Plant Cell Physiol. 32(6): 873–880 (1991)

On the Putative Role of Microtubules in Gravitropism of Maize Coleoptiles

Peter Nick [1], Eberhard Schäfer [2], Rainer Hertel [2] and Masaki Furuya [1]

[1] *Frontier Research Program, Riken Institute, Hirosawa 2-1, Wako-shi, Saitama, 351-01 Japan*
[2] *Institut für Biologie II/III der Universtität, Schänzlestr. 1, D-7800 Freiburg i.Br, Federal Republic of Germany*

The antimicrotubular drug ethyl-*N*-phenylcarbamate (EPC), at 1 to 10 mM, strongly inhibits gravitropism in coleoptiles of *Zea mays* L. Under the same conditions, phototropism remains essentially unimpaired. Propyzamide, a antimicrotubular drug specific for plant microtubules, at 0.02 to 0.2 mM causes qualitatively identical results, but to a weaker extent. Immunofluorescence data show that even only partial elimination of cortical microtubules in the outer epidermis is correlated with complete inhibition of gravitropism. Phototropism can proceed to some extent even after complete removal of cortical microtubules. Together with previous work the data indicate a complex role of the microtubular cytoskeleton in gravitropism: (1) late transduction of both, photo- and gravitropism and (2) early transduction of gravity, possibly by perceiving and transducing pressure or displacement of statoliths.

Key words: Ethyl-*N*-phenylcarbamate (EPC) — Gravitropism — Microtubules — Phototropism — Propyzamide — *Zea* (phototropism/gravitropism).

Cortical microtubules adjacent to the outer cell wall of the outer epidermis of maize coleoptiles reorient from transverse to longitudinal during the response to phototropic or gravitropic stimulation (Nick et al. 1990). This lead to the hypothesis that microtubules might be involved in the late transduction of the photo- and gravitropic response (Nick et al. 1990). To further test this hypothesis and to provide additional information the effect of microtubule-eliminating drugs may be analysed. It is known that colchicine inhibits elongation in maize coleoptiles (Bergfeld et al. 1988). Preliminary experiments showed that colchicine can inhibit phototropism at 5 mM. However, colchicine is known to alter the fluidity of the plasma membranes (Wunderlich et al. 1973), its effect is irreversible (Brown and Bouck 1973), and its action in plant material is not very specific (Gunning and Hardham 1982). For this reason, two other drugs, EPC and propyzamide were chosen for this study. By using two different drugs it may be possible to increase the likelihood that the observed effects can be attributed to the elimination of microtubules rather than unknown side-effects of a given drug.

EPC is a herbicide, which eliminates microtubules in epicotyl cells of *Vigna* (Shibaoka and Hogetsu 1977). Since it binds tubulin reversibly, it had been used for tubulin isolation by affinity chromatography with different higher plant species (Mizuno et al. 1981, Mizuno and Suzaki 1990). In-vitro polymerisation of mung-bean tubulin was completely inhibited by EPC concentrations exceeding 0.3 mM. However, already preformed microtubules were not disrupted (Mizuno and Suzaki 1990). The in-vivo effect of EPC (Shibaoka and Hogetsu 1977) is therefore likely to be caused by prevention of tubulin polymerisation rather than actual disruption of microtubules.

Propyzamide (also known as pronamide) causes mitotic arrest (Carlson et al. 1975, Izumi et al. 1983). As with EPC, its effect is reversible (Izumi et al. 1983). It eliminates cortical microtubules in roots of wheat and maize (Bartels and Hilton 1973) and in tobacco-cell cultures (Akashi et al. 1988). It inhibits in-vitro polymerisation of tobacco-cell tubulin at 0.1 mM, but does not destroy polymerised microtubules (Akashi et al. 1988). Thus, its in-vivo effect may be explained in a similar way to that of EPC. However, propyzamide acts more specifically than EPC, since the polymerisation of animal micro-

Abbreviations: EPC, ethyl-*N*-phenylcarbamate; DMSO, dimethylsulfoxide.

tubules is not impaired by the drug (Bartels and Hilton 1973, Robinson and Herzog 1977, Akashi et al. 1988).

