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Acrylamide inhibits gravitropism and affects microtubules in rice coleoptiles

C. Gutjahr[1,*] and **P. Nick**[2]

[1] Institut für Biologie II, Albert-Ludwigs-Universität Freiburg, Freiburg im Breisgau
[2] Botanisches Institut 1, Universität Karlsruhe, Karlsruhe

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Summary. To find components which participate in gravitropic signal transmission, we screened different cell biological inhibitors for their effect on gravitropic bending of rice coleoptiles. Acrylamide, which is known to affect intermediate filaments in mammalian cells, strongly inhibited gravitropic bending at concentrations that did not inhibit growth of coleoptile segments. This inhibition was reversible. Investigating the acrylamide effect further, we found that it interferes with an event that occurs around 15 min after the onset of stimulation. We also observed that acrylamide inhibits polar indolyl-3-acetic acid transport. Furthermore, acrylamide efficiently eliminated microtubules, whereas actin filaments remained intact. To our knowledge this is the first report of effects of monoacrylamide in plant cells.

Keywords: Acrylamide; Auxin transport; Gravitropism; Microtubule; *Oryza sativa* L.; Rice.

Abbreviations: BFA brefeldin A; EPC ethyl-N-phenylcarbamate; IAA indolyl-3-acetic acid; IF intermediate filament; MT microtubule; NPA 1-N-naphthylphthalamic acid.

Introduction

Gravitropism follows the basic scheme of stimulus physiology that divides the syndrome into three principal steps: stimulus perception, signal transmission, and reaction. Gravity is perceived by the physical changes of masses within the cell. Among the different theories of gravity perception, such as the protoplast pressure model (Wayne and Staves 1996), the starch statolith model (Nemec 1900, Haberland 1900, Kiss and Sack 1990, Kuznetsov and Hasenstein 1996) has been widely accepted for higher plants (Kiss 2000). Statoliths are dense amyloplasts that sediment to the physiological bottom of statocysts (statolith-containing cells). It is hypothesized that they put weight either on the endoplasmic reticulum or on parts of the plasma membrane (Sack 1997) and/or pull on cytoskeletal elements (Sievers et al. 1991, Baluška and Hasenstein 1997, Godbolé et al. 2000). This might activate mechanosensitive Ca^{2+} channels (for a review, see Chen et al. 1999). The response of asymmetric growth in roots and shoots is explained by the Cholodny–Went theory (Went 1926, Cholodny 1927; for a review, see Muday 2001, but see Trewawas 1992): Upon gravitropic stimulation, auxin is transported laterally in the direction of the gravity vector such that an auxin gradient is established across the organ. This gradient leads to differential growth and curvature. Although there are many reports on the role of Ca^{2+}, calmodulin, K^+, proton fluxes, and membrane potential changes upon gravitropic stimulation (for a review, see Fasano et al. 2002), the signal transduction processes that translate the physical signal of statolith sedimentation into the chemical message of auxin transport have remained elusive.

To obtain an indication of possible factors involved in transmission events between statolith sedimentation and the lateral auxin gradient, we screened 19 substances for their possible effects on gravitropic bending of rice coleoptiles. Rice coleoptiles were used for this study because they are endowed with a very efficient gravitropic response even when they are submersed. Moreover, their cuticle is thin and therefore readily permeated by a broad

* Correspondence and reprints (present address): Laboratoire de Génétique Végétale, Sciences III, Université de Genève, 30 Quai Ernest-Ansermet, 1211 Genève, Switzerland.
E-mail: carogutj@hotmail.com

range of pharmacological agents. Among the inhibitors were Ca^{2+} channel inhibitors, physiologically active ions, hormones, hormone transport inhibitors, and cytoskeletal drugs. Cytoskeletal drugs were included to possibly identify new functions of the plant cytoskeleton. Gravitropism represents a physiological marker activity that is highly sensitive to alterations of the cytoskeleton. For instance, a gradient in the orientation of cortical microtubules (MTs) is an essential element of the effector system (Nick et al. 1990, Himmelspach and Nick 2001), and a highly dynamic MT population seems to participate in gravitropic sensing (Nick et al. 1991, Godbolé et al. 2000). Microfilaments seem to negatively regulate gravitropic bending, since actin-depolymerizing drugs enhance gravitropic curvature (Nick et al. 1997, Yamamoto and Kiss 2002). Among the different inhibitors, acrylamide inhibited gravitropic bending most strongly without affecting the effectual apparatus of cell growth.

Acrylamide is widely used to identify functions of intermediate filaments (IFs) in mammalian cells, since it causes IF disorganization while leaving other cytoskeletal components mostly intact (Durham et al. 1983, Eckert 1985, Shabana et al. 1994, but see Aggeler and Seely 1990). This effect is attributed to inhibition of a protein kinase dependent on cyclic AMP that is responsible for signaling to IFs (Eckert and Yeagle 1988). To our knowledge, monoacrylamide has not yet been used to probe for potential functions of IFs in plants. Polyacrylamide is used in agriculture to prevent soil erosion (Seybold 1994), to increase the water-holding capacity of sandy soils (Silberbush et al. 1993), or as a coating material for controlled fertilizer release (Abraham and Pillai 1995). Residual monoacrylamide is very slowly taken up by plant roots and metabolized in the plant via acrylic acid (Raid 1998). In phytotoxicity tests, Raid (1998) found that growth of suspension culture cells from 15 different crop species was reduced by 50% at acrylamide concentrations between 1 and 10 mM, depending on the respective species. Apart from this test, nobody has yet, to our knowledge, looked for specific effects of monoacrylamide on the physiology of plant cells.

The aim of this study was thus to test the specificity of the acrylamide effect on gravitropism. We could show that acrylamide interferes with a gravitropic event that occurs between 15 and 30 min after the onset of stimulation, i.e., around the time when the lateral auxin gradient emerges (Gutjahr et al. 2005), it inhibits auxin transport, and it destroys MT bundles in the epidermis but leaves actin filaments intact.

Material and methods

Plant material, treatment of seedlings and coleoptiles

Seedlings of rice (*Oryza sativa* L. subsp. *japonica* cv. Nihonmasari) were raised for 5 days at 25 °C in photobiological darkness (wrapped in black cloth and placed in light-tight black boxes) under aerial conditions.

For curvature assays, rice seedlings were raised as described by Riemann et al. (2003). Five rice seeds were fixed, embryo up, with medical glue ("B401"; Factor II Inc., Lakeside, Ariz, U.S.A.), 5 mm below the edge of a microscopy slide (Fig. 1). The slides were placed in conventional staining trays in Plexiglas boxes (95 by 95 by 60 mm). The Plexiglas boxes were filled with deionised water so that the seeds were only partially covered to ensure optimal oxygen access and germination.

For elongation assays and indolyl-3-acetic acid (IAA) transport measurements, seeds were sown equidistantly, embryo up, on floating plastic meshes in Plexiglas boxes (95 by 95 by 60 mm, 25 seeds per box) as described by Nick and Furuya (1993).

