.

Cell axis in a.,b., and d. are parallel to the length of the page.
Bar = 0.3 um.

suppressed the lateral expansion caused by these microtubule inhibitors. As colchicine and cremart are considered to disrupt microtubules by different mechanisms (Gunning and Hardham 1982), it seems unlikely that gibberellin would protect microtubules by interfering with the actions of these two differently acting agents. Gibberellin probably protected microtubules by stabilizing them. The result that gibberellin protected microtubules from disruption by low temperature may support this idea. Recently, Durnam and Jones (1982) reported that the number of microtubules in cells of lettuce hypocotyl segments decreased during incubation of the segments in water, but not when the segments were incubated in gibberellin solution. This result also indicates that gibberellin stabilizes microtubules.

The result that gibberellin stabilized microtubules agrees with reports that gibberellin reversed colchicine-induced growth inhibition (Das et al. 1966., Fragata 1970), but not with reports that colchicine reversed gibberellin-induced stem elongation (Atsmon and Lang 1968, Shibaoka 1972, Sawhney and Srivastava 1974). Fragata (1970) reported that gibberellin reversed the effect of colchicine when the level of colchicine in the tissue was low, but not when it was high. Probably, gibberellin always stabilized the microtubules, but colchicine reverses its effects when enough amount is present to disrupt the stabilized microtubules. Whether or not gibberellin protects microtubules in plant materials in which colchicine reverses the effect of gibberellin can be found by testing gibberellin ability to protect

microtubules from disruption by low temperature.

Low temperature treatment has been reported to increase the frequence of C-shaped terminations of microtubules in Azolla root cells (Hardham and Gunning 1978). In onion leaf sheath cells, the effect of low temperature seems not to be restricted to the terminations. As normal microtubule cross sections are hard to observe in low-temperature-treated onion leaf sheath cells, the C-shaped profile seems to extend over the entire lenghts of the microtubules. In cells from low-temperature-treated seedlings, C-shaped or no microtubules were observed, suggesting that the C-shaped microtubules were associated with disassembly in onion leaf sheath cells as in Cu^{2+}-treated Heliozoan axopodium (Roth and Shigenaka 1970).

I found that the microtubules in cells from gibberellin-treated seedlings run in the direction strictly transverse to the cell axis, while those in cells from water-treated seedlings they run in a relatively wide range of directions. According to the concept of Gunning et al. (1978), transverse microtubule arrays might develop by activation of microtubule-organizing centers (MTOCs) at the longitudinal cell edges. This concept leads me to the hypothesis that gibberellin activates MTOCs along the longitudinal cell edges. To check this hypothesis, I examined the microtubule arrangement near the longitudinal edges of cells from both water- and gibberellin-treated seedlings. I found no MTOC complex having the morphology reported by Gunning et al. (1978). This is probably because I did not examine the cells in the postcytokinesis

phase when the complexes would have been most conspicuous (Gunning et al. 1978).

Although I could not find the MTOC complex, the fact that microtubules near cell edges are more stable than those near the faces of the walls (Mita and Shibaoka 1983) still suggests the presence of MTOCs along the cell edges. A method for locating MTOCs in expanding cells should be devised. It would be also instructive to examine the effect of gibberellin on the microtubule-plasma membrane cross-linking, because microtubules which are cross-linked to the plasma membrane have been thought to be more stable than those which are not (Hepler and Palevitz 1974).

As reported for azuki bean epicotyl cells (Shibaoka 1974), gibberellin caused a predominance of transversely oriented microtubules in onion leaf sheath cells. Thus, gibberellin acted conversely to the stimulus of long-day conditions which reduced the number of microtubules and disturbed their transverse orientations (Mita and Shibaoka 1983). This leads me to the assumption that the changes in microtubule arrangement during bulb development induced by the stimulus of long-day conditions may be brought about by decreasing the endogenous gibberellin content. But, the result in Chapter III that an inhibitor of gibberellin biosynthesis caused disorientation of microtubules in leaf sheath epidermal cells, and that in Chapter I that stimulus of long-day conditions caused no disorientation of microtubules in the epidermal cells although it caused disorientation of microtubules

in cortical cells, indicate that this assumption is not valid. Probably, leaf sheaths of onion plants whose basal parts are swelling in response to the seasonal daylength change are being supplied with sufficient amounts of gibberellin.