If microtubules are involved in the late tropistic transduction chain common to both gravi- and phototropism (Nick et al. 1990), treatment by EPC and propyzamide should cause inhibition of both. If differences were observed in drug sensitivity between gravi- and phototropism, a more complex role of microtubules has to be assumed. The intention of this paper is to show that, in fact, such differences exist and to discuss how they might be explained.

Materials and Methods

Plant material—Seedlings of *Zea mays* L. (Brio42. HT, Hokkai Seikan, Sapporo, Japan) were raised for 4 days in petri dishes as described by Nick and Schäfer (1988): After soaking in running tap water for 2 h they were sown, embryo up, on thick household tissue soaked with deionised water. To suppress mesocotyl growth and nutations (Kunzelmann and Schäfer, 1985) plants were grown for 3 d under red light (1 Wm^{-2}) at 25°C. They remained in the dark for a further day and were then used for the experiments.

Chemicals and drug application—EPC, purchased from Wako Chemical (Tokyo, Japan) was first dissolved in a small amount of ethanol and then made up with water to a stock solution of 500 mM. Propyzamide, a kind gift from Prof. Shibaoka, Osaka, was dissolved in DMSO to 200 mM and frozen. Working solutions were obtained by dilution with water. To account for influences of the solvent DMSO, its concentration was kept at 1% in all experiments involving propyzamide including the controls. Straight seedlings were selected and transferred in controlled orientation to petri dishes with thick household tissue soaked with the testing solutions. The amount of solution was chosen such that the roots were in contact with the liquid, but the paper did not slide during tilting the dishes. Preincubation time was 2 h for the time course experiments. It was extended to 4 h for the dose-effect experiments, in order to allow sufficient time for the uptake of the drugs even for low concentrations. During incubation, plants remained under symmetric red light (1 Wm^{-2}).

Gravitropic stimulation—Plants were gravitropically stimulated by tilting the petri dishes containing the plants by 30°. The direction of the gravity stimulus was parallel to the shorter coleoptile cross section.

Phototropic stimulation—A unilateral blue-light *pulse* of 1.9 μmol m^{-2} (30 s) was used, which elicits maximal first positive phototropism. The maximum curvature induced by this treatment (30°) was of about the same size as the curvature produced by the gravitropic stimulation described above. Again, the direction of the stimulus was parallel to the shorter coleoptile cross section.

Response evaluation—Light was measured by means of a photoradiometer (YSI Model 65A, Yellow Springs Instrument Co.; Yellow Springs, Ohio, U.S.A.). Photographs were taken at time intervals during the tropistic response for the evaluation of the bending response. Curvature is defined as the angle between the median of the coleoptile tip and the tangent in the node. The experiments were performed in a saturating red-light background (1 W m^{-2}). Points represent averages from 10 seedlings. Cortical microtubules in the outer epidermis were assayed at variable time intervals after treatment with EPC or propyzamide. For this purpose, immunofluorescence staining of microtubules in epidermal strips was used as described in Nick et al. (1990) with minor modifications.

Results

Effect of EPC on gravitropism—As a first qualitative test, a putatively saturating concentration (10 mM) of EPC was applied to maize seedlings 2 h prior to continuous gravistimulation by 30° from the vertical (Fig. 1a). The control developed detectable curvature about 1 h after onset of stimulation and regained the vertical position from about 5 h onwards. There was, however, no detectable gravitropic response of the EPC-treated seedlings.

Fig. 2a shows the dose-effect relation for the inhibition of gravitropic bending (preincubation time 4 h): 1 mM EPC reduced curvature to almost the same degree as 10 and 25 mM, whereas 0.1 mM caused only slight inhibition compared to the control. This shows that inhibition of microtubule polymerisation effectively inhibits gravitropism. It remained unclear, however, whether this was due to an inhibition of output (i.e., growth or lateral auxin transport), or to loss of gravitropic perception.

