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Auxin Redistribution during First Positive Phototropism in Corn Coleoptiles[1]

Microtubule Reorientation and the Cholodny-Went Theory

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ABSTRACT

In red-light grown corn (*Zea mays* L. cv Brio42.HT) coleoptiles, cortical microtubules adjacent to the outer cell wall of the outer epidermis reorient from transverse to longitudinal in response to auxin depletion and after phototropic stimulation in the lighted side of the coleoptile. This was used as an *in situ* assay of cellular auxin concentration. The fluence-response relation for the blue light-induced reorientation is compared with that for first positive phototropism and the dose-response relationship for the auxin-dependent reorientation. The result supports the theory by Cholodny and Went, claiming that phototropic stimulation results in auxin displacement across the coleoptile. In terms of microtubule orientation, this displacement becomes even more pronounced after preirradiation with a weak blue light pulse from above.

To explain phototropism, two principal models have been proposed. (a) The hypothesis by Blaauw (2) proposed that curvature is brought about due to growth inhibition by BL[2] on the lighted side of the plant. No interaction between individual cells is required. However, the existence of mutants in *Arabidopsis*, lacking growth inhibition by BL but still able to perform normal phototropism (13), does not speak in favor of this hypothesis.

(b) In contrast, the Cholodny-Went theory (5, 19) suggested that tropistic stimulation evoked the formation of a transorgan polarity redistributing growth-promoting factors ("auxins") from the lighted toward the shaded side of the plant.

In fact, a redistribution of growth from the lighted toward the shaded side has been demonstrated for first positive phototropism in corn coleoptiles (8) as well as for pea epicotyls (1). However, investigations of auxin contents yielded contradictory results (18). Careful feeding experiments with traces of radioactively labeled IAA (17) support the Cholodny-Went theory for gravitropism of corn coleoptiles. Nevertheless, the discussion between the two schools of thought is still open.

Recently, reorientation of cortical microtubules in the outer epidermis of corn coleoptiles has been described (15). This response is triggered by auxin, phototropic, and gravitropic stimulation. Auxin depletion resulted in a longitudinal orientation, addition of IAA in a transverse orientation. In the case of phototropism, microtubules became longitudinal only in the lighted flank. Those in the shaded side were found to be transverse. For gravitropism, microtubule reorientation from transverse to longitudinal was restricted to the upper coleoptile flank. The time course of microtubule reorientation was identical regardless of whether BL or auxin were used as inducing factors. These authors presented a hypothesis that phototropic stimulation causes a gradient in auxin concentration across the coleoptile, which is accompanied by corresponding restructuring of the cytoskeleton in the epidermis. Cortical microtubules, by virtue of their guiding influence upon cellulose-microfibril deposition, could then determine the mechanical properties of the epidermis, which eventually might lead to differential growth.

It is by no means clear to what degree the observed correlations of phototropic stimulation, auxin (which is in this context operationally defined as microtubule-reorienting factor), microtubules, and curvature are the manifestation of a real causality. Nevertheless, it is possible to use microtubule reorientation as a marker for the auxin activity of a given cell. It should be possible to construct a calibration curve for this response by screening microtubule orientation after incubation with different concentrations of IAA. The fluence-response relation for phototropism and microtubule orientation can then be compared with this calibration curve yielding an estimated fluence-response curve for auxin content in both flanks of the coleoptile.

The crucial point about this approach is that it surveys the responses of individual cells. Photoaffinity labeling using auxin derivatives has been used successfully for an *in situ* assay of auxin localization (9). The approach chosen here is supplementary. It provides an *in situ* assay of auxin activity. It should be emphasized that the original theory by Cholodny and Went (5, 19) was based upon a bioassay measuring auxin activity rather than auxin content. It is obvious that this method will yield only qualitative or half-quantitative results. Nevertheless, it should allow a formal discrimination be-

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[2] Abbreviations: BL, blue light (450 nm); RL, red light (660 nm).

tween the two principal models for phototropism. It was with this intention that the study was carried out.

MATERIALS AND METHODS

Plant Material

Seedlings of corn (*Zea mays* L. cv Brio42.HT, Hokkai Seikan, Sapporo, Japan) were raised for 4 d in plant-tissue culture vessels (9.5 cm × 9.5 cm × 13.5 cm, Flow Laboratories Inc., McLean, VA). The seeds were soaked in running tap water for 2 h and then sown, embryo up, on thick household tissue (Kim-Towel, Kimberley-Clark, Tokyo, Japan) soaked with deionized water. To suppress mesocotyl growth and nutations (10), plants were kept for 3 d under RL (0.5 W m^{-2}) at 25°C. At this time they were selected for straightness and length, and aligned in parallel by turning each seedling individually. They remained in the dark for another day and were, after further selection, used for the experiments.