All manipulations of seedlings described in the following paragraphs were performed under green safelight (λ_{max} = 550 nm).

Determination of gravitropic curvature: dose–response curves and reversibility of acrylamide effect

Seedlings fixed on microscopy slides and grown in darkness for 5 days were submerged in different solutions in conventional slide-holders used for histology. Deionized water was used for all solutions. Gravitropic stimulation was provided by tilting the whole slide holder by 90°. To determine curvature angles, the glass slides with seedlings were photocopied and the angles measured with a protractor (Fig. 1). The temperature in the green-light room ranged from 22 to 28 °C. All points in a given graph were recorded at the same time and thus at the same temperature.

For dose–response curves, seedlings were preincubated vertically for 1 h in acrylamide, brefeldin A (BFA), 1-N-naphthylphthalamic acid (NPA), or ethyl-N-phenylcarbamate (EPC) solutions of different concentrations and then gravitropically stimulated for 2 h. Deionized water served as control. Acrylamide (Rothiphorese Gel A; Roth, Karlsruhe, Federal Republic of

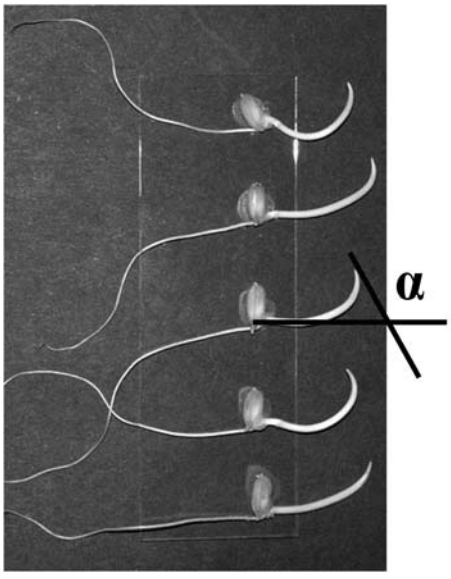

Fig. 1. Rice coleoptiles after 2 h of gravitropic stimulation at 90°. Curvature angles were measured between two lines drawn (always by the same person in the same way) along the mesocotyl (most vertical part of the coleoptile, below the node) and the outer edge of the bent coleoptile tip

Germany) concentrations ranged from 14 to 700 mM, BFA concentrations from 1 to 30 μM, NPA concentrations from 0.1 to 30 μM, and EPC concentrations from 0.1 to 5 mM. NPA was a kind gift form Dr. W. Michalke (Institute of Biology III, Albert-Ludwigs-Universität Freiburg, Freiburg, Federal Republic of Germany) and synthesized according to Thompson et al. (1973), BFA was purchased from Sigma-Aldrich (Neu-Ulm, Federal Republic of Germany) and EPC (common name phenyl urethane) from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

To test the reversibility of the acrylamide effect, seedlings were incubated vertically for 30 min in 100 mM acrylamide or in deionized water as a control. After 3 washings for 5 min each in distilled water (1 liter per 20 seedlings), the coleoptiles were gravitropically stimulated for 2 h at 90° in water for the control and washout samples or in 100 mM acrylamide for the acrylamide sample.

Elongation assays with segments and intact coleoptiles

The growth of coleoptile segments was measured as described by Wang and Nick (1998). Segments with a length of 10 mm were excised, 3–13 mm below the tip, from seedlings grown on floating plastic meshes for 5 days in complete darkness and incubated under continuous rotation on a topover shaker in complete darkness for 5 h in 140 mM acrylamide, 10 μM BFA, 1 μM NPA, 1 mM EPC, or several other inhibitors (names and concentrations are listed in Table 1) or deionized H_2O as a control. Length increments after 5 h were determined under a stereomicroscope.

For elongation assays of intact coleoptiles, seedlings were fixed on microscopy slides and grown in complete darkness. Coleoptile lengths were measured under green safelight, and the complete seedlings were submerged for 5 h in (1) 5 mM morpholineethanesulfonic acid (MES) buffer, pH 7.0, as a control, (2) the same buffer with 0.1 μM IAA (Sigma-Aldrich), (3) with 140 mM acrylamide, or (4) with a combination of IAA and acrylamide. The pH was measured during the incubations with and without acrylamide and IAA, and found to be stable at 7.0. Subsequently, the coleoptile length was measured again and the growth increment calculated.

Viability check using the trypan blue dye exclusion test

For this test (Phillips 1973), coleoptiles were immersed for 5 min in trypan blue (Sigma-Aldrich), which was diluted 100-fold, and then rinsed with water. As blue staining of cells indicates cell death, the number of blue cells on two opposing flanks of coleoptiles treated with acrylamide was scored. The mean number (two independent experiments, n = 8) of blue cells obtained from treated coleoptiles was compared to that from control coleoptiles (two independent experiments, n = 8) that were incubated for 2 h in water instead of acrylamide before staining.

Relation of "after-bending" to stimulation time

Seedlings were preincubated vertically for 30 min in 140 mM acrylamide, 1 μM NPA, 10 μM BFA, or 1 mM EPC or in water as a negative control. Subsequently, they were gravitropically stimulated for 5, 10, 15, 30, 45, or 60 min in the same solutions, as described above. After gravitropic stimulation, they were rinsed and placed vertically in Plexiglas boxes for 1 h such that their roots were submerged in water. The "after-bending" resulting from the stimulus perceived in presence of the respective drug ("gravitropic memory" according to Brauner and Hager 1957) was measured as described above. In a control experiment (Fig. 3E), the coleoptiles were not preincubated, but 14 mM acrylamide were added simultaneously with the onset of gravitropic stimulation.

Asymmetric inhibition of curvature by acrylamide

A mixture of acrylamide and lanolin (Roth) with an acrylamide content of 10% and a water content of 30% was spread onto the whole length of the upper or lower flank of coleoptiles. The coleoptiles were gravitropically stimulated in a moist chamber for 3 h. As a control, a water-lanolin mixture (30% water) and coleoptiles without lanolin were used. The curvature angle was then measured.

Measurement of longitudinal IAA transport

The following procedure is a modified version of the method used by Godbolé et al. (2000). Five-day-old seedlings grown on floating plastic meshes in complete darkness were selected for straightness. Segments (5 mm long) were excised 3–8 mm below the tip, the primary leaf was carefully removed and the segments were incubated for 1h in the following solutions: (1) 5 mM MES buffer, pH 7.0, as a control; (2) 0.1 μM IAA in 5 mM MES buffer, pH 7.0; (3) 140 mM acrylamide in 5 mM MES buffer, pH 7.0; (4) 140 mM acrylamide plus 0.1 μM IAA in 5 mM MES buffer, pH 7.0.