Effect of EPC on phototropism—To address this question, a phototropically optimal pulse stimulation (1.9 μmol m^{-2}, 30 s) was administered unilaterally after preincubation with EPC (10 mM, beginning from 2 h prior to irradiation). The phototropic curvature which developed (Fig. 1b) was somewhat smaller (a maximum of about 20° at 2.5 h instead of 30° as in water-treated control plants). However, whereas curvature began to decrease in the control seedlings from 2 h after the pulse, it remained constant in the EPC-treated plants. Therefore, in the plants subjected to the drug, the final curvature was larger than in the controls. This demonstrates that phototropic curvature is only slightly impaired by the drug. In contrast, gravitropic straightening, observed in the control plants, was apparently suppressed by the EPC treatment. This indicates that the gravitropic input and not the curvature/growth output was upset by the antimicrotubular drug.

The dose-effect relation for phototropism (preincubation for 4 h) is given in Fig. 2b: Maximal curvature (2 h after the light pulse) was not dramatically affected by 0.1 or

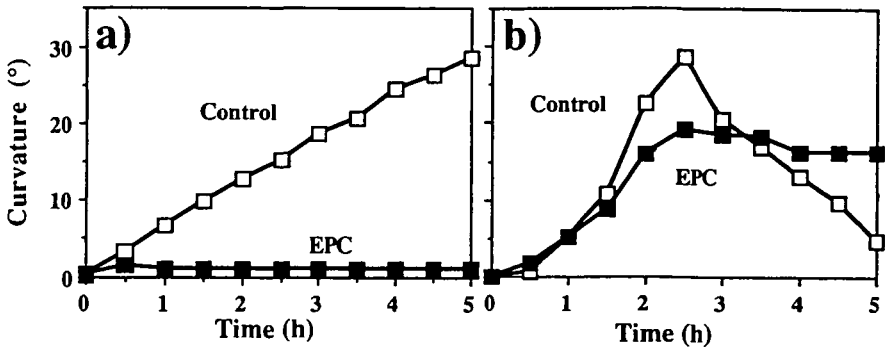

Fig. 1

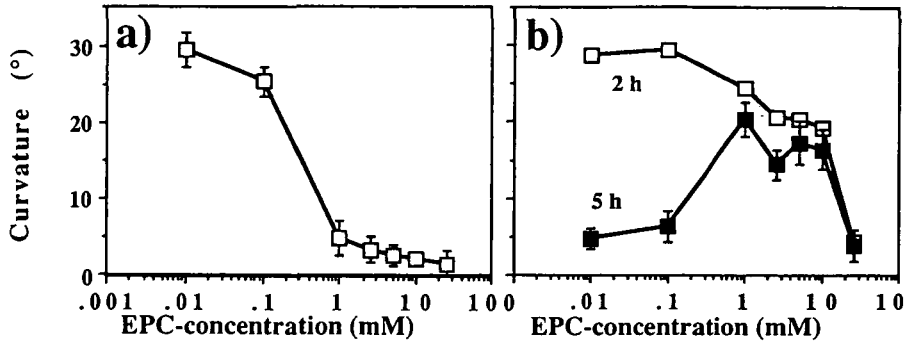

Fig. 2

Fig. 1 Gravitropism (a) and first positive phototropism (b) in presence and absence of 10 mM EPC. Plants were preincubated for 2 h in water or 10 mM EPC. At t=0, gravitropic stimulation at an angle of 30° was applied continuously (a). In (b) a phototropic stimulus (1.9 μmol m^{-2}, 30 s) was given at t=0. The experiment comprises the data of about 30 seedlings per treatment, the standard errors were between 1° and 2°.

Fig. 2 Dose-effect relation of gravitropism (a) and first positive phototropism (b) for EPC. Plants were incubated various EPC concentrations for 4 h and then stimulated gravi- or phototropically as described in Fig. 1. The gravitropic curvature 5 h after the onset of stimulation (a) and the phototropic curvature 2 h (maximal curvature) and 5 h (effect of gravitropic straightening) after the light pulse (b) is indicated.

