Cytoskeletal Drugs and Gravity-Induced Lateral Auxin Transport in Rice Coleoptiles

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Abstract: Gravity-induced events such as amyloplast sedimentation and lateral auxin transport were probed with cytoskeletal drugs in coleoptiles of rice (*Oryza sativa* L.). Amyloplast sedimentation was retarded by taxol. Lateral transport of auxin (^{3}H-indoleacetic acid) was strongly inhibited by EPC (ethyl N-phenylcarbamate), but only partially inhibited by taxol. 1 mM EPC reduced gravitropism while phototropism was not affected. The findings suggest that microtubules may transduce pressure or proximity of amyloplasts to the auxin exporter in the plasmalemma.

Key words: Amyloplasts, cytoskeletal drugs, gravitropism, lateral auxin transport, microtubules, rice (*Oryza sativa* L.).

Introduction

A molecular explanation of gravitropism is still lacking (reviews Björkman, 1988[2]; Sack, 1997[25]). There are, however, several events that are generally believed to participate in perception and transduction of gravity in plants. The starch–statolith hypothesis has been supported by a wealth of correlative evidence (Hertel et al., 1969[8]; Kiss et al., 1989[10]; Sack, 1991[24]; Kuznetsov and Hasenstein, 1996[11]). The amyloplasts may press upon a mechanosensitive receptor of still unknown nature (Björkman, 1988[2]), and this may lead to up–down asymmetry in the perceiving cell.

A second generally accepted process in gravitropism is the lateral transport of auxin leading to a net displacement of auxin towards the lower flank of gravitropically stimulated organs. Such a displacement was proposed in 1927 by Cholodny (see in Trewavas, 1992[31]), and has been well established by measurements of the distribution of endogenous auxin from the tip of gravitropically stimulated coleoptiles (Dolk, 1936[6]) or by measuring the lateral asymmetry of applied radiolabelled IAA (see Parker and Briggs, 1990[21]; Masson, 1995[12]). The last step of the gravitropic response is asymmetric growth that is believed to result from the asymmetric distribution of auxin (as discussed in Trewavas, 1992[31]).

The transduction from mechanical force of the statoliths to mechanosensitive ion channels or auxin transporters in the plasmalemma remains to be elucidated. One may postulate a role of the cytoskeleton as a pressure transducer to a mechanosensitive element in the plasma membrane (e.g., Nick et al., 1991[18]).

In moss protonemata, microtubules have been proposed to mediate gravitropic transduction triggered by the sedimenting amyloplasts (Schwuchow et al., 1990[26]; Walker and Sack, 1990[33]). In maize coleoptiles, the microtubule-eliminating drugs, ethyl N-phenylcarbamate (EPC) and propyzamide, have been shown to impair gravitropic bending at concentrations that still allow for phototropism (Nick et al., 1991[17]). This study suggested a role for a dynamic population of microtubules in the transduction of gravity.

In an attempt to obtain an insight into the role of the microtubular cytoskeleton in the gravitropic response, the effect of some drugs was tested on sedimentation of amyloplasts, on lateral IAA transport and on gravitropic bending: ethyl N-phenylcarbamate (EPC) which blocks the polymerization of tubulin (Mizuno and Suzaki, 1990[13]; Morejohn, 1991[14]), and taxol, a drug that suppresses tubulin depolymerisation and causes bundling of microtubules (Parness and Horwitz, 1981[22]; Rowinsky et al., 1991[23]; Morejohn, 1991[14]). Rice coleoptiles were used because they respond swiftly to gravity (Iino et al., 1996[9]) and permit simple assays of lateral IAA transport. In rice, mutants that affect gravitropism (Abe et al., 1994[1]; Godbolé et al., 1999[7]) or the cytoskeleton (Nick et al., 1994[14]; Wang and Nick, 1998[34]) have been isolated that should allow a better understanding of the interaction of the cytoskeleton and gravity perception.