For transport measurements, 5 vertically oriented coleoptile segments were sandwiched between two agar blocks (8 by 2 by 1 mm, 1.5% agar). Care was taken that the entire cut surfaces were in contact with the agar block and that the segments did not touch each other laterally to avoid IAA bypasses. The donor block at the apical surface contained 0.1 μM IAA and 0.1 μCi (1 Ci = 37 GBq) [^{14}C]IAA (American Radiolabeled Chemicals, St. Louis, Mo., U.S.A.), but no drugs. After incubation for 2 h in a moist chamber at 22 ± 1°C, donor and receiver blocks and segments were collected separately in 5 ml of Rotizint scintillation fluid (Roth) with 500 μl of 5 mM Tris buffer and moderately shaken overnight for equilibration. Radioactivity in counts per minute was determined by scintillation counting (LS 5000CE; Beckmann, Munich, Federal Republic of Germany). Basal values were determined with non-radioactive segments and plain agar blocks. To assure that only [^{14}C]IAA that was actually taken up by coleoptiles was taken into account as transported, the basal value was subtracted from counts per minute for segments and receptor blocks, and transport calculated in the following manner:

$$\text{transport}\ [\%] = [\text{cpm(receptor)}/(\text{cpm(receptor)} + \text{cpm(segments)})]100,$$

where cpm(receptor) is the number of counts recovered in the receptor block and cpm(segments) is the counts measured in the segment.

Inverted segments incubated in 5 mM MES buffer, pH 7.0, for 1 h were used as a negative control for basipetal transport.

Visualization of actin filaments and MTs

Actin filaments were labelled by transient expression of YFP-talin, as described by Holweg et al. (2004). The same cell was viewed before and after 1 h incubation of the whole coleoptile in 140 mM acrylamide.

For MT staining, 6-day-old coleoptiles grown on plastic meshes in complete darkness were used. Segments with a length of 10–15 mm were excised 3 mm below the tip and incubated for 1 h at 25 °C in 140 mM acrylamide or water as a control. MTs were subsequently stained as described by Toyomasu et al. (1994) with minor modifications. The blocking reagent was goat normal serum (Sigma, Karlsruhe, Federal Republic of Germany). Mouse monoclonal antiserum directed against α-tubulin (DM1A; Sigma) and diluted 50-fold in Tris-buffered saline (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.25% [v/v] Triton X-100) was used as the primary antibody. The secondary antibody was from sheep, directed against mouse immunoglobulin G and labeled with fluorescein isothiocyanate (FITC) (Sigma).

Cells were viewed under a confocal laser scanning microscope (DM RBE; Leitz, Bensheim, Federal Republic of Germany) using a True Confocal Scanner Leica TCS 4D (Leica, Heidelberg, Federal Republic of Germany) with an argon-krypton laser at 488 nm excitation, a beam splitter at 510 nm, and a 515 nm emission filter. Pictures were further processed by Photoshop 5.5 (Adobe Systems, Mountain View, Calif., U.S.A.).

Statistics

Means and standard errors were calculated by the Microsoft Excel 2000 statistical package. P values were calculated by a Mann–Whitney U test for nonparametric data by the SPSS 11.5 statistical program.

Results

Acrylamide inhibits gravitropism

Searching for factors that might play a role in gravitropic signal transduction, we screened 19 different cell biological inhibitors for their effect on gravitropic curvature of rice coleoptiles (Table 1). Acrylamide (widely used in mammalian cells to impair intermediate filaments, but so far not tested as an inhibitor in plant cells) inhibited gravitropic curvature most strongly (Table 1). As can be seen from the dose–response curve in Fig. 2A, increasing concentrations of acrylamide progressively inhibited gravitropic curvature. At a concentration of 140 mM, curvature was only 15% of the control. To see whether the effectual apparatus of elongation growth was harmed, we tested the effect of 140 mM acrylamide on elongation growth of coleoptile segments that are – by the nature of the experiment – depleted of endogenous auxin. The growth of these segments was not inhibited, even over a long time interval of 5 h (Table 1 and Fig. 2A, inset). In contrast, Ca^{2+} channel inhibitors (Gd^{3+}, La^{2+}, verapamil) that also reduced gravitropic curvature were found to virtually eliminate elongation growth (Table 1). The same was true for abscisic acid, tungstate, and trifluoperazine (Table 1). Only 4 compounds out of the 19 substances screened inhibited gravitropic curvature under conditions that left segment growth (which was independent of endogenous, transported auxin) more or less untouched (Table 1). Apart from acrylamide (Table 1 and Fig. 2A), the others were BFA, an inhibitor of vesicle transport (Table 1 and Fig. 2B), NPA, an inhibitor of auxin efflux (Table 1 and Fig. 2C), and EPC, a blocker of tubulin assembly (Table 1 and Fig. 2D).

Since acrylamide enters plant cells extremely slowly (Raid 1998), we used concentrations in the range of

Table 1. 20 substances tested for their effect on rice coleoptile gravitropic curvature and on coleoptile segment growth

Substance	Effect[a]	Source	Solvent[b]	Gravitropic curvature (deg)[c]	Segment increm. (mm/5 h)[c]
H_2O	control	Roth, Karlsruhe		122 ± 4	0.85 ± 0.11
10 mM K^+	physiol. active ion	Roth, Karlsruhe	H_2O	97 ± 5	0.28 ± 0.07
1 mM Ca^{2+}	physiol. active ion	Merck, Darmstadt	H_2O	86 ± 5	0.08 ± 0.04
1 mM EGTA	Ca^{2+} chelator	Roth, Karlsruhe	H_2O	65 ± 12	0.96 ± 0.09
500 μM trifluoperazine	calmodulin inhibitor	Sigma-Aldrich, Neu-Ulm	H_2O	66 ± 14	0.20 ± 0.06
1 mM La^{2+}	Ca^{2+} channel inhibitor	Fluka, Buchs	H_2O	86 ± 6	0.20 ± 0.08
500 μM Gd^{3+}	Ca^{2+} channel inhibitor	Sigma-Aldrich, Neu-Ulm	H_2O	99 ± 6	0.2 ± 0.07
1 mM verapamil	Ca^{2+} channel inhibitor	Sigma-Aldrich, Neu-Ulm	H_2O	79 ± 3	0.09 ± 0.05
100 μM ruthenium red	Ca^{2+} channel inhibitor	Roth, Karlsruhe	H_2O	70 ± 6	0.14 ± 0.06
20 μM cyclopiaonic acid	ER Ca^{2+} channel inhibitor	Sigma-Aldrich, Neu-Ulm	DMSO	95 ± 6	0.61 ± 0.12
1 μM abscisic acid	hormone	Sigma-Aldrich, Neu-Ulm	EtOH	77 ± 7	0.22 ± 0.06
10 μM norflurazon	ABA inhibitor	Novartis, Basel	EtOH	69 ± 7	0.90 ± 0.14
50 μM tungstate	ABA inhibitor	Sigma-Aldrich, Neu-Ulm	EtOH	86 ± 8	0.30 ± 0.08
1 μM naphthylphthalamic acid	auxin efflux inhibitor	W. Michalke, Freiburg	DMSO	35 ± 11	1.04 ± 0.1
100 nM epi-β-brassinolide	hormone	Sigma-Aldrich, Neu-Ulm	EtOH	161 ± 16	ND[d]
10 mM butanedione monoxime	myosin inhibitor	Sigma-Aldrich, Neu-Ulm	H_2O	42 ± 6	ND
20 μM taxol	microtubule stabilizer	Sigma-Aldrich, Neu-Ulm	DMSO	89 ± 5	0.47 ± 0.1
1 mM ethyl-N-phenyl carbamate	microtubule inhibitor	Wako Pure Chemical Industries, Osaka	EtOH	35 ± 5	0.76 ± 0.15
140 mM acrylamide	?	Roth, Karlsruhe	H_2O	18 ± 6	0.94 ± 0.08
10 μM brefeldin A	vesicle transport inhibitor	Sigma-Aldrich, Neu-Ulm	DMSO	20 ± 4	0.85 ± 0.14
3 mM ascorbic acid	ROS scavenger, reducing agent	Sigma-Aldrich, Neu-Ulm	H_2O	15 ± 6	0.54 ± 0.07