1 mM EPC, whereas 10 mM yielded a reduction of maximal curvature by about 10°. 25 mM EPC practically abolished any curvature. If the curvature 5 h after phototropic induction is compared (to analyse the extent of gravitropic straightening) the angle was small for 0.1 mM EPC, indicating that gravitropic straightening had taken place. For 1 to 10 mM EPC the angle was much larger than for the water controls (compare Fig. 1b) and still was close to the maximum curvature 2 h after induction. This demonstrates that gravitropic straightening has been inhibited by 1 and 10 mM EPC, whereas the phototropic response itself still could proceed. For 25 mM EPC the curvature was again small, but this was caused by the fact that the phototropic response itself was suppressed as seen by the small curvature 2 h after induction.

Effect of propyzamide on gravitropism—The effect of propyzamide on gravitropism (Figs. 3a and 4a) is qualitatively similar to that of EPC: after preincubation for 2 h with 0.2 mM propyzamide the gravitropic response was reduced by about 50% (Fig. 3a).

The dose-response relation of this effect for 4 h preincubation (Fig. 4a) demonstrates that curvature was already somewhat reduced by 0.02 mM propyzamide, 0.2 mM caused a 50% reduction and 2 mM practically abolished the gravitropic response.

Effect of propyzamide on phototropism—The time course of phototropic curvature (Fig. 3b) for 0.2 mM propyzamide shows that the maximal phototropic curvature was smaller by 10° compared to the controls. However, the gravitropic straightening was impaired to a much more dramatic extent. As for EPC, this lead to larger final curvatures compared to the controls.

The dose-effect relation is given in Fig. 4b: Maximal curvature was not affected by 0.02 mM propyzamide, 0.2 mM brought a reduction by 10° and 2 mM reduced maximal curvature to less than 15% of that for the water controls.

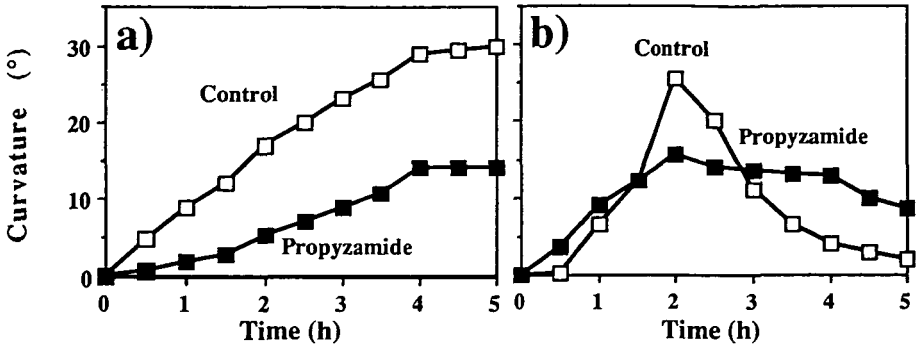

Fig. 3

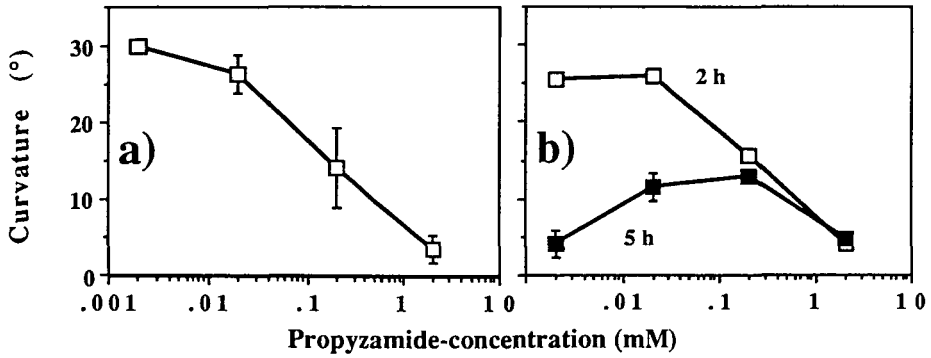

Fig. 4

Fig. 3 Gravitropism (a) and first positive phototropism (b) in presence and absence of 0.2 mM propyzamide. Plants were preincubated in water or 0.2 mM propyzamide, the DMSO content in both groups was 1%. Stimulations as in Fig. 1.