Light Conditions and Phototropic Stimulation

The RL used for raising the plants was also used as experimental background light. It was obtained from daylight white fluorescent tubes (Toshiba FL 20.5, Toshiba, Tokyo) in combination with a red acrylic filter (Shinkolite A102, Mitsubishi Rayon, Tokyo). Monochromatic BL for phototropic stimulation was isolated from a stereo microscope light source (Olympus, Tokyo) behind a heat filter and an interference filter (λ_{max} 450 nm, half-band width 32 nm, T_{max} 78%, No. 8910–1113 13 P77, Olympus, Tokyo). Filter spectra were measured using a spectrophotometer (DU-50, Beckman, Tokyo). Fluence rate could be varied by the width of a diaphragm built into the light source and the lamp voltage. The set-up was adjusted such that the inhomogeneity within the illuminated light field was less than 10%. The energy flux was measured using a radiophotometer (YSI model 65 A, Yellow Springs Instrument Co., Yellow Springs, OH). Light direction was parallel to the shorter coleoptile cross-axis with the caryopsis being situated in the shaded side of the plant. Irradiation time amounted to 30 s to avoid any effects of sensory adaptation (6). During the time from irradiation until response evaluation, plants stood under RL (0.5 W m^{-2}) at 25°C.

Hormone Treatment

Coleoptile segments (10 mm long, 2 mm below the tip) were excised and the primary leaf discarded. The segments were incubated under RL (0.5 W m^{-2}) at 25°C in various solutions of IAA for variable time intervals. During incubation, they were gently shaken to allow aeration to occur. Growth of coleoptile segments was simultaneously assayed as described earlier (15).

Response Evaluation

Phototropic curvature was determined using a simple xerographic method (14). Cortical microtubules were stained by means of immunofluorescence. Coleoptile segments (isolated as described above; in case of phototropically stimulated plants, the lighted side was marked by an incision) were fixed for 45 min at 25°C in microtubule-stabilizing buffer (0.1 M 1,4-piperazine-diethanolsulfonic acid, 1 mM $MgCl_2$, 5 mM ethylene glycol-bis-(β-aminomethyl ether)-*N,N,N',N'*-tetra-acetic acid, pH 6.8) containing 2.5% (v/v) formaldehyde, 10% (v/v) DMSO, and 10 μg/mL pepstatin A and leupeptin. After three washings in the same buffer without formaldehyde and DMSO, epidermal strips were peeled from the flat sides of the coleoptile. Those from the lighted and the shaded flanks were collected separately. They were then incubated for 1 h at 37°C with a mouse monoclonal antibody against β-tubulin from calf brain (Transformation Research Inc., Framingham, MA) diluted 1:100 in PBS (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 0.1% [w/v] NaN_3, 1 mg/mL BSA, pH 7.3). Then the strips were washed three times with PBS and incubated with a fluorescein isothiocyanate-labeled secondary antibody (anti-mouse immunoglobulin G, Sigma, Tokyo) diluted 1:20 in PBS for 1 h at 25°C. Three washings with the same buffer followed. Strips were then kept overnight at 4°C in PBS supplemented with 0.1% (w/v) Triton X-100 and 10 mM dithioerythrol, and mounted in an antifading agent (Citifluor, Amersham, Tokyo) with the outer side of the epidermis facing upwards. They were viewed under a fluorescence microscope (BHX, Olympus, Tokyo) and photographs taken on Kodak Trix X Pan 400 ASA film (Kodak, Rochester, NY).

Microtubule orientation at the outer cell face was determined as the angle between the array of microtubules and the longitudinal cell axis. Because their orientation was not always homogeneous within a given cell, it was determined for short cell-surface sections, two to three cell widths in length. Within such patches, microtubules were fairly parallel, such that a preferential direction could be attributed to them. Frequency distributions were constructed comprising the data from 20 to 45 plants (corresponding to two to four independent experiments). The average deviation from the transverse position was calculated from these frequency distributions. In cells with oblique microtubules, "left-turns" and "right-turns" were distinguished by negative or positive angles, respectively. However, no significant distribution asymmetries were observed. Therefore, the distinction between left-turns and right-turns was omitted for the calculation of average orientations.