General discussion

Results shown in Chapter I through III show that swelling of onion leaf sheaths is always accompanied with changes in the arrangement of cortical microtubules and those in Chapter IV that when the swelling is suppressed by gibberellin the transverse arrangement of cortical microtubules is kept unchanged. These results strongly suggest that cortical microtubules are involved in controlling the onion bulb development. Probably, cortical microtubules control the direction of cell expansion by controlling the direction of newly depositing cellulose microfibrils also in onion plants. But, to make clear whether or not cortical microtubules are really involved in controlling bulb development, the direction of cellulose microfibrils in the innermost layer of the cell wall should be examined. Isodiametrical cell expansion has been known to occur when cells declease their activities in cellulose biosynthesis (Hara et al., 1973). The change in the activity in cellulose biosynthesis during onion bulb development should, therefore, be examined.

Except in the case in which swelling is caused by microtubule-disrupting agents, the swelling causes only under long-day conditions. A "bulbing hormone" which is considered to be produced only under long-day conditions seems to be essential for bulb formation to occur (Heath and Holdsworth, 1948). Although the existence of bulbing hormone has not been convincingly demonstrated, the fact that the

stimulus of long-day conditions is received by leaf blades, while swelling causes in leaf sheath strongly suggest the existence of such hormone. Isolation and characterization of such bulbing hormone is indispensable for clarifying the mechanism in which long-day conditions causes onion bulb development. Isolation of physiologically active substances can not be schieved without establishing an appropriate bioassay method. In establishing the bioassay method, the results in Chapter II and III will be useful. The result that intact young seedling whose leaf sheaths are sufficiently supplied with gibberellin do not show swelling even under long-day conditions seems to indicate that we can not use intact seedling as plant materials in assaying bulbing hormone; endogenous gibberellin will suppress the effect of exogenously supplied bulbing hormone. We should use root-excised seedling for assaying bulbing hormone.

As bulb development caused by the stimulus of long-day conditions accompanied with the decrease in the number of cortical microtubules, bulbing hormone must have an ability to disrupt microtubules or to inhibit microtubule polymerization. Thus, possibly, the method for detecting microtubule-disrupting agents will be utilized for detecting bulbing hormone.

The stimulus of long-day conditions seems to induce the production of dormancy inducing substance, because onion bulbs formed under long-day conditions do not start growth for about 40 to 50 days after the completion of bulb development (Aoba, 1964).

Many kinds of dormancy-inducing substances have already been isolated from plant tissues. Abscisic acid is well known dormancy-inducing substance in winter-resting buds of woody plants (Wareing and Saunders, 1971). A growth inhibitor isolated from Lunularia cruciata, lunularic acid (Valio and Schwabe, 1970), is supposed to regulate summer dormancy of this liverwort (Schwabe and Nachnony-Bascomb, 1963). A substance which has a structure similar to that of lunularic acid, batatasin III (Hashimoto et al., 1974),has been isolated from dormant bulbils of Dioscorea batatas and is supposed to induce dormancy of the bulbils (Hasegawa and Hashimoto, 1973). From dormant bulbs of Narcissus (Piozzi et al., 1968) and Lycoris (Okamoto et al., 1968), a growth inhibitor has been isolated and named lycoricidinol. An antimitotic activity has been reported with this inhibitor (Piozzi et al. 1968., Okamoto et al., 1968).

As bulb or bulbil formation is always accompanied with the onset of dormancy, it is tempting to speculate that dormancy-inducing substances have an ability to initiate bulb or bulbil formation. Thus, it seems worthwhile for us to examine whether or not well-known dormancy-inducing substances have an ability to induce onion bulb formation.

Root excision caused swelling of leath sheath cells of onion seedlings (Chapter II). The results shown in Chapter III and IV suggest that roots suppress the swelling by supplying leaf sheaths with gibberellin. Although the wide distribution of gibberellins in plant

kingdom has well been established, little has been studied with gibberellin in onion plants. No information, therefore, is available as to what kinds of gibberellins are present in onion tissues and by what tissues are onion gibberellins produced. Studies on gibberellins and gibberellin metabolism in onion plants are needed to clarify the mechanism by which onion plants control bulb development.

Although detailed studies on onion gibberellin have to be made before we conclude that endogenous gibberellins play an important role in controlling onion bulb formation, the results in Chapter IV clearly show that exogenously supplied gibberellin does control swelling of onion leaf sheaths. Disruption of cortical microtubules by colchicine and cremart brings about swelling of onion leaf sheaths and swelling caused by these drugs is suppressed by exogenously supplied gibberellin. The most important result in Chapter IV, I think, is that gibberellin stabilizes cortical microtubules. Probably, gibberellin suppresses the swelling caused by the microtubule-disrupting agents by stabilizing cortical microtubules. The finding that gibberellin stabilizes cortical microtubules raises a new question, a question of how gibberellin stabilizes cortical microtubules. As mentioned in "Discussion" of Chapter IV, stability of cortical microtubules are considered to be affected by the presence of microtubule-organizing center (MTOCs) and cross-bridges between microtubules and plasma membrane. Thus to answer this question, biochemical studies on proteins which are responsible for MTOC- or cross-linking-activity should be carried out.