Fig. 4 Dose-effect relation of gravitropism (a) and first positive phototropism (b) for propyzamide. Plants were incubated various concentrations of propyzamide for 4 h and then stimulated gravi- or phototropically as described in Fig. 1. The gravitropic curvature 5 h after the onset of stimulation (a) and the phototropic curvature 2 h (maximal curvature) and 5 h (effect of gravitropic straightening) after the light pulse (b) is indicated.

Again, the gravitropic straightening was more sensitive: curvature 5 h after induction amounted to about 12° for 0.02 and 0.2 mM propyzamide. This is three times greater than in the water controls and shows that, whereas the phototropic response itself could still take place at those concentrations, gravitropic straightening was very slow. For 2 mM curvature is very small (comparable to that 2 h after induction), because the phototropic response itself has been severely damaged.

Balancing experiments—Generally speaking, the sensitivity difference between photo- and gravitropism, although visible, was less pronounced for propyzamide than for EPC. This lead to the idea of the balancing experiments (Fig. 5a, b) which were supposed to amplify the observed differences. The strength of stimulations was chosen such that they caused the same maximal effect (a curvature of about 30°). The resulting pattern is essentially the same for the two drugs: for low concentrations final curvature is negative, indicating that the gravitropic response dominated—the phototropic response is transient (compare Figs. 1a, 3a). For high concentrations seedlings remained straight, and coleoptiles did not show any curvature. Measurements of coleoptile length (data not shown) confirmed the suspicion that growth as such was inhibited. The most interesting results, however, were obtained with intermediate (1 to 10 mM EPC, 0.02 to 0.2 mM propyzamide) concentrations: here the phototropic response clearly prevails, whereas gravitropism appears to be suppressed. It should be noted that for 0.02 mM of propyzamide the inhibition of gravitropism (Fig. 4a) was very small, although detectable. Nevertheless, a strong effect could be already seen for gravitropic straightening (Fig. 4b, 5h curve). In the balancing experiment, too, a strong effect was observed (Fig. 5b). In both cases, phototropism and gravitropism are directly counteracting. Under such conditions even small differences in drug sensitivity will become readily visible, which was the idea behind the design of the balancing experiment. This cannot be due to an

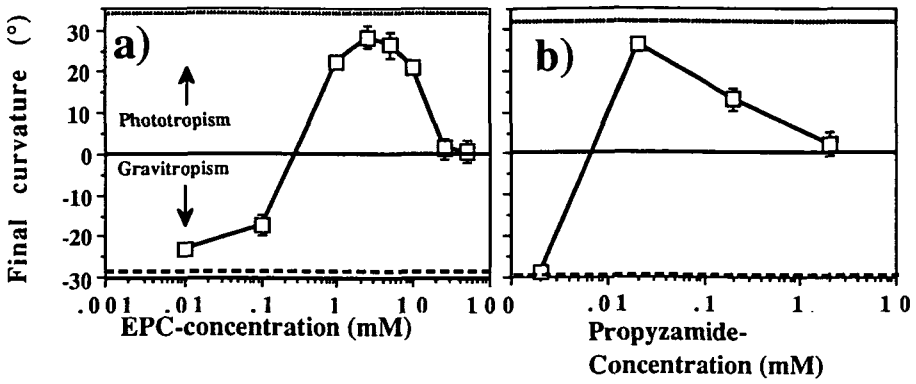

Fig. 5 Dependence of photogravitropic balancing on the concentration of EPC (a) or propyzamide (b). Plants were incubated with different concentrations of EPC (a) or propyzamide (b) for 4 h. Then they were subjected to phototropical pulse stimulation (see Fig. 1b) and gravitropic counterstimulation at an angle of 30° (see Fig. 1a). Twelve hours after the light pulse the final curvature was determined. Negative values indicate bending according to the gravitropic stimulation, positive values bending towards the light. The dotted lines show the maximal curvature of water-treated plants induced by phototropic induction and omission of the gravitropic counterstimulus. The dashed lines show the curvature of water-treated plants if the gravitropic treatment is not preceded by phototropic induction.

elimination of growth, since a phototropic response was still possible under those conditions. It must rather be due to the inability to perceive or transduce the gravitropic stimulus properly.