Materials and Methods

Radiolabelled indole-3-acetic acid (^{3}H-IAA, 925 GBq/mmol) was purchased from Amersham Life Sciences, Braunschweig, Germany; NPA (1-N-naphthylphthalamic acid) was synthesized according to Thomson et al. (1973[30]); EPC (ethyl N-phenylcarbamate) was obtained from Wako Chemical, Tokyo, Japan. All other chemicals were purchased from Roth, Karlsruhe, Germany, or from Sigma, Deisenhofen, Germany.
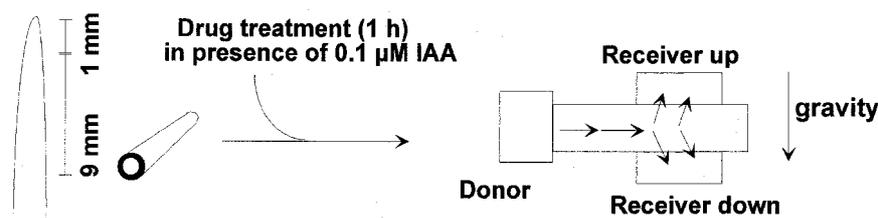
Fig. 1 For lateral IAA transport, the 9 mm coleoptile segments, on the surface of a glass slide, were sandwiched between two plain agar blocks (4 × 4 × 1 mm) placed at the basal 4 mm of the segments such that one block was on the upper flank and the other one on the low-er flank of the horizontally placed segment; a donor block was positioned at its apical cut surface. The slides were placed on their sides and incubated for 150 min. Lateral export had to take place through the cuticle.

Caryopses of rice (*Oryza sativa* L. ssp. *japonica,* cv. Nihonma-sari) were dehusked, surface-sterilized in 5% sodium hypo-chlorite or in 30% H_2O_2, sown on plastic mesh floating on wa-ter and cultivated in complete darkness at high humidity at 25 °C, as described in Nick and Furuya (1993[18]). For cytologi-cal analysis and for assays of lateral IAA transport, coleoptiles were allowed to grow in air for 5 d.

In order to measure amyloplast displacement, excised rice coleoptiles of about 15 mm in length were placed, with the gravity vector pointing from tip towards base, in Eppendorf tubes containing water (control) or aqueous solutions of var-ious cytoskeletal drugs, and kept for 30 min in the same orien-tation. They were then inverted for various time intervals, fixed in place by flooding in fixative (3% v/v glutaraldehyde and 10% w/v paraformaldehyde) for 20 min at room tempera-ture, and washed twice for 5 min in water. The apical 5 mm were excised and tangential sections of 20–40 μm thickness were made of these segments on a cryotome (2800 Frigocut-E, Reichert-Jung, Nussloch, Germany) at – 20 °C, after embed-ding the segments in Tissuetek (Miles Scientific, Naperville, IL). The sections were then stained with Lugol solution (10% w/v KI, 2% w/v I_2), and photographed under bright-field illumi-nation (Axiophot, Zeiss, Oberkochen, Germany). Distribution of the amyloplasts was scored over the long axis of a cell; cells were classified into 5 groups according to amyloplast location (see legend Table **1**).

For auxin transport assays, coleoptiles, about 15 mm in length, were selected for straightness; 9 mm long segments were ex-cised (see Fig. **1**, left) and incubated for 1 h in water containing either 0.1 μM IAA or, alternatively, in 0.1 μM IAA supplemented with various concentrations of NPA or cytoskeletal drugs such as EPC. In order to avoid a gravitropic response during that period, this pre-incubation took place on a shaker. During the transport experiment itself no drugs were applied.

As shown in Fig. **1**, for the transport test the pretreated cole-optile segments were placed on a microscope slide and sand-wiched between two plain agar blocks (4 × 4 × 1 mm, 1.5% agar) placed at the basal 4 mm of the segments such that one block was on the upper flank and the other one on the lower flank of the horizontally placed segment; a donor block (4 × 4 × 1 mm, 1.5% agar; containing 0.1 μM IAA and trace amounts, approximately 10000 cpm, of ^{3}H-IAA) was posi-tioned at its apical cut surface. The slides were placed on their sides and incubated in a moist chamber for 150 min. The cuticle of the rice coleoptile allowed significant lateral IAA export.