We should also examine both in vitro and in vivo whether gibberellin modifies tubulin molecules or not.

Besides such biochemical studies, cytological studies should also be made to examine whether gibberellin increases the number of MTOCs or not. To localize MTOCs, we should examine changes in the arrangement of cortical microtubules at short intervals. For such a study, an indirect immunofluorescence staining method using anti-tubuline antibody will be useful.

As mentioned also in "Discussion" of Chapter IV, the direction of cortical microtubules is supposed to be controlled by MTOCs, thus, the study to localize MTOCs will give us also an answer to the question of how gibberellin arranges microtubules transverse to the cell axis.

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References

Aoba, T. (1964) Studies on bulb formation and dormancy in onion. Bull. Yamagata Univ., Agr. Sci. 4: 265-363.

Atsmon, D. and A. Lang (1968) Gibberellin-induced elongation and meristematic activity. (Abstr.) Isr. J. Bot. 17: 225.

Das, V. S. R., J. V. S. Rao and K. V. M. Rao (1966) Colchicine-gibberellin interaction in the control of elongation of Cucumis hypocotyl. Cur. Sci. 35: 471-472.

Durnam, D. J. and R. L. Jones (1982) The effects of colchicine and gibberellic acid on growth and microtubules in excised lettuce hypocotyls. Planta 154: 204-211.

Fragata, M. (1970) The mitotic apparatus, a possible site of action of gibberellic acid. Natureissenschaften 57: 139.

Green, P. B. (1962) Mechanism for plant cellular morphogenesis. Science 138: 1404-1405.

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

Green, P. B., R. O. Erickson and P. A. Richmond (1970) On the physical basis of cell morphogenesis. Ann. N. Y. Acad. Sci. 175: 712-731.

Gunning, B. E. S. and A. R. Hardham (1982) Microtubules. Annu. Rev. Plant Physiol. 33: 651-698.

Gunning, B. E. S., A. R. Hardham and J. E. Hughes (1978) Evidence for initiation of microtubules in discrete regions of the cell cortex in Azolla root-tip cells, and an hypothesis on the development of cortical arrays of microtubules. Planta 143: 161-179.

Hara, M., N. Umetsu, C. Miyamoto and K. Tamari (1973) Inhibition of the biosynthesis of plant cell wall materials, especially cellulose biosynthesis, by coumarin. Plant Cell Physiol. 14: 11-28.

Hardham, A.R. and B. E. S. Gunning (1978) Structure of cortical microtubule arrays in plant cells. J. Cell Biol. 77: 14-34.

Hasegawa, K and T. Hashimoto (1973) Quantitative changes of batatasins and abscisic acid in relation to the development of dormancy in yam bulbils. Plant Cell Physiol. 14: 369-377.

Hashimoto, T and K. Hasegawa (1974) Structure and synthesis of batatasin, dormancy-inducing substances of yam bulbils. Phytochemistry 13: 2849-2852.

Heath, O. V. S. (1943) Studies in the physiology of the onion plant. Part II. Ann. Appl. Biol. 30: 308-319.

Heath, O. V. S. (1945) Formative effects of environmental factors as exemplihied in the development of the onion plant. Nature 155: 623-626.

Heath, O. V. S. and M. Holdsworth (1948) Morphogenetic factors as exemplified by the onion plant. Soc. Exp. Biol. Symp. 2: 326-350.

Hepler, P. K. and B. A. Palevitz (1974) Microtubules and micrifilaments.
Annu. Rev. Plant Physiol. 25: 309-362.

Hogetsu, T. and H. Shibaoka (1978) Effects of colchicine on cell shape and on microfibril arrangement in the cell wall of Closterium acerosum.
Planta 140: 15-18.

Izumi, K., I. Yamaguchi, A. Wada, H. Oshio and N. Takahashi (1984) Effects of a new plant growth retardant S-3307 on growth and gibberellin content of rice plant.
Plant Cell Physiol. : In Press

Kato, T. (1964) Physiological studies on the bulbing and dormancy of onion plant III.
J. Jap. Soc. Hort. Sci. 33: 53-61.

Kirschner, M. W. (1980) Implications of treadmilling for the stability and polarity of actin and tubulin polymers in vivo.
J. Cell. Biol. 86: 330-334.