Cortical microtubules—If plants were incubated with 10 mM EPC (Fig. 6a–d), microtubules looked normal up to 40 min after incubation onset. At this time, the transverse orientation of microtubules was more distinct than at 0 min. This can be attributed to the effect of the red light irradiation during drug incubation (manuscript in preparation). At 80 min, cells with shortened and partially disintegrated microtubules prevailed. After 120 min in the great majority of cells only fluorescent spots aligned to the cell wall could be detected. For 1 mM EPC, even after 4 h microtubules were partially disrupted in most cells and appeared to be less distinct than normal. Nevertheless, if plants were phototropically stimulated at this time (4 h after the onset of drug incubation), oblique or longitudinal microtubules were found in the lighted side, if checked after 2 h (6 h after the onset of the drug treatment) (Fig. 6j). However, no reorientation from transverse to longitudinal was found in response to gravitropic stimulation (Fig. 6i). The results for 0.02 mM propyzamide (Fig. 6e–h, k, l) appear to be similar.

Discussion

Microtubules and tropism—a complex relation—The experiments with EPC and propyzamide, reported here, can be summarised by the following statements: (1) Gravitropism and gravitropic straightening are inhibited at lower drug concentrations (1 to 10 mM EPC, 0.02 to 0.2 mM propyzamide) than phototropism. (2) High concentrations of the drugs (25 mM EPC, 2 mM propyzamide) suppress both gravi- and phototropism. (3) Microtubules in the outer epidermis, where growth is regulated, are eliminated by drug concentrations, which still allow phototropic (but not gravitropic) bending (Fig. 6). (4) Gravitropism is affected under conditions, where cortical microtubules in the outer epidermis are still undamaged in most of the cells and capable of reorientation in response to phototropic stimulation (compare Figs. 2a, 6j and 4a, 6l).

The following conclusions can be drawn from the relation between cortical microtubules in the epidermis and tropism: (1) Cortical microtubules in the outer epidermis are not necessary for the fast phototropic response (compare Figs. 1b, 6a–d and 3b, 6e–h). (2) A different, more drug-sensitive population of microtubules is involved in signal transduction of gravitropism (but not phototropism). This population presumably triggers an early step, occuring before the confluence of photo- and gravitropic transduction chains. (3) The suppression of phototropism at very high drug concentrations (25 mM EPC, 2 mM propyzamide) is not due to elimination of cortical microtubules in the outer epidermis, since those disappear already at lower concentrations, where phototropism still functions.

The first conclusion is not congruent with the working hypothesis proposed in the Introduction (Nick et al. 1990). Although there is a good correlation between microtubule orientation, auxin and tropism, this does not mean that there is a causal relationship. In a forthcoming paper further grounds against a simple causal chain (light or gravi-