RESULTS

Dose-Response Curve for IAA-Induced Microtubule Reorientation

To determine the dose-response relation correctly, first the time course of reorientation was measured for treatments, which were weak (incubation in 0 μM IAA), intermediate (incubation in 1 μM IAA), or strong (incubation in 100 μM IAA) with respect to the growth of segments. Microtubules are transverse or somewhat oblique at the start of the treatment (Fig. 1). They become more or less longitudinal within 1 h if no IAA is added. For 1 μM IAA, this tendency is much less pronounced. This is due to an inhomogeneous response even of immediately neighboring cells (Fig. 2A). For 100 μM

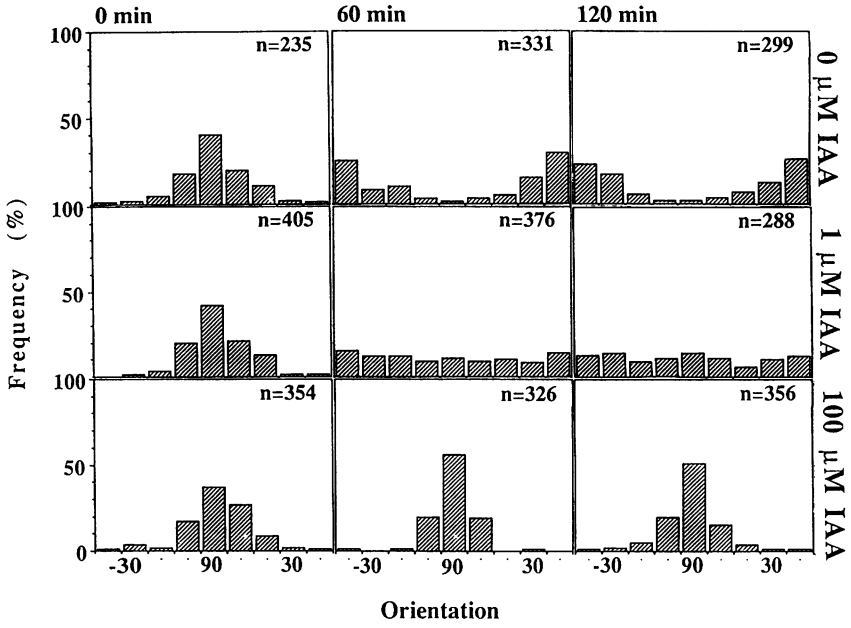

Figure 1. Frequency distribution over microtubule orientation at different stages of incubation with IAA under 0.5 W m^{-2} RL. n, Number of surface areas counted. The abscissa indicates microtubule orientation with 90° denoting transverse, +0° and −0° denoting longitudinal microtubules.

IAA, the opposite is observed; microtubules reach a clear transverse array. These data show that the reorientation response has come to a final state at 60 min and does not change afterwards, which is consistent with previous data (15). For the dose-response study, an incubation time of 2 h was chosen, because even for moderate treatments that should suffice to reach the final state. The frequency distributions for an incubation time of 2 h (Figs. 1 and 3) reveal that for IAA concentrations of 50 μM or more, microtubules are transverse, whereas IAA concentrations of 0.5 μM or less produce longitudinal microtubules. The average deviation from the transverse position was calculated and plotted *versus* the concentration of IAA used for incubation, yielding a calibration curve for microtubule orientation *versus* the concentration of exogenous auxin (Fig. 4a). Taking into account the large SDs caused by the inhomogeneity in the response of neighboring cells (Fig. 2A), this curve can be regarded as correlated with the dose-response curve of growth (Fig. 4b). Both curves appear to shift to higher concentrations when compared with data from the literature on endogenous auxins (18). The reason for this is unknown—it might be due to the fact that the penetration of IAA into the tissue is hampered by the cuticle.

Fluence-Response Curve for BL-Induced Microtubule Reorientation

The fluence-response curve for first positive phototropism as measured 2 h after induction (Fig. 5a) exhibits the familiar bell-shaped appearance with a lower threshold at about 0.1 μmol m^{-2}, a maximum of more than 30° at 2 μmol m^{-2}, and zero curvature for fluences higher than 20 μmol m^{-2}. The reorientation of microtubules in the lighted flank of the coleoptile (Fig. 6) initiates from a predominantly transverse to slightly oblique state and has developed toward a more longitudinal orientation after 1 h. The response is strong for 2 μmol m^{-2} BL, less distinct for 0.5 μmol m^{-2}, and barely detectable for 0.1 μmol m^{-2}. The frequency distributions are consistent with the view that a final state has been reached at 60 min and is maintained thereafter, congruent with earlier data (15). Phototropic curvature is maximal at about 2 h after induction. Therefore, a waiting time of 2 h after induction seemed to be feasible.