[a] ER, endoplasmic reticulum; ABA, abscisic acid; ROS, reactive oxygen species
[b] DMSO, dimethyl sulfoxide; EtOH, ethyl alcohol
[c] Results are means with standard errors (n = 20)
[d] ND, not determined

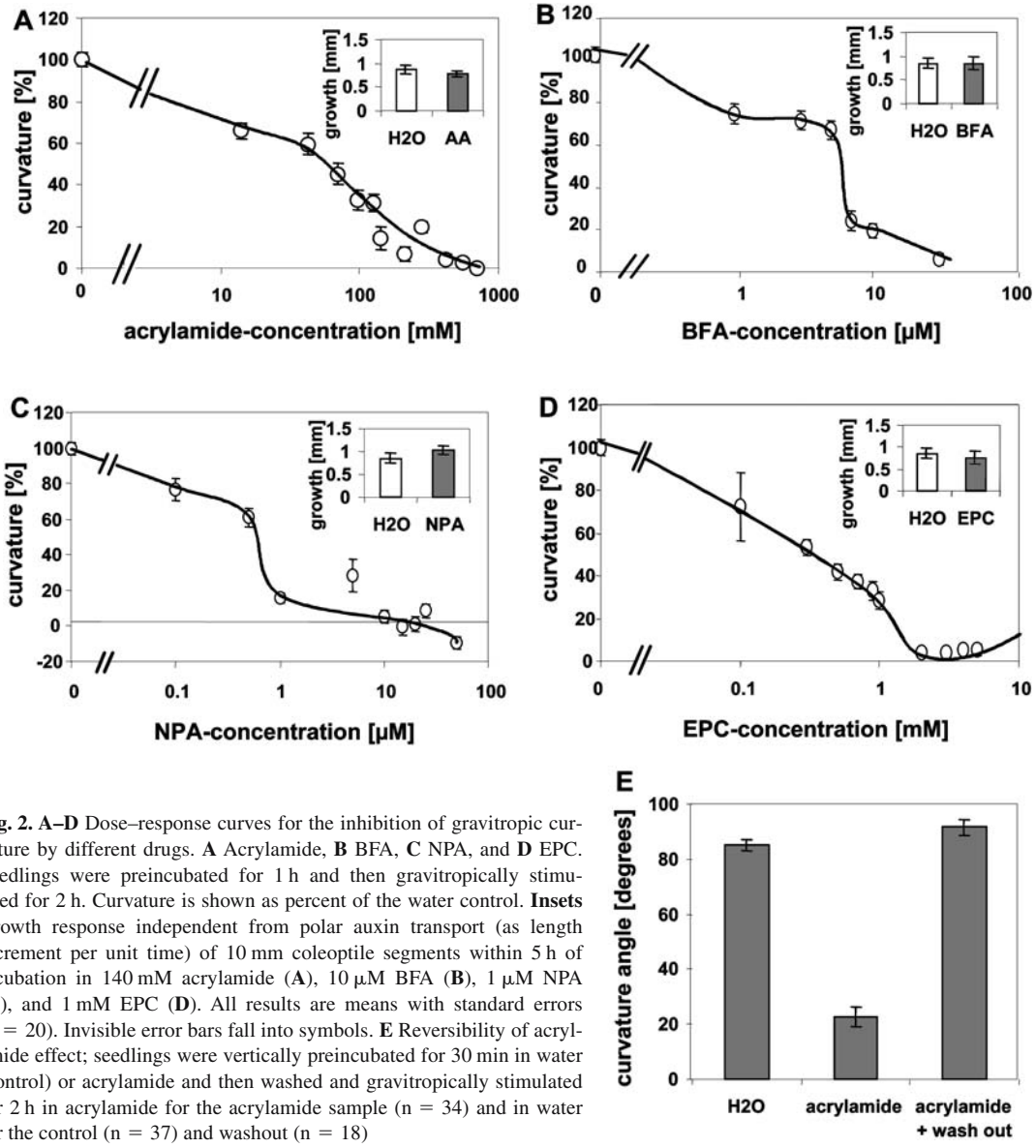

Fig. 2. A–D Dose–response curves for the inhibition of gravitropic curvature by different drugs. **A** Acrylamide, **B** BFA, **C** NPA, and **D** EPC. Seedlings were preincubated for 1 h and then gravitropically stimulated for 2 h. Curvature is shown as percent of the water control. **Insets** Growth response independent from polar auxin transport (as length increment per unit time) of 10 mm coleoptile segments within 5 h of incubation in 140 mM acrylamide (**A**), 10 µM BFA (**B**), 1 µM NPA (**C**), and 1 mM EPC (**D**). All results are means with standard errors (n = 20). Invisible error bars fall into symbols. **E** Reversibility of acrylamide effect; seedlings were vertically preincubated for 30 min in water (control) or acrylamide and then washed and gravitropically stimulated for 2 h in acrylamide for the acrylamide sample (n = 34) and in water for the control (n = 37) and washout (n = 18)

tenfold those that are used in mammalian cells. These concentrations cause an alkalization of the medium. For instance, 140 mM acrylamide produced a pH of 7.8, which remained stable for the course of the experiment. We tested the possibility that curvature is inhibited by this basic pH; however, there was no significant difference in gravitropic curvature between coleoptiles incubated in water (pH 7.0) or at pH 7.8 (data not shown). We also used trypan blue dye exclusion to test whether the cells remained viable during the 2 h of incubation in 140 mM acrylamide and we did not observe any decrease of viability during this period (data not shown). Figure 2E shows that the acrylamide effect on gravitropic bending is reversible. After treatment with acrylamide for 30 min and subsequent washout, coleoptiles bent to the same degree as the controls which had only been treated with water.