Konrad-Hawkins, E. (1964) Developmental studies on regenerates of Callithamnion roseum Horvey.
Protoplasma 58: 42-59.

Lang, J. M., W. R. Eisinger and P. B. Green (1982) Effects of ethylene on the orientation of microtubules and cellulose microfibrils of pea epicotyl cells with polylamellate cell walls.
Protoplasma 110: 5-14.

Ledbetter, M. C. and K. P. Porter (1963) A "microtubule" in plant cell fine structure.
J. Cell Biol. 19: 239-250.

Magruder, R. and H. A. Allard (1937) Bulb formation in some American and European varieties of onions as affected by length of day.
J. Agr. Res. 54: 719-752.

Marchant, H. J. and E. R. Hines (1979) The role of microtubules and cell-wall deposition in elongation of regenerating protoplasts of Mougeotia.
Planta 146: 41-48.

Miginiac, E. (1978) Photoperiodic plant.
Bot. Mag. Tokyo. Special Issue, I : 159-173.

Mita, T and H. Shibaoka (1983) Changes in microtubules in onion leaf sheath cells during bulb development.
Plant Cell Physiol. 24: 109-117.

Mita, T and H. Shibaoka (1984) Gibberellin stabilizes microtubules in onion leaf sheath cells.
Protoplasma 119: 100-109.

Newcomb, E. H. (1969) Plant microtubules.
Annu. Rev. Plant Physiol. 20: 253-288.

Okamoto, T., Y. Torii and Y. Isogami (1968) Lycoricidinol and lycoricidine, new plant-growth regulators in the bulbs of Lycoris radiata Herb.
Chem. Pharm. Bull. 16: 1860-1864.

Piozzi, F., C. Fuganti, R. Modelli, and G. Ceriotti (1968)
Narciclasine and narciprimine.
Tetrahedron. 24: 1119-1131.

Preston, R. D. (1974) The physical biology of plant cell walls.
p. 383-409. Chapman and Hall, London.

Probine, M. C. and R. D. Preston (1962) Cell growth and structure and mechanical properties of the wall in internodal cell of Nitella opaca.
J. Exp. Bot. 13: 111-127.

Reynolds, E. S. (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy.
J. Cell Biol. 17: 208-212.

Roelofsen, P. A. (1966) Ultrastructure of the wall in growing cells and its relation to the direction of the growth.
Advan. Bot. Res. 2: 69-149.

Roth, L. E. and Y. Shigenaka (1970) Microtubules in the Heliozoan axopodium. II. Rapid degradation by cupric and nickelous ions.
J. Ultrastruct. Res. 31: 356-374.

Sawhney, V. K. and L. M. Srivastava (1974) Gibberellic acid induced elongation of lettuce hypocotyls and its inhibition by colchicine.
Can. J. Bot. 52: 259-264.

Schwabe, W. W. and S. Nachmony-Bascomb (1963) Growth and dormancy in Lunularia cruciata (L.) Dum. II. The response to daylength and temperature.
J. Exp. Bot. 14: 353-378.

Schwabe, W. W. and I. F. M. Valio (1970) Growth and dormancy in Lunularia cruciata (L.) Dum.
J. Exp. Bot. 21: 112-121.

Shibaoka, H. (1972) Giggerellin-colchicine interaction in elongation of azuki bean epicotyl sections.
Plant Cell Physiol. 13: 461-469.

Shibaoka, H. (1974) Involvement of wall microtubules in gibberellin promotion and kinetin inhibition of stem elongation. Plant Cell Physiol. 15: 255-263.

Spurr, A. R. (1969) A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26: 31-43.

Stebbins, G. L. and S. S. Shah (1960) Developmental studies of cell differentiation in the epidermis of monocotyledons II. Cytological features of stomatal development in the Gramineae. Develop. Biol. 2: 477-500.

Sumida, S. and M. Ueda (1976) Effect of O-ethy O-(3-methyl-6-nitrophenyl) N-sec-butylphosphorothioamidate (S-2846), an experimental herbicide, on mitosis in Allium cepa. Plant Cell Physiol. 17: 1351-1354.

Takeda, K. and H. Shibaoka (1981) Effects of gibberellin and colchicine on microfibril arrangement in epidermal cell walls of Vigna angularis Ohwi et Ohashi epicotyls. Planta 151: 393-398.

Torrey, J. G. (1976) Root hormones and plant growth. Annu. Rev. Plant Physiol. 27: 435-459.

Wareing, P. F. and P. F. Saunders (1971) Hormone and dormancy. Annu. Rev. Plant Physiol. 22: 261-288.