Frequency distributions in both the lighted and shaded flanks of the coleoptile were measured after phototropic pulses of varying strength (Fig. 7). Two observations are noteworthy. (a) In the shaded side, microtubules maintain

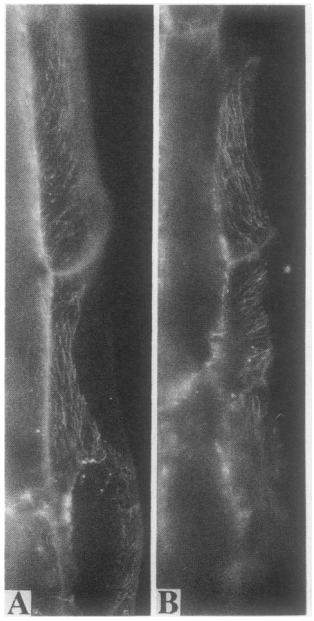

Figure 2. Immunofluorescence images of cortical microtubules in the outer epidermis under 0.5 W m^{-2} RL. A, Situation 2 h after the onset of incubation with 1 mM IAA. B, Situation in the lighted side of the coleoptile 2 h after irradiation with 0.5 μmol m^{-2} BL.

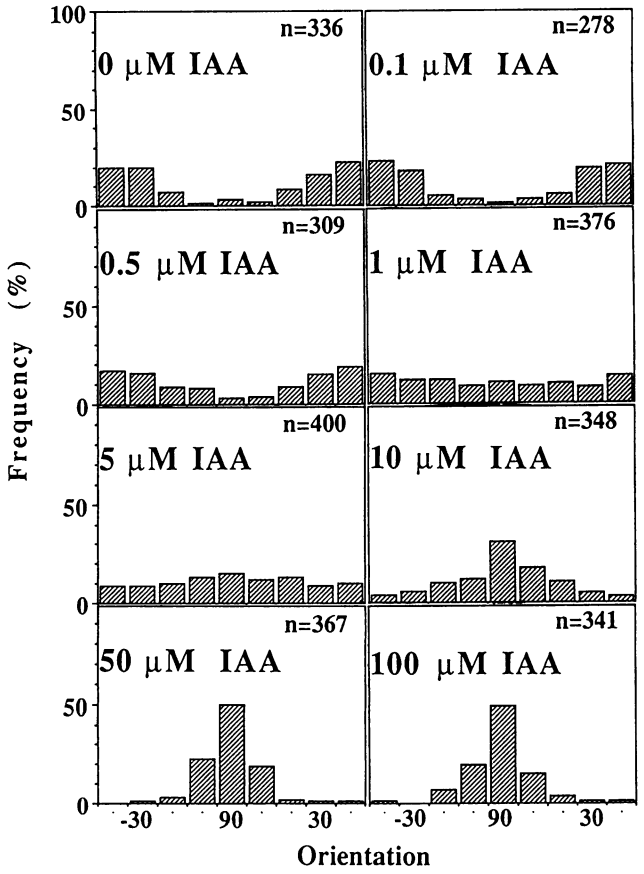

Figure 3. Frequency distribution over microtubule orientation 2 h after the onset of incubation in various concentrations of IAA.

their initial orientation for fluences up to 2 μmol m^{-2}. However, they become slightly more transverse if a phototropically optimal pulse (2 μmol m^{-2} BL) is given. If the fluence is increased even further, a situation prevails in which some cells have transverse, other cells oblique, and still other cells longitudinal microtubules. It should be underlined, however, that even for 20 μmol m^{-2}, when zero curvature is reached, they do not become predominantly longitudinal.

(b) In the lighted side, microtubules behave as in the dark for 0.1 μmol m^{-2} BL (the lower threshold for phototropic bending). For 0.5 μmol m^{-2} BL, some cells have transverse, other cells oblique, and still other cells longitudinal microtubules. The inhomogeneity between neighboring cells appears to be even more dramatic than for incubation with 1 μM IAA (Fig. 2). Cells with longitudinal microtubules dominate for 2 μmol m^{-2} BL. If the fluence is increased further, this tendency is reversed: for 5 μmol m^{-2}, again a mixed orientation is observed, and for 20 μmol m^{-2} (when zero curvature is reached), the situation again is similar to that observed for weak phototropic stimulation.

If the average microtubule deviation from the transverse position is determined (Fig. 5b), a bell-shaped fluence-response curve emerges for the lighted side, which resembles the bell-shaped fluence-response curve of first positive phototropism (Fig. 5a). For the shaded side, there is not much change visible, except a slight tendency toward the transverse orientation for 2 μmol m^{-2} and a not very pronounced tendency toward oblique orientations for higher fluences.