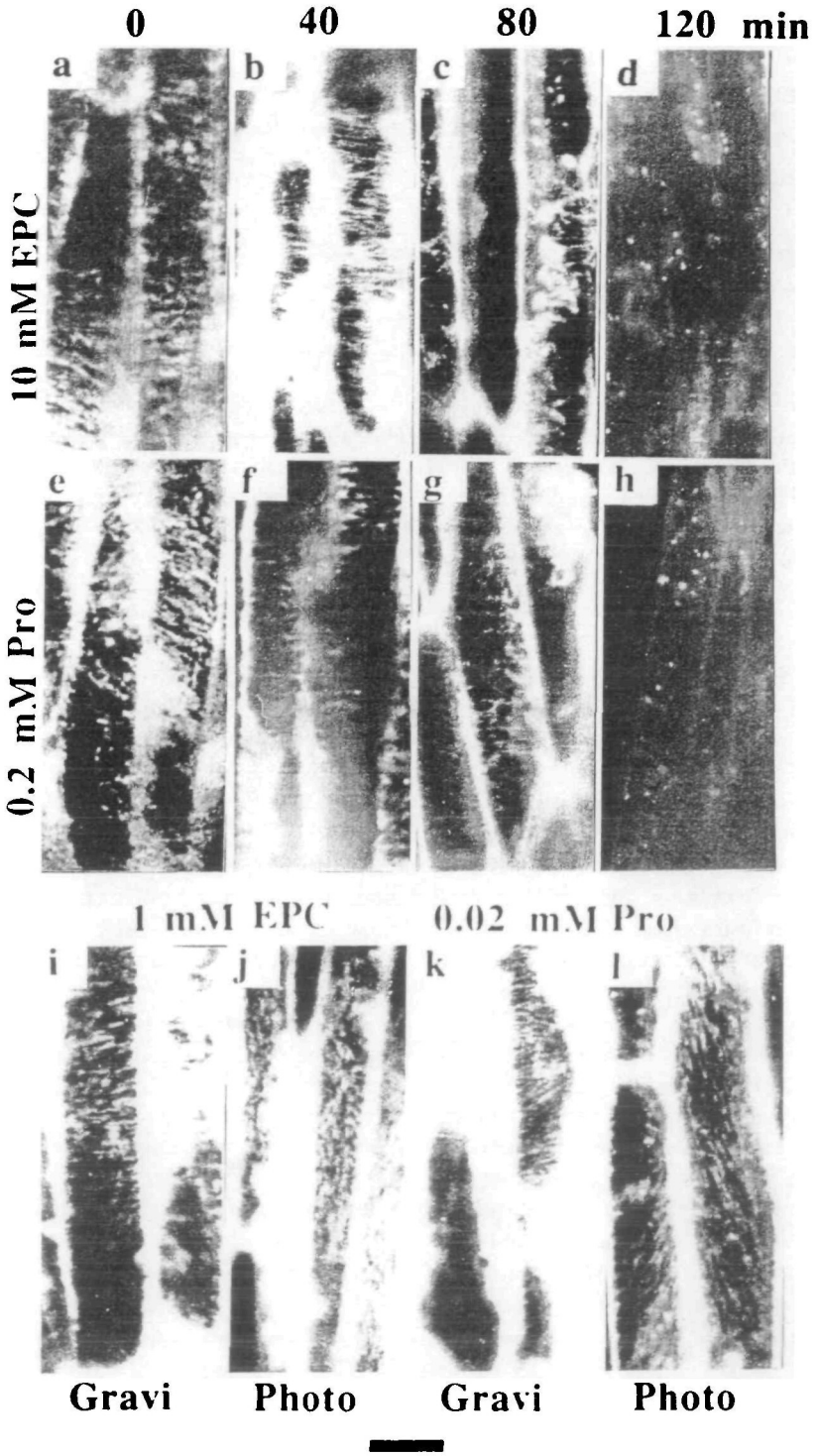

Fig. 6 Elimination of cortical microtubules in the outer epidermis by EPC (a–d, i, j) or Propyzamide (e–h, k, l). (a–d) 10 mM EPC at 0 min (a), 40 min (b), 80 min (c), and 120 min (d) after the onset of drug incubation. (e–h) 0.2 mM propyzamide at 0 min (e), 40 min (f), 80 min (g), and 120 min (h) after the onset of drug stimulation. (i–l) microtubule response to photo- and gravitropic stimulation. Plants were incubated for 4 h with either 1 mM EPC (i, j) or 0.02 mM propyzamide (k, l). Then they were stimulated gravitropically (i, k) and the response checked 2 h later in the upper side, where reorientation should occur. (j, l) show the situation for phototropic stimulation given at 4 h assayed 2 h later in the lighted side. The bar corresponds to 10 μm.

ty-auxin-microtubules-microfibrils-growth) will be presented (manuscript in preparation).

The second conclusion shows that a different, highly drug-sensitive population of microtubules is mediating gravitropic, but not phototropic transduction. It should be underlined that the gravity-sensing cells containing the statoliths are parenchymal and not epidermal. It is therefore likely that this drug-sensitive population of microtubules is located in the parenchyma and not in the outer epidermis.

The third conclusion could be understood by invoking an even more drug-resistant microtubule population necessary for phototropism. However, it may be more reasonable to think of unspecific side-effects of such concentrations similar to those produced by colchicine (Wunderlich et al. 1973).