Gravitropic sensitivity is confined to the apical 3 mm of rice coleoptiles (Gutjahr et al. 2005), whereas gravitropic curvature takes place a considerable distance away, nearer to the base. This means that stimulus perception and response are spatially separated, such that the stimulus must be transmitted over several millimeters. When a coleoptile is gravitropically stimulated for a relatively short period and then returned to the vertical position, there is no curvature. However, the spatial signal has already been generated and can be transmitted to the effector tissue causing a so-called after-bending ("Nachkrümmung"; Brauner and Hager 1957). To obtain further insight into the event

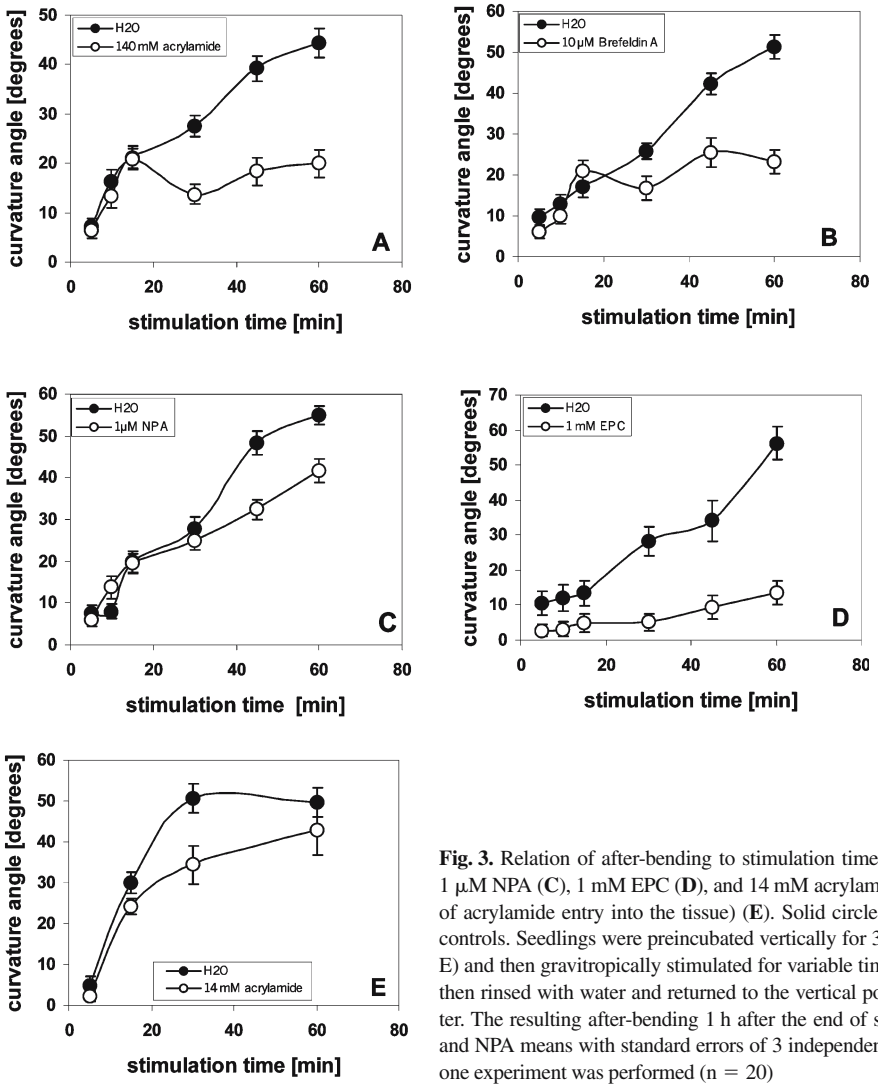

Fig. 3. Relation of after-bending to stimulation time for 140 mM acryl-amide (**A**), 10 µM BFA (**B**), 1 µM NPA (**C**), 1 mM EPC (**D**), and 14 mM acrylamide without preincubation (as control for velocity of acrylamide entry into the tissue) (**E**). Solid circles, inhibitor-treated seedlings; open circles, water controls. Seedlings were preincubated vertically for 30 min (except for the experiment shown in panel E) and then gravitropically stimulated for variable time intervals as indicated on the x-axis. They were then rinsed with water and returned to the vertical position such that the roots were covered with water. The resulting after-bending 1 h after the end of stimulation was measured. For acrylamide, BFA, and NPA means with standard errors of 3 independent experiments are shown (n = 60), for EPC only one experiment was performed (n = 20)

affected by acrylamide, we used the after-bending to determine the stage of the gravitropic reaction that is inhibited by acrylamide. For this purpose, the gravitropic reaction was dissected by means of stimulation-time kinetics. Coleoptiles were gravitropically stimulated in a solution of 140 mM acrylamide for variable time intervals. To assure that the stimulation-time kinetics were not influenced by the speed of tissue penetration at the given concentration, we preincubated the coleoptiles for 30 min in acryl-amide in a vertical position before initiating gravitropic stimulation. In preliminary experiments, where acryl-amide was added simultaneously with the onset of stimul-ation, we had observed that even a tenfold lower concentration of 14 mM significantly inhibited gravitropic bending within 30 min (Fig. 3E). This means that after a period of 30 min a concentration of acrylamide sufficient to inhibit its gravitropic target event should have entered the tissue. After stimulation, the after-bending that had developed in the vertical position after 1 h was measured and plotted against the stimulation time. In water-treated controls, the "after-bending" increased steadily with the stimulation time. The acrylamide-treated coleoptiles displayed the same bending as the control for stimulation times of up to 15 min. However, for times exceeding 15 min, after-bending did not increase further, but remained in the range of a stimulus of 15 min duration, similar to the experiment without preincubation (Fig. 3A, E). It should be noted that under the conditions of this experiment no curvature could be observed prior to 30 min after the onset of stimulation. Significant after-bending (assessed 1 h after the coleoptiles had been turned to the vertical) was already observed for stimulation times of

5–10 min (Fig. 3A–E). Thus, the gravitational signal is already perceived after 5 min of stimulation. The event that is primarily inhibited by acrylamide must therefore occur between 15 and 30 min after the onset of stimulation. From this we conclude that gravitropic signal perception is acrylamide-insensitive, whereas a later phase of the gravitropic reaction, which could well be a step in signal transmission, is inhibited.

To further characterize the physiological target of acrylamide in the context of gravitropism, we compared the relation of after-bending to stimulation time for acrylamide with those for other inhibitors with known effects on gravitropism, namely, BFA (vesicle transport), NPA (auxin efflux), and EPC (MT assembly) (Fig. 3B–D). Inhibitor concentrations were chosen such that they inhibited gravitropic curvature to the same degree as 140 mM acrylamide (the respective dose–response curves are shown in Fig. 2B–D). For BFA, we chose a concentration of 10 μM; for NPA, 1 μM; and for EPC, 1 mM. As for acrylamide, coleoptiles were preincubated vertically for 30 min to allow the entry of the respective inhibitors before the start of gravitropic stimulation. In the case of BFA, the relation of after-bending to stimulation time was very similar to that for acrylamide (Fig. 3B). For NPA, the inhibition became detectable from about 30 min of stimulation time and was not as pronounced as that of acrylamide and BFA – moreover after-bending increased further when stimulation times increased (Fig. 3C). In contrast, EPC inhibited gravitropic curvature from the very beginning of the gravitropic reaction (Fig. 3D), indicating that it interferes with early stimulus perception (see also Godbolé et al. 2000).