Estimation of Auxin Content in Relation to BL Fluence

The calibration curve for microtubule reorientation in relation to auxin content (Fig. 4a) can be used to correlate the response to phototropic stimulation to the response after incubation with IAA in a kind of *in situ* assay. The basic principle is to calculate the average microtubule deviation produced by a given phototropic treatment and look for the IAA concentration that produces the same average microtubule deviation. This calculation assumes that the orientation of the microtubules is cell autonomous. This assumption appears to be sound because even within a given cell, microtubule orientation can change (Fig. 2). The resulting estimated fluence-response curve for auxin activity ("auxin" defined operationally as "microtubule-reorienting factor") in the lighted and the shaded flank (Fig. 5c) is characterized by a dramatic drop of auxin activity in the lighted side for fluences inducing maximal phototropism. If the fluence is raised further, it recovers in the lighted side and approaches the original dark level. In the shaded side, the effects display a mirror image of those in the lighted side: auxin activity

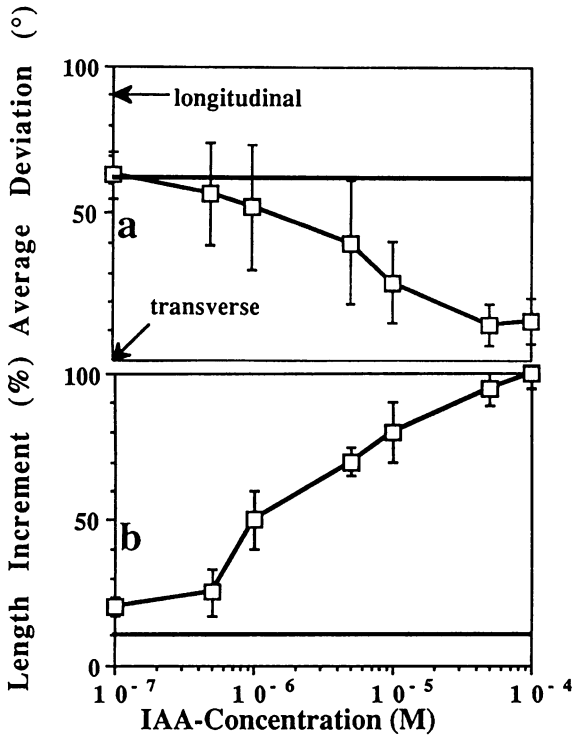

Figure 4. a, Calibration curve for microtubule reorientation *versus* IAA concentration. The curve was obtained by calculating the average deviation of microtubule orientation from the transverse position after incubation for 2 h in various concentrations of IAA. Error bars denote the SD values as calculated from the frequency distributions. b, Length increment of coleoptile segments after incubation for 2 h in various concentrations of IAA relative to a saturating IAA treatment. Bold lines denote the values obtained for incubation in water.