Donors, receivers and segments were collected separately, placed in 5 ml of Rotiszint scintillation fluid (from Roth, Karls-ruhe, Germany), and radioactivity expressed as cpm was deter-mined by liquid scintillation counting (in a LS 5000CE from Beckman, München, Germany) with a counting efficiency of approximately 30% for tritium. The counting error for the cpm values is negligible since samples were counted for 3 × 20 min each.

The lateral transport asymmetry was defined as the difference between cpm in receiver$_{down}$ minus cpm in receiver$_{up}$, divided by the sum of radioactivity (cpm) found in both receivers. If not stated otherwise, the transport interval extended over 150 min at 23 °C ± 2 °C with the receiver blocks in place throughout this period. When the set-up was mounted, con-tact between the blocks was carefully avoided, and negative controls, using a piece of teflon tubing instead of the cole-optile, did not reveal any spill-over of radioactivity that might produce artifactual auxin asymmetry (data not shown). To fur-ther safeguard against this possibility, negative controls with coleoptiles pretreated in NPA were included with the experi-ments.

For a control of longitudinal ^{3}H-IAA transport, segments for each treatment, taken after the lateral transport assay de-scribed above, were cut in apical and basal halves, and their radioactivity (cpm) was determined.

In order to obtain a measurable phototropic response, sub-mersed coleoptiles were used to assess photo- and gravitro-pism, according to a modified cultivation and test protocol (Neumann and Iino, 1997[16]). Single caryopses, embryos down, were sown in four-sided clear cuvettes on agar (0.7%, 10 mm depth); the cuvettes were covered with parafilm, placed in plastic boxes and cultured in the dark at 25 °C for 2.5 d. Then, cuvettes with straight coleoptiles of uniform length were se-lected under green safe light and filled with deionized water, and closed with parafilm. The seedlings were kept submersed in a vertical position for 6 h before phototropic or gravitropic stimulation. At this point, the water was exchanged for water containing EPC or taxol, or plain water as a control, and cuvettes were closed again with parafilm. For assessment of the phototropic response, cuvettes with coleoptiles in vertical orientation and in the respective inhibitor solutions were con-

Table 1 Sedimentation of amyloplasts in the gravity-sensing cells of rice coleoptiles. 1 h pretreatment with water (control), with 1 mM EPC, or with 10 µM taxol, followed by inversion. 79–88 cells from at least 8 coleoptiles were scored for each treatment with respect to amyloplast distribution immediately (0 sec) and 45 and 120 sec after inversion of the coleoptiles. The positions of individual amyloplasts were not quantified, but their predominant localization or clustering was estimated by eye. Values give % frequencies of cells with predominant amyloplast localization in five different zones of the cells. *Basal, up:* cells with clear clustering of amyloplasts in the basal, now upper, end (no displacement); *between basal and centre:* predominant localization in the second quarter; *centre:* cells with amyloplasts spread over the two central quarters; *between centre and apical, down:* predominant localization in the third quarter; and *apical, down:* cells with clustering in the apical, now lower, end (completed displacement)

		0 sec	45 sec	120 sec
Control	basal, up	65	6	0
		24	23	0
	centre	11	52	42
		0	19	47
	apical, down	0	0	11
+ EPC	basal, up	57	0	0
		28	34	9
	centre	15	44	32
		0	22	48
	apical, down	0	0	11
+ Taxol	basal, up	75	59	45
		18	29	35
	centre	7	8	17
		0	3	3
	apical, down	0	1	0

Table 2 Effect of 5 µM NPA and 1 mM EPC on lateral and longitudinal [³H]IAA transport. Lateral auxin transport was measured for 150 min with 9 mm coleoptile segments as described in Fig. 1. cpm in Receiver$_{up}$, facing upwards, and cpm in Receiver$_{down}$, facing downwards, are shown along with the standard errors of the mean. The sample size was 18 replicates in the control, 10 with NPA and 14 with EPC. Lateral transport asymmetry is defined as (Receiver$_{down}$ – Receiver$_{up}$)/(Receiver$_{down}$ + Receiver$_{up}$). After the lateral transport, two segments for each treatment were cut in apical and basal halves, and their radioactivity (cpm) was determined. The ratio b/a, of [³H]IAA appearing in the basal half over the cpm in the apical half, is taken as a measure of longitudinal transport