Acrylamide inhibits IAA transport

We addressed the question of whether acrylamide acts at the level of the lateral auxin gradient, which drives the differential growth responsible for gravitropic bending. Acrylamide was mixed with lanolin and spread on either the upper or lower flank of coleoptiles that were subsequently gravitropically stimulated. Curvature was much more strongly inhibited when the acrylamide-lanolin mixture was applied to the lower flank of the coleoptile than when it was applied to the upper flank (80% greater inhibition, Fig. 4A). In a control experiment, lanolin mixed with H_2O was applied. Here the curvature difference between coleoptiles treated on the lower and upper flanks was much smaller (35% greater inhibition when the lower flank was treated, Fig. 4A).

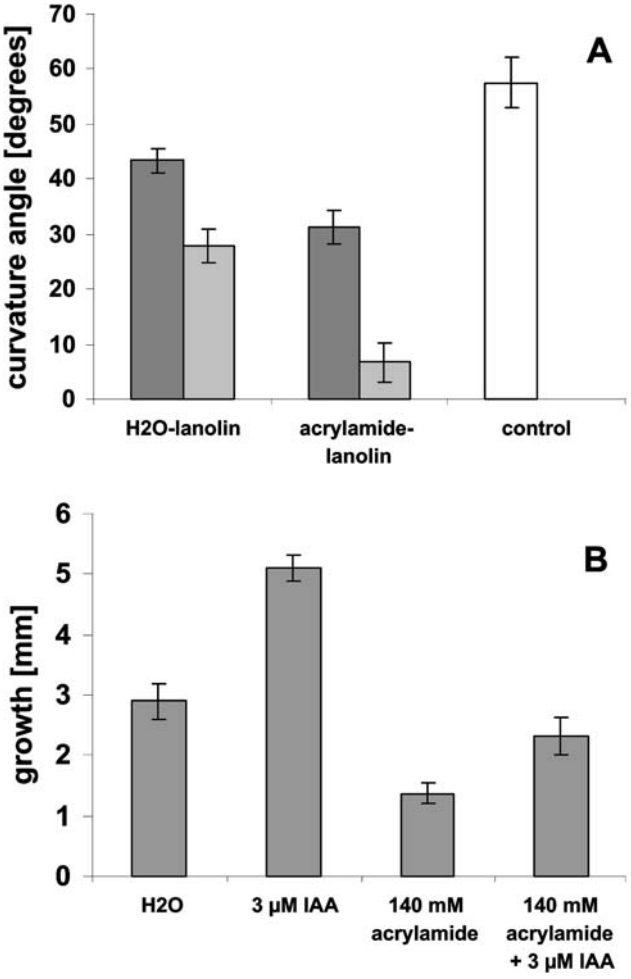

Fig. 4. A Differential inhibition of the gravitropic curvature by acrylamide. A 10% acrylamide-lanolin mixture was spread along the upper and lower flanks of coleoptiles. A H_2O-lanolin mixture and coleoptiles without lanolin (white bar) served as controls. The coleoptiles were stimulated for 3 h in a moist chamber and the curvature angle was measured. Means with standard errors of 3 independent experiments (n = 60) are shown. Dark gray, upper flank; light gray, lower flank. **B** Inhibition of IAA-induced growth of intact coleoptiles by acrylamide: Growth increment of intact coleoptiles within 5 h of incubation in the solutions indicated on the x-axis is shown. Error bars indicate standard errors, n = 20

BFA has been shown to affect the localization of PIN proteins, which are thought to be components of the IAA transport machinery (Robinson et al. 1999, Geldner et al. 2001). The similarity of the stimulation-time kinetics (Fig. 3) for acrylamide, BFA, and the IAA efflux inhibitor NPA and the result of the lanolin-acrylamide treatment suggest that acrylamide somehow interferes with auxin signaling. Four possibilities exist: IAA-induced growth at the level of the response, auxin sensitivity, polar IAA transport, or even IAA metabolism (synthesis, conjugation, degradation) could be inhibited by acrylamide. To discriminate between these possibilities we tested the effect of acrylamide (1) on the

growth of intact coleoptiles (no washout of endogenous auxin in contrast to coleoptile segments) that were kept vertically and (2) on longitudinal IAA transport.

The elongation assay tested whether a possible growth inhibition by acrylamide can be restored by applying exogenous IAA. Figure 4B shows that 140 mM acrylamide inhibited the growth of intact coleoptiles by about 50%. Reduced growth could be restored by simultaneously adding 3 μM IAA, such that the growth rate was similar to that of untreated coleoptiles. This again shows that acrylamide does not destroy the growth machinery of rice coleoptiles as such, and also that the growth block is related to a limited availability of, or reduced sensitivity to, endogenous auxin.

To distinguish between these two possibilities, we determined auxin transport directly. Coleoptile segments were incubated in 140 mM acrylamide for 1 h before measurements were started. Since IAA is washed out from coleoptile segments upon decapitation, we also tested how the addition of 0.1 μM IAA (corresponding to the endogenous concentration of IAA in this tissue, see Riemann et al. [2003]) influences auxin transport. Longitudinal transport relative to the water-treated control is given in the following. After treatment with 140 mM acrylamide, IAA transport was strongly and significantly reduced to only 31% ± 11% of the control value ($P = 0.002$). When 0.1 μM IAA as added, the effect of acrylamide was slightly weaker (IAA transport was reduced to 55% ± 20% of the control value). However, this difference was not significant at the 95% level ($P = 0.09$). Exogenous IAA also slightly enhanced IAA transport in the absence of acrylamide (to 126% ± 8% of the water-treated control value, $P = 0.115$).

Acrylamide destroys MTs but not actin filaments

Coleoptile gravitropism and auxin transport are very sensitive to manipulations of the cytoskeleton (Nick et al. 1991, Wang and Nick 1998, Godbolé et al. 2000). Therefore, we tested whether acrylamide changes the stability or distribution of MTs and/or actin filaments in epidermal cells, i.e., the effector cells for the gravitropic response. MTs were visualized by immunostaining, and actin was visualized by transient expression of YFP-talin using biolistic transformation. Holweg et al. (2004) have shown that YFP-talin does not stabilize actin filaments. It is, therefore, possible to observe effects of external stimuli or inhibitor substances on actin filaments in vivo.