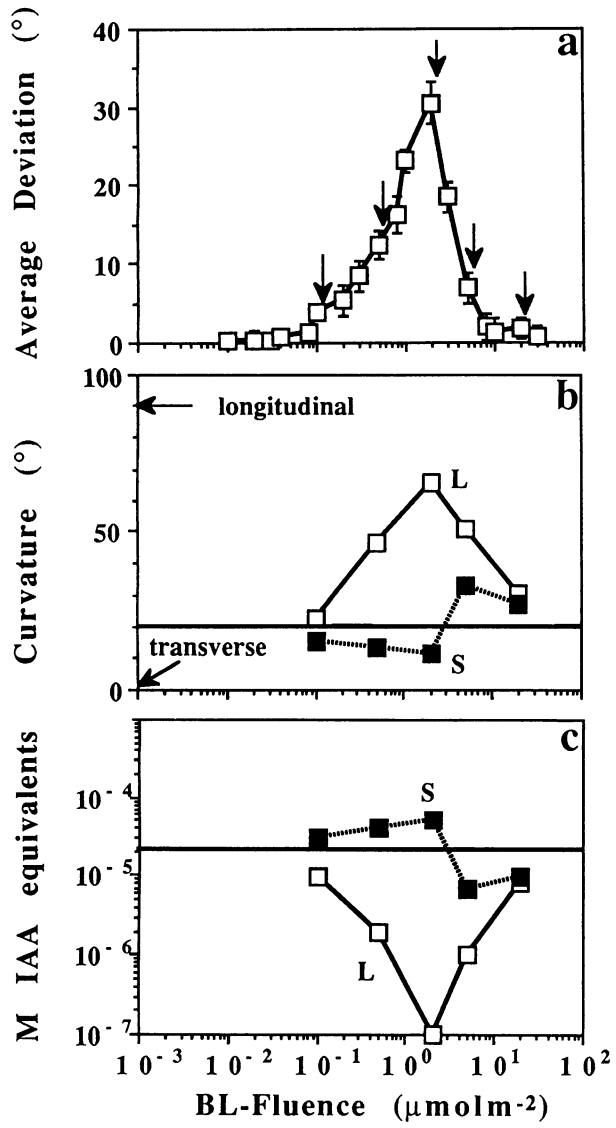

Figure 5. Fluence-response relation for first positive phototropism (a), average microtubule orientation in the lighted (L) and shaded (S) side (b), and estimated auxin activity in IAA equivalents in the lighted and the shaded side of the coleoptile (c). Plants were kept for 2 h under RL (0.5 W m^{-2}) after phototropic stimulation in the shorter coleoptile cross-section. Bold lines give the value for the unirradiated dark controls. Arrows in (a) indicate fluences for which microtubule orientation has been screened.

increases for phototropically optimal stimulation and drops back to almost the dark level for higher fluences. In relative terms, the increase is less conspicuous than the sharp decrease in the lighted side. In absolute terms, however, it appears that the decrease in the lighted and the increase in the shaded side are roughly equivalent (it should be kept in mind that the scale in Fig. 5c is logarithmic).

Reduction of Auxin Content by BL Preirradiation and Subsequent Phototropic Stimulation

The microtubule reorientation toward the transverse position, although detectable in the shaded side of phototropically stimulated coleoptiles for 2 μmol m^{-2} (Fig. 7), was not very conspicuous. Considering the SD values caused by the inhomogeneity between neighboring cells (Fig. 2), one may doubt whether the effect is significant at all. Thus, an experiment yielding a more qualitative outcome is warranted. The small response might be caused by the fact that auxin content in the native tissue is near to saturation with respect to transverse microtubule orientation. One may ask whether it is possible to decrease this initial auxin content. Decapitation is not a good method, because it also removes the zone sensitive to BL (6, 12). However, it is known that irradiation with symmetric BL has the same effect (18), and previous experiments (16) showed that microtubules turn from horizontal to longitudinal in response to a BL pulse administered from above. Thus, the following experimental schedule was developed. After prestimulation with a pulse of 0.5 μmol m^{-2} BL from above and a waiting time of 20 min, plants were irradiated unilaterally with a pulse of 2 μmol m^{-2}, which should induce maximal curvature. Curvature and microtubule orientation were then checked 2 h after phototropic stimulation. A waiting time of 20 min between prestimulation and phototropic induction was inserted to allow sufficient time for the sensory adaptation elicited by the pretreatment to fade away (6). The observed large curvature (26.5°) showed that this was successful.

The frequency distribution of microtubule orientation (Fig. 8, upper row) reveals a mixed orientation (some cells having transverse, other cells oblique, still other cells longitudinal microtubules), if the subsequent unilateral stimulation is omitted. If it is included (Fig. 8, lower row), microtubules are clearly longitudinal in the lighted side and clearly transverse in the shaded side. Auxin concentration, as estimated from microtubule orientation, is increased in the shaded side from 10^{-5} M to 5×10^{-5} M IAA equivalents. In the lighted side, it decreases from 5×10^{-5} M to 10^{-7} M IAA equivalents.

DISCUSSION

The comparison of phototropically induced microtubule reorientation with auxin-dependent microtubule reorientation favors the following conclusions. (a) In the native, nonirradiated tissue, the level of auxin appears to be high and near saturation with respect to its capacity to force microtubules into the transverse array (Fig. 1, situation for 0 min). (b) Irradiation with unilateral BL effective in evoking phototropic bending causes a decrease of auxin in the coleoptile flank facing the light (Figs. 5–7). This is compatible with the hypothesis of Blaauw (2) and the Cholodny-Went theory (5, 19). (c) In the shaded flank of the coleoptile, the level of auxin increases to a degree comparable with the depletion in the lighted flank (Figs. 5–7). This increase is rendered more conspicuous if the overall auxin level is somewhat lowered by a preirradiation with a weak BL pulse from above (Fig. 8). The Blaauw theory predicts a slight decrease of auxin content in the shaded side because some BL should reach the shaded side and exert its growth-inhibiting effect. The Cholodny-Went theory predicts that auxin content should increase to more or less the same extent as it decreases in the lighted flank.