	Lateral Transport			Longitudinal Transport		
	Receiver$_{up}$	Receiver$_{down}$	Asymmetry	Apical half	Basal half	b/a
Control	64 ± 14	146 ± 40	0.40	1004	462	0.46
+ NPA	61 ± 15	65 ± 14	0.03	1209	66	0.05
+ EPC	105 ± 28	112 ± 36	0.03	1132	273	0.24

tinuously illuminated for 150 min from one side with blue light (fluence rate 1 µmol m^{-2} s^{-1}). For assessment of the gravitropic response, the cuvettes with the coleoptiles were placed horizontally for 150 min in the dark. Finally, all coleoptiles, still in the cuvettes, were photographed, and the angle of phototropic or gravitropic bending was measured on the enlarged projected negatives.

Results

Effects of cytoskeletal drugs on amyloplast mobility

For a measurement of statolith movement, rice coleoptiles were examined microscopically. The inner tissues in the apical 5 mm were rich in amyloplasts and their abundance decreased towards the base. Amyloplasts were especially numerous in the bundle sheath, but also all over the ground parenchyma. In all of these cells, the amyloplasts were found to be highly mobile. Upon inversion of the coleoptile they sedimented to the cell's apical half within 120 sec (Table **1**, Control).

When coleoptiles were pretreated with EPC the velocity of amyloplast sedimentation was found to be similar to the controls (Table **1**, + EPC). Pretreatment with 20 µM cytochalasin D again resulted in similar amyloplast sedimentation (data not shown). In contrast, upon pretreatment with taxol, which causes bundling of microtubules, amyloplasts moved more slowly with most of them still in the upper half of the cells after 120 sec (Table **1**, + Taxol).

Effects of cytoskeletal drugs on auxin transport

Lateral transport of ³H-IAA in untreated rice coleoptile segments became detectable 20–40 min after the beginning of horizontal stimulation and persisted for longer periods (data not shown). NPA, the known and potent auxin transport inhibitor (Morgan, 1964[15]), and the antimicrotubular drug EPC were tested in parallel assays on lateral and basipetal IAA transport (Table **2**). Pretreatment with 5 µM NPA strongly diminished the amount of auxin arriving in basal half of segments and abolished the lateral asymmetry almost completely. Pretreatment with 1 mM EPC eliminated the lateral ³H-IAA asymmetry almost completely, similar to the specific auxin transport inhibitor NPA (Table **2**, left). However, while 5 µM NPA blocked basipetal auxin transport, EPC reduced longitudinal transport by less than 50 % (Table **2**, right). This finding was supported by further longitudinal auxin transport experiments using a standard donor segment receiver set-up with 9 mm rice coleoptile segments (10 replicates). Transport after 150 min yielded 706 ± 69 cpm in the control receiver blocks, and 419 ± 52 cpm after 1 mM EPC treatment, i.e., only a 41 % inhibition of longitudinal IAA transport.

The dose–response curve of EPC on lateral auxin asymmetry was estimated (data not shown). Concentrations above 300 µM appeared to inhibit efficiently; no inhibition, occasionally even a stimulation, of lateral transport was observed at 10 µM EPC.

A weaker (30 to 40%) inhibition of lateral ³H-IAA transport was seen after treatment of coleoptiles with 1 mM solutions of oryzalin or colchicine, two compounds that can dissociate microtubules (Morejohn, 1991[14]; Blancaflor and Hasenstein, 1996[3]).

Taxol, the drug causing microtubular bundling and slowing down statolith sedimentation (Table **1**), was also tested for its effects on lateral auxin transport. In tests similar to those shown in Table **2**, pretreatment of coleoptile sections with 10 µM taxol, a concentration close to the limit of solubility, reduced lateral ³H-IAA transport only partially, to 65 % of the

control. Using 0, 2, 5 and 10 μM taxol, with at least 6 replicates each, the lateral auxin asymmetry was as follows: control, 0.43; taxol at 2 μM, 0.33; at 5 μM 0.28, and at 10 μM 0.28. Upon longitudinal transport, 10 μM taxol had no inhibitory effect (data not shown).