After 1 h incubation in 140 mM acrylamide, MT bundles disappeared (Fig. 5A) and only small, punctate remnants persisted. In sharp contrast, actin microfilaments

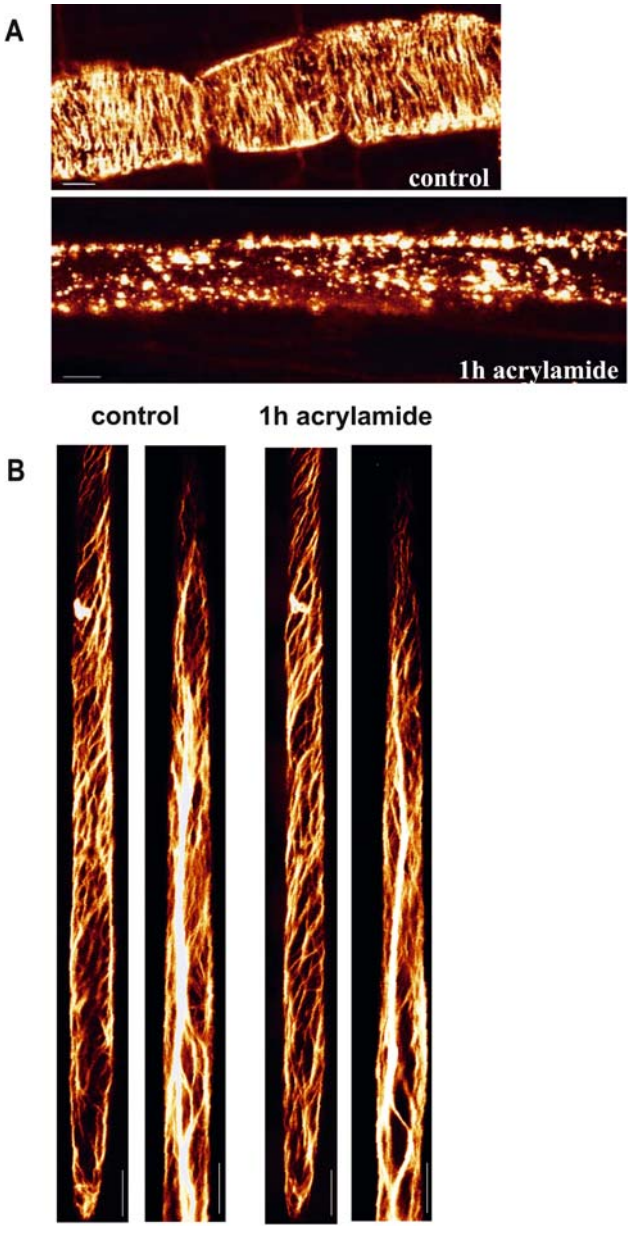

Fig. 5A, B. Effect of acrylamide on cytoskeletal elements in epidermal cells. **A** MTs. Segments of 6-day-old rice coleoptiles were incubated in 140 mM acrylamide for 1 h and in water as a control. Subsequently, semithin tangential sections were cut with a vibratome and stained by immunofluorescence. Cortical MTs disappear almost completely after treatment with 140 mM acrylamide. The central part of a long epidermal cell is shown for the acrylamide treatment and 1.5 short epidermal cells for the control. Bars: 10 μm. **B** Actin filaments. Epidermal cells of 5-day-old rice coleoptiles were transformed with YFP-talin by particle bombardment and used for the experiment 1 day later, when the label was fully expressed. The two poles of the same long epidermal cell are shown before (left) and after (right) treatment with 140 mM acrylamide for 1 h. For acrylamide treatment, the whole coleoptile was submerged in acrylamide. It can be clearly seen that acrylamide has no effect on actin filaments. Bars: 10 μm

were not harmed and even remained dynamic after 1 h of acrylamide treatment (Fig. 5B).

Discussion

Acrylamide inhibits gravitropic bending

Testing the effect of different cytoskeletal drugs on gravitropic bending of rice coleoptiles, we found that acrylamide, an inhibitor of mammalian cell IFs, inhibits gravitropism strongly and specifically, as it does not affect the machinery of cell growth per se. From the dose–response curve of acrylamide effect on gravitropic bending (Fig. 2A), we chose 140 mM for the working concentration because it inhibits gravitropic bending almost entirely (by 85%). However, even at a tenfold lower concentration, a significant inhibition of 40% can be achieved (Fig. 2A). Nevertheless, the concentrations are much higher than the 5–10 mM usually applied to animal cells (e.g. Durham et al. 1983, Eckert 1985, Shabana et al. 1994). Aggeler and Seely (1990) showed that concentrations greater than 10 mM are reversibly cytotoxic for rabbit synovial fibroblasts. With trypan blue staining, we saw that a concentration of 140 mM acrylamide does not affect the viability of rice coleoptile cells within 2 h. It is also known from other inhibitor substances, e.g., 2,3-butanedione monoxime, a myosin inhibitor, that the concentrations needed for plant cells are more than 10 times higher than those needed for animal cells (see, for instance, Holweg et al. 2003).

One reason for the need for higher concentrations in plant cells could be that acrylamide can not enter through the plant cell wall with the same ease as it enters animal cells. Another reason might be degradation by reactive oxygen species, which are abundantly produced in the cell wall (Schopfer et al. 2002). Raid (1998), who produced kinetics of C^{14}-acrylamide uptake in lettuce and wheat suspension cell cultures, found that the lettuce cells needed 10 days to take up 20% of the radioactivity from a 10 μM acrylamide solution and the wheat cells needed 8 days to take up 25%. After the first day of incubation, both cell cultures had only taken up around 5% of the radioactivity given in the medium. In our experiments, preincubation times amounted to 30 or 60 min, thus a high concentration was needed to assure sufficient acrylamide uptake. To use longer incubation times as a strategy to increase the intracellular concentration of acrylamide was not feasible in our system, because the whole physiology of rice coleoptiles changes fundamentally after anoxia (see, for instance, Pijon and Furuya 1967, Kutschera et al. 1991). The crucial point is, however, that concentrations that inhibit gravitropism, leave the cell elongation machinery completely untouched (Fig. 2A). Furthermore, coleoptiles bent again after acrylamide had been washed out (Fig. 2E). This is difficult to reconcile with a general poisoning of the target cells. The presence of well-organized actin microfilaments after treatment with acrylamide (Fig. 5B) is a further indication of the viability of the acrylamide-treated tissue.