The data presented here clearly show a bell-shaped fluence-response curve for the orientation of microtubules in

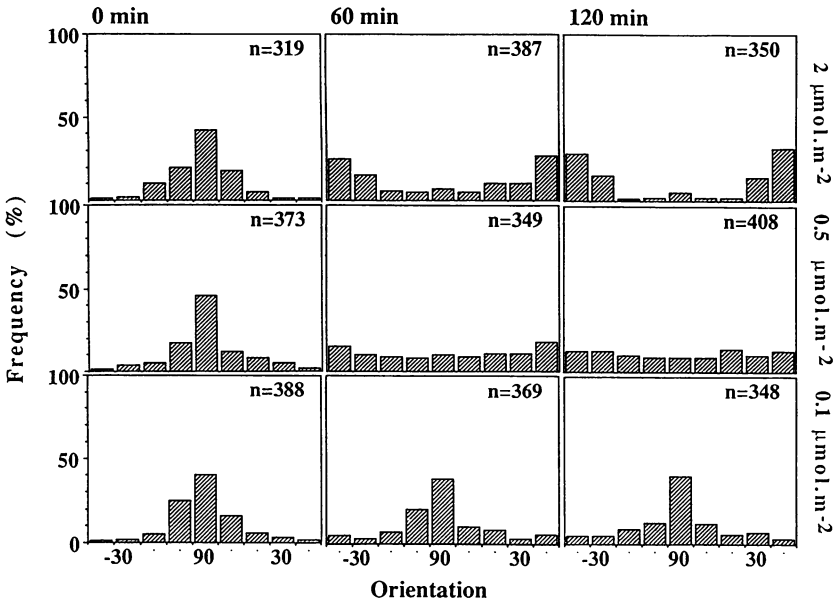

Figure 6. Frequency distribution over microtubule orientation in the lighted side of the coleoptile at various time intervals after phototropic stimulation with various fluences of BL. Abscissa values of 90° stand for transverse, +0° and −0° for longitudinal microtubules. n, The number of surface areas counted.

the lighted side. Furthermore, fluence changes lead to responses that are opposite in sign for the lighted and the shaded flank. It is felt that such a counterdirected response is easier to reconcile with the Cholodny-Went theory than with the hypothesis of Blaauw. It is also easier to reconcile with recent findings in *Arabidopsis* that growth inhibition by BL can be genetically separated from phototropism (13).

The *in situ* assay used for this study has the advantage of not interfering with the system one wants to test (in contrast with studies using radioactively labeled IAA). Moreover, extraction efficiency, a major issue in the debate about radioactive feeding experiments (18), is not relevant to the assay used here. Nevertheless, the *in situ* evaluation, too, has certain "weak points." (a) The calibration is done with external IAA. It is not clear to what degree this can be equaled to internal auxin (3). It might be that factors other than IAA may influence the orientation of microtubules. A further problem is posed by the fact that the cuticle hinders the penetration of IAA into the tissue. This might explain the relatively high IAA concentrations necessary to saturate growth and microtubule response (Fig. 4). This means that the estimated values shown in Figure 5c are certainly higher compared with the real situation. These problems, however, do not question the qualitative finding that the (operationally defined) auxin is redistributed from the lighted toward the shaded side of the coleoptile.

(b) The assay is based upon a comparison of average microtubule orientations (Figs. 4 and 5, b and c). There is, however, large inhomogeneity in the response of individual cells. Even immediately neighboring cells can behave quite differently (Fig. 2). Is this inhomogeneity due to an inhomogenous distribution of auxin or is it due to inhomogeneity in the cellular response to auxin? This question can be solved only by a combination of an *in situ* assay for auxin activity with an *in situ* assay of auxin localization.

However, the same criticisms hold true for the conventional radioactive feeding-assay: (a) it works with labeled