Cytochalasin D (at 20 μM) inhibited lateral transport of auxin only by about 30%. Longitudinal auxin transport was inhibited to the same extent, consistent with previous results obtained with cytochalasin B in maize coleoptiles (Cande et al., 1973[4]).

Cytoskeletal drugs on gravitropic and phototropic bending

Effects of EPC and taxol on gravitropism and on 2nd positive phototropism of rice coleoptiles were measured according to Neumann and Iino (1997[16]) and compared (Fig. **2**). Seedlings were grown and tested under submersed conditions in order to obtain an easily measurable phototropic response. Treatment with EPC resulted in a distinct reduction of gravitropic curvature, whereas the phototropic response remained at the control level. Taxol treatment only partially reduced gravitropic curvature. Again, the phototropic response was the same as in the controls (Fig. **2**). This suggests that the inhibition of gravitropic curvature by EPC and the partial inhibition by taxol was not caused by a direct action on the capacity for asymmetric growth.

In order to obtain significant phototropic bending (see Neumann and Iino, 1997[16]) the tropism experiments shown in Fig. **2** were performed with submersed rice coleoptiles, where growth and curvature responses are increased, up to 5 times for phototropism, compared to coleoptiles growing in air. In addition to these curvature tests with submersed coleoptiles (Fig. **2**), time-course studies of gravitropic bending of air-grown, 5 d old rice coleoptiles were also performed. After 90 min horizontal exposure, controls curved 23° ± 9°, while barely detectable 0° ± 2° bending was measured following treatment with 0.5 mM EPC. After 3 – 4 h, a recovery of the response under EPC was observed, accompanied by a partial reappearance of microtubules (Nick, unpubl.).

Discussion

Microtubular drugs interfere with various steps of the presumed gravitropic transduction chain. The results presented will be discussed in the context of the following hypothesis proposed to account for the transduction from mechanical force of statoliths to local stimulation of auxin export. Movement and/or pressure of amyloplasts in the gravitational field is received by elements of the cytoskeleton, such as actin filaments or cortical microtubules, which transmit the stimulus either via mechanosensitive ion channels (Ding and Pickard, 1993[5]), or directly, to the auxin efflux carriers in the adjacent plasmalemma region.

Concerning actin microfilaments, cytochalasin D at concentrations that destroy these structures, did not visibly influence amyloplast sedimentation, or specifically impair gravitropically induced auxin asymmetry, or block gravitropic curvature in rice coleoptiles (data not shown). This agrees with evidence from Staves et al. (1997[29]) that cytochalasin D did not inhibit root gravitropism in three species. It can be inferred that actin
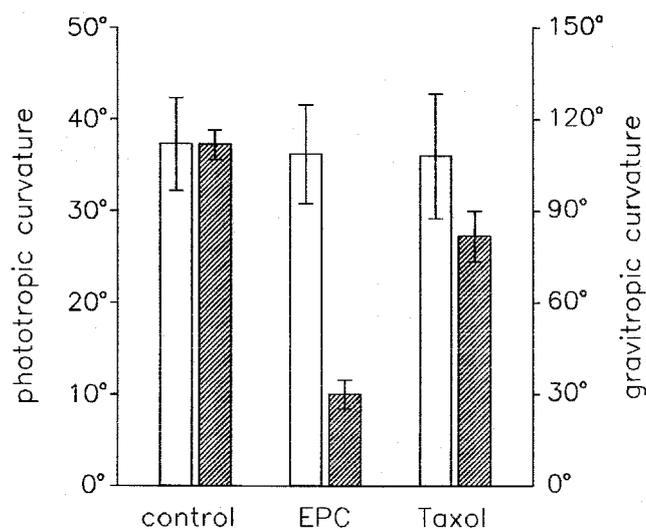
Fig. 2 Effect of microtubular drugs on gravitropic and phototropic curvature of submersed rice coleoptiles. EPC or taxol were applied to the surrounding water. Phototropic curvature (2nd positive) was assayed according to Neumann and Iino (1997[16]), with continuous, unilateral broad-band blue illumination (fluence rate 1 μmol m^{-2} s^{-1}). Values represent the mean curvature (and standard errors of the mean) after 150 min of gravitropic (hatched bars) or phototropic (open bars) stimulation. n = 8.

filaments are not essential to transmit the gravitational stimulus to the auxin transport system in the plasmalemma.