Acrylamide affects gravitropism after 15 min of stimulation

To determine the mode of acrylamide action, we first tried to determine the stage of the gravitropic reaction that is inhibited. The dependence of after-bending on stimulation time shows that the inhibiting effect of acrylamide starts after 15 min of gravitropic stimulation (Fig. 3A). It is around the same time interval that the lateral auxin gradient emerges (Gutjahr et al. 2005). We can exclude the possibility that the onset of acrylamide action is limited by its slow uptake by the coleoptile. When a tenfold lower concentration of acrylamide was added directly at the onset of stimulation (i.e., without preincubation for 30 min), we found that the block of gravitropic curvature became manifest within 30 min (Fig. 3E). Thus, a preincubation for 30 min in the working concentration of 140 mM is more than sufficient to ensure that enough acrylamide has entered the target cells to block its target gravitropic event. The stimulation-time relation for BFA was very similar to that for acrylamide (Fig. 3B). BFA is known to affect different components of vesicle secretion (for a review, see Nebenführ et al. 2002). IAA transport is inhibited as a side effect, which is attributed to a disturbed localization of the IAA transporter as a consequence of altered vesicle transport (Robinson et al. 1999, Geldner et al. 2001). Like acrylamide and BFA, NPA also inhibits the gravitropic reaction (Figs. 2C and 3C). However, whereas the dose–response curves of BFA and NPA are qualitatively similar (Fig. 2B, C), the stimulation-time kinetics differ somewhat: While BFA inhibition remained at the same level for 30 min of stimulation and beyond, curvature of NPA-treated coleoptiles still increased with increasing stimulation time. It had been proposed earlier (Geldner et al. 2001) that phytotropins like NPA might inhibit auxin efflux through affecting vesicle trafficking. However, this was directly tested in tobacco cells and shown not to be the case (Petrášek et al. 2003). Instead, NPA appears to bind to a protein that directly interacts with the efflux car-

rier (Morris et al. 1991, Cox and Muday 1994). From this mode of action, one would expect that NPA interferes with gravitropic signal transmission downstream from BFA. The relatively similar pattern produced by the three inhibitors, acrylamide, BFA, and NPA and the temporal coincidence of the lateral auxin gradient with the appearance of inhibition in the after-bending time course suggest that their common target is either IAA transport or IAA-induced growth. However, since acrylamide produces a pattern that is closer to that of BFA than that of NPA, it is more likely to act at the level of secretory pathways than directly on efflux carriers, as proposed for NPA.

Acrylamide inhibits IAA transport and destroys MTs

The next step was to discriminate between inhibition of IAA transport and inhibition of the IAA response. We measured IAA transport directly with ^{14}C-labelled IAA. Our measurements show that acrylamide reduced IAA transport by 70%. Consistent with this finding, acrylamide interferes with gravitropically induced asymmetric growth (Fig. 4A). Moreover, it inhibits the growth of intact coleoptiles, which depends on polar auxin transport (in contrast to auxin-depleted coleoptile segments), and this inhibition can be partly counterbalanced by exogenous IAA (Fig. 4B).

Interestingly, the acrylamide effect on IAA transport is mitigated when 0.1 μM IAA are present in the incubation medium (see Results section). This concentration corresponds roughly to the endogenous auxin content of rice coleoptiles observed under these conditions (Riemann et al. 2003). The reason for this mitigation might be that coleoptiles are stressed to a lesser extent when loss of IAA into the incubation medium is prevented. Alternatively, the activity of the IAA efflux system could be regulated by auxin. In fact, it has been shown for pea shoots and maize coleoptiles that auxin stimulates its own transport (summarized in Morris 2000) and it is speculated that auxin regulates the distribution of its own carrier systems.

Treatment with 140 mM acrylamide destroys MTs but leaves actin filaments intact (Fig. 5A, B). Thus, the observed reduction in basipetal auxin transport after acrylamide treatment cannot be explained in terms of affected microfilaments. On the other hand, microfilaments have been proposed to be involved in auxin efflux (for reviews, see Muday 2000, Muday and Murphy 2002), and the BFA-induced mislocalization of the PIN1 protein can be prevented by inhibitors of actin assembly (Geldner et al. 2001). However, in addition to actin, MTs are essential for the maintenance of polar auxin transport. Godbolé et al. (2000) found a specific and strong inhibition of IAA transport after treatment with MT-eliminating drugs, such as EPC. Of course, it cannot be ruled out at the present stage that acrylamide affects IAA transport and MTs independently. Nevertheless, the idea that acrylamide inhibits IAA transport through an elimination of MTs provides at least a plausible working model.

How does acrylamide eliminate MTs? An outlook

Acrylamide treatment eliminates MTs and inhibits IAA transport. But how acrylamide acts on MTs remains an open question. EPC binds to tubulin monomers and thereby inhibits MT assembly, so that MTs disappear progressively as a consequence of their innate turnover (Mizuno and Suzaki 1990). This compound inhibits the gravitropic reaction from the very beginning (Fig. 3D). It probably affects a population of MTs that is involved in early signal perception (Godbolé et al. 2000). Because the acrylamide effect starts at a much later time point (Fig. 3A), it is improbable that it acts in the same way as EPC, i.e., by binding tubulin heterodimers. (A possible influence of acrylamide uptake kinetics on the time-point of MT disappearance was excluded by the preincubation of the coleoptiles prior to gravitropic stimulation [see Fig. 3E].) To detect a potential affinity of acrylamide for tubulin heterodimers we will perform EPC-sepharose affinity chromatography (Wiesler et al. 2002) and test whether the bound rice tubulin can be eluted by acrylamide.

It seems more likely though that acrylamide affects MTs through associated proteins rather than by binding tubulin heterodimers. In mammalian cells, acrylamide disturbs the organization of IFs by inhibiting a protein kinase dependent of cyclic AMP that phosphorylates IFs (Eckert and Yeagle 1988). Fry et al. (1998) showed that a tyrosine-kinase inhibitor (7-acrylamido-4-anilino-quinazolin) used for potential combat of cancer acts by nucleophilic addition of its acrylamide residue to a cysteine in the ATP-binding pocket of the tyrosine kinase. In mammalian cells, acrylamide thus seems to act on protein kinases.

Are protein kinases involved in IAA transport or MT organization in plants? Bernasconi (1996) could suppress IAA efflux from zucchini hypocotyls with different tyrosine kinase inhibitors. Baskin and Wilson (1997) have shown that treatment of *Arabidopsis thaliana* roots with serine-threonine phosphatase and protein kinase inhibitors disorganizes cortical MTs. It is known from animals that phosphorylation is a central signal in the regulation of the activity of MT-associated proteins (Cassimeris and Spittle

2001). It would thus be a next step to test whether acrylamide interferes with phosphorylation signals.

To our knowledge, there are no reports of MT damage following acrylamide treatment in animal cells. It is widely used as specific inhibitor of IF distribution (Eckert 1985). Mizuno (1995) found a 50 kDa protein (p50) that forms IF-like structures and promotes the assembly of MTs in vitro. However, antibodies against p50 did not stain cortical MTs. Apart from unique fibrillar, IF-like structures, they only stained the spindle and phragmoplast in dividing cells. Goodbody et al. (1989) found that animal anti-IF antibodies coaligned with cortical MTs in carrot and maize suspension cells. Interestingly, vimentin IFs have been also found to accompany MTs in animal cells (Ball and Singer 1981). Future studies will have to resolve whether acrylamide interferes with phosphorylation signals that control MT stability either directly or through the mediation of IF-like MT-associated proteins.

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