Microtubules as gravity "sensors"—a speculative outlook—It appears as if a highly drug-sensitive population of microtubules triggers early gravity transduction. This finding is not an isolated phenomenon and it is worth to reconsider some work done in mosses and algae, suggesting that microtubules are involved in gravitropic perception: In an analysis of *Chara* rhizoids, Friedrich and Hertel (1973) implied that microtubules might perhaps receive the pressure from statoliths. Colcemid, given at a certain narrow range of concentrations, did not affect growth, but prevented curvature of horizontally exposed rhizoid tips. The very same rhizoids grew horizontally, but did not bend.

Using protonemata of the moss *Ceratodon*, Hartmann (1984) found that colchicine prevented gravitropism, while —at the same time—allowing red-light phototropism to develop. Further analysis of *Ceratodon* gravitropism (Schwuchow et al. 1989, Walker and Sack 1990) has confirmed a central role for microtubules.

This role of microtubules is not just a general or trivial involvement of (any) cytoskeletal elements, because it has been shown (Wendt et al. 1987, Mulkey et al. 1987) that other anti-cytoskeletal drugs, for instance cytochalasin (eliminating actin microfilaments), do not inhibit perception of gravity.

The gravitropic transduction chain presumably starts with the pressure—and perhaps some displacement—of statoliths, e.g., plastids with or without starch. This "sensor" ("susceptor" in Björkman's (1988) terminology), in turn, has to act on some structure (the "receptor" according to Björkman), such as the cytoskeletal net.

After the input, provided by the interaction of the statoliths with the cytoskeleton, the action might be transduced to ion channels and/or auxin secretion pumps (Hertel and Leopold 1963). This idea connects the Nemec-Haberlandt statolith theory of 1900 with the Cholodny-Went auxin theory of 1928 (Went and Thimann, 1937).

The role of heavy plastids seems to be well established (see e.g., Hertel et al. 1969, for the maize coleoptile, and Kiss et al. 1989, for *Arabidopsis*). Concerning the second step in the stimulus-response chain, the "receptor" role cannot be played by the plasma membrane, since the statoliths do not touch it. Furthermore, recent evidence renders a direct statolith action on the endoplasmic reticulum unlikely (Wendt et al. 1987).

The idea of microtubules as gravity-"receptors" might also explain the effect of calmodulin inhibitors on gravitropism (Biro et al. 1982 for coleoptiles; Stinemetz (1989), Evans et al. (1986) and Björkman and Leopold (1987) for maize roots). Anti-calmodulin substances such as chlorpromazin inhibited responses to gravity such as curvature, lateral movement of calcium and voltage differences. On the other hand, in those cases where it was tested, growth and longitudinal auxin transport remained more or less unaffected. It might be that chlorpromazin is not acting via calcium transport, but as inhibitor for Ca-calmodulin elicited microtubule depolymerisation.

A comment should be added concerning the function of microtubules: Gravity perception is inhibited at a lower inhibitor concentration than is growth. The same is true for the *Chara* rhizoid. This may be explained by two microtubule populations of different sensitivity (Akashi et al. 1988). Alternatively, the inhibition of orderly growth might require a complete loss of the structural microtubule net (high inhibitor requirement). In contrast, gravity sensing may be already disturbed, when the speed of microtubular elongation and shrinking is lowered—which might occur at non-saturating concentrations of EPC or propyzamide.

Our hypothesis suggests that statoliths may be "pressed" into the microtubular structure, which is subject to local turnover. This is how they might "gravistimulate". Unfortunately, the microtubules in the parenchyma are not easy to access by the technique used here. As to substantiate our, admittedly speculative, hypothesis, it is essential to clarify the morphological relation between amyloplasts and cytoskeleton in the statocytes and how this relation is affected by low concentrations of microtubule-eliminating drugs. Herefore, a rapid and non-denaturing fixation method (e.g., quick freezing) in combination with electron microscopy will be necessary. Furthermore, microtubular dynamics (Sullivan 1988), the interaction of microtubules with the plasma membrane, and the role of calmodulin in this process have to be clarified.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 206), and by a grant to P.N. from the Science and Technology Agency Japan. Valuable information from Dr. M. L. Evans concerning the manuscript, and from Prof. H. Shibaoka and Dr. K. Mizuno concerning EPC and propyzamide (and the kind gift of this drug) is gratefully acknowledged.

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(Received February 23, 1991; Accepted June 14, 1991)