Taxol, which leads to bundling of microtubules, decreases the mobility of amyloplasts (Table **1**). This could indicate a physical contact between the amyloplast statoliths and microtubules in the gravity-sensing cells. Such a contact has been demonstrated in moss protonemata (Wacker et al., 1988[32]).

Only partial inhibition of lateral auxin transport was observed after treatment with taxol. The concentrations used were similar to those reported to suppress tubulin depolymerisation in cytosolic extracts from maize and rice coleoptiles (Nick et al., 1995[20]).

It may be concluded that microtubule bundles are sufficient to transmit the gravitational stimulus from the amyloplasts to the auxin transport system. The decrease in amyloplast sedimentation rate observed after taxol treatment (Table **1**) was in the order of a few minutes while the lateral auxin transport was measured over more than two hours.

While taxol leads to bundling of microtubules and to slower statolith movement, drugs with depolymerizing action – such as EPC or oryzalin – should allow faster sedimentation. In rice coleoptiles, amyloplast movement was already fast in controls, and an acceleration in the presence of EPC could not be demonstrated (Table **1**). In the cress root cap, however, where amyloplast sedimentation is slower than in rice, the rate of sedimentation was elevated after microtubule elimination with oryzalin (Sievers and Hejnowicz, 1994[28]).

The gravitropically-induced lateral auxin transport is severely impaired after treatment with 1 mM EPC (Table **2**). Beyond the published findings (Nick et al., 1991[17]) on EPC inhibition of

gravicurvature, the results presented here show that lateral IAA transport is blocked by this drug while basipetal auxin transport can still continue. EPC affects the graviresponse of the auxin transport process presumably prior to and independent of cell elongation. It should be noted that the dose–response relation of EPC on gravity-induced lateral IAA transport is comparable with the action of this drug upon polymerization of plant tubulin *in vitro* (Mizuno and Suzaki, 1990[13]).

EPC inhibits gravitropism while leaving phototropism unimpaired (Fig. **2**). The latter can be interpreted as a marker for the capacity for asymmetric growth. The observed difference between photo- and gravitropic bending of rice coleoptiles in terms of drug sensitivity is consistent with previous observations in maize coleoptiles (Nick et al., 1991[17]) and reconfirms the conclusion that the microtubular drug EPC affects the graviresponse prior to and independent of cell elongation.

From these results with EPC it can be inferred that complete depolymerization of microtubules severely impairs the transduction of force from the amyloplasts to auxin transport at the plasmalemma. Comparing the action of taxol and of EPC, it appears that microtubule bundles are adequate in the transduction chain from statoliths to asymmetric auxin flow, but depolymerization of microtubules apparently eliminates an essential element from this chain.

But, even among depolymerizing drugs, there might be different inhibitory effects on IAA lateral transport, probably caused by different drug binding sites that can be attributed to specific tubulin isotypes (Yamamoto et al., 1998[35]). This could explain results from Blancaflor and Hasenstein (1996[3]) who found very little, if any, inhibition of gravitropic bending in maize roots treated with oryzalin that eliminated the bulk of microtubules.

Recently, mutants of gravity responses have been analysed in *Arabidopsis,* and the gene *ARG1,* affecting an early step in the perception has been cloned and sequenced (Sedbrook et al., 1999[27]). Interestingly, the encoded protein has membrane domains and resembles a protein interacting with cytoskeletal elements, suggesting a role for the cytoskeleton in early gravitropic transduction. A further, phylogenetic argument can be adduced from the role of microtubules in amyloplast-controlled moss protonemal gravitropism. Schwuchow et al. (1990[26]) and Walker and Sack (1990[33]) report that in presence of oryzalin microtubules disappear, and protonemata show no measurable curvature upon horizontal exposure.

Acknowledgements

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