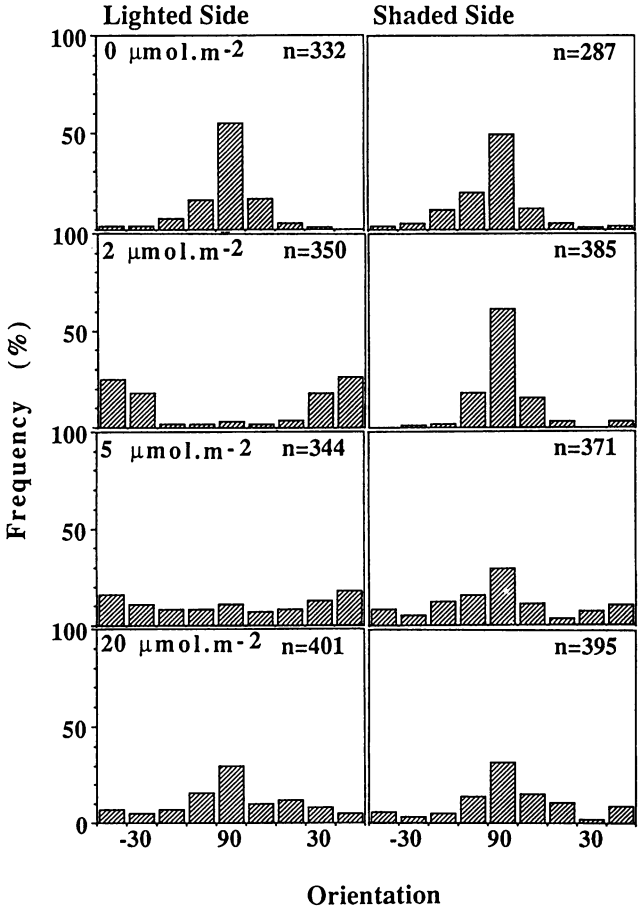

Figure 7. Frequency distribution over microtubule orientation for the final states (2 h after induction) of the response after phototropic pulse stimulation of variable strength. The situation in the lighted side is shown on the left, the situation in the shaded side on the right.

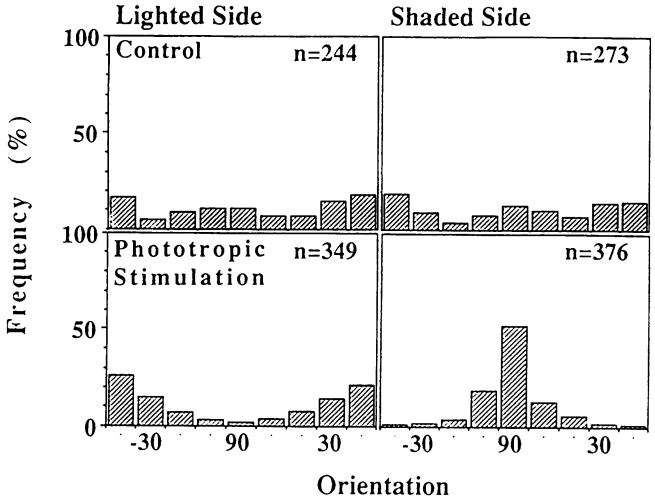

Figure 8. Frequency distribution over microtubule orientation after preirradiation with 0.5 μmol m^{-2} BL from above without (upper row) and with (lower row) subsequent phototropic stimulation with 2 μmol m^{-2} BL. The time interval between preirradiation and phototropic stimulation amounted to 20 min and the response was evaluated 2 h after phototropic stimulation (140 min after preirradiation).

exogenous IAA, which might not behave in the same way as endogenous auxin; (b) it has to rely on extraction of tissue, *i.e.* it does not yield much information about the actual distribution of auxin between different cells.

Notwithstanding the fact that the relation between auxin and microtubules is anything other than clear, an important conclusion about phototropic transduction can be extracted from the data presented here. The hypothesis by Blaauw (2) explains curvature in terms of strictly localized growth inhibition by the locally perceived BL. In fact, it is possible to explain the bell-shaped fluence-response curve of first positive phototropism in such terms. If fluence grows beyond saturation for growth inhibition, the shaded side will be increasingly inhibited as well, which results in a drop of curvature. In the extreme case, the BL will be saturating in both flanks, causing the zero curvature observed for strong light pulses.

If it is true that microtubule orientation is correlated to growth (15), one would expect the following in terms of the Blaauw theory. In the lighted side, microtubules remain longitudinal even following strong light pulses (5 and 20 μmol m^{-2}) because the action of BL should be saturated. Microtubules in the shaded side should become increasingly longitudinal as well, until the difference with the lighted side disappears, which results in zero curvature. Instead of Figure 5b, one would rather expect two sigmoidal curves shifted by the absorption gradient of BL across the tissue (11). Figure 5b is clearly not compatible with this view. The bell-shaped fluence-response curve of phototropism (Fig. 5a) is mainly due to a bell-shaped fluence-response curve for average microtubule orientation in the lighted flank of the coleoptile (Fig. 5b). This means that the gradient of light across the tissue is sensed earlier in transduction and transformed into a global, systemic signal, which determines the orientation of microtubules in both sides of the plant. Bluntly stated, the cells in the lighted side "know" how much light the cells in the shaded side perceived and vice versa. At least they appear to do so if their cytoskeletons are analyzed. The existence of such systemic signals ("phototropic polarity") was shown in an elegant experiment by Johannes Buder (4) and they are implicit elements of the Cholodny-Went theory—the displacement of auxin across the tissue is driven by this phototropic polarity (7). The bell-shaped curves in Figure 5, b and c mirror the growth and disappearance of this phototropic polarity.

ACKNOWLEDGMENT

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