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The auxin response of actin is altered in the rice mutant *Yin-Yang*

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Summary. The rice mutant *Yin-Yang* has been selected during a screen for resistance to cytoskeletal drugs and is characterized by alterations in epidermal cell length and a precocious onset of gravitropism. The elongation response of coleoptile segments to auxin does not reveal changes of auxin sensitivity in *Yin-Yang*. However, in contrast to the wild type, cell elongation in *Yin-Yang* is highly sensitive to the actin-polymerisation blocker cytochalasin D. This increased sensitivity to cytochalasin D requires optimal concentrations of auxin to become manifest. The auxin response of actin microfilaments in epidermal cells differs between wild type and mutant. In the wild type, the longitudinal microfilament bundles become loosened in response to auxin. In the mutant, these bundles disintegrate partially and are replaced by a network of short filaments surrounding the nucleus. Several aspects of the mutant phenotype can be mimicked in the wild type by treatment with cytochalasin D. The mutant phenotype is discussed in terms of signal-dependent changes of actin dynamics and the putative role of actin during cell elongation.

Keywords: Actin; Auxin; Cell elongation; Gravitropism; Microfilaments; *Oryza sativa*.

Abbreviations: CD cytochalasin D; EPC ethyl-N-phenylcarbamate.

Introduction

Signal-triggered reorganization of the cytoskeleton is a common theme in the morphogenetic response of plant cells. In gramineous coleoptiles, for instance, the response of cell elongation to light is accompanied by a reorientation of cortical microtubules from transverse into longitudinal arrays (Nick et al. 1990, Zandomeni and Schopfer 1993), and by changes in the bundling of actin microfilaments (Waller and Nick 1997). Both responses are specific for the epidermis, i.e., the tissue, where elongation growth is controlled (Kutschera et al. 1987).

The link between cortical microtubules and cell shape is thought to be mediated by the guided deposition of cellulose that is directed by the microtubules (Giddings and Staehelin 1991). A transverse array of microtubules and, consequently, cellulose microfibrils seems to be a prerequisite for cell elongation (Green 1980), whereas longitudinal or oblique arrays of microtubules and microfibrils are characteristic for cells with isotropic or preferentially lateral growth. Transverse rings of cellulose microfibrils are expected to counteract lateral expansion and, indirectly, to reinforce longitudinal growth (Green 1980).

In contrast to microtubules, the connection between actin microfilaments and cell growth has remained obscure, so far. Nevertheless, actin seems to play a role for the control of cell growth as indicated by several observations. (1) Inhibitors of the actin-myosin system flip the axis of cell growth from elongation to radial expansion in Arabidopsis roots (Baskin and Bivens 1995). (2) Reorganization of microfilaments mediates the phototropic response of tip-growing moss caulonemata (Meske et al. 1995). (3) Phytochrome-triggered inhibition of cell elongation in maize mesocotyls is accompanied by rapid bundling of actin microfilaments, whereas the stimulation of cell elongation by far-red light in maize coleoptiles is accompanied by a loosening of actin bundles (Waller and Nick 1997). (4) In oat coleoptiles, auxin-induced

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cell elongation was shown to require intact actin microfilaments (Thimann et al. 1992, Thimann and Biradivolu 1994). (5) In soybean root cells the rigidity of actin changes in response to aluminum (Grabski and Schindler 1995) and in response to auxin and cytokinin (Grabski and Schindler 1996) accompanied by corresponding changes in growth rate.

These observations, although clearly demonstrating that actin does interfere with the control of cell elongation, lead to seemingly contradicting conclusions about the underlying mechanism: The correlation between bundling of actin microfilaments and inhibition of cell elongation (Thimann et al. 1992; Grabski and Schindler 1995, 1996; Waller and Nick 1997) seems to indicate that microfilaments impede cell growth. On the other hand, when actin microfilaments are eliminated by cytochalasin D, an inhibition of growth is observed, suggesting that microfilaments support rather than limit cell elongation.

Recently, cytoskeletal mutants have been selected in rice (Nick et al. 1994). During the characterization of these mutants, a new mutant line, *Yin-Yang*, was isolated. In this mutant, the responses of actin microfilaments to auxin are observed to be abnormal as described in the present work. The mutant phenotype is discussed in the context of the potential functions of actin in the control of cell elongation and with respect to signal-dependent changes of actin dynamics.

Material and methods

Plant cultivation

Seeds of wild-type and mutant rice were dehusked and surface-sterilized in 30% H_2O_2 for 10 min and then sown equidistantly, embryo up, on floating plastic meshes in plexiglass boxes (95 mm × 95 mm × 60 mm, 15 seeds for each box) as described earlier (Nick and Furuya 1993). In this setup, the seedling germinated under aerial conditions, floating on the surface of the medium. Any submergence of the seedlings was carefully avoided, because the physiology of rice is completely altered unter anaerobic conditions. Since the elongation of rice seedlings is extremely sensitive to light (Furuya et al. 1969), the cultivation occurred in complete darkness: The plexiglass boxes were placed in light-tight black boxes covered with black cloth and kept in a dark room at 25 °C until the seedlings were used for the experiments. In some experiments (Table 1), the seedlings were cultivated on 1 mM of ethyl-N-phenylcarbamate (EPC) or on 5 mM colchicine.

Estimation of cell length

Epidermal cell length and cell number were determined from micrographs of epidermal strips with the autofluorescence of the cell wall as natural label. For coleoptiles, the strips covered the whole length from the very tip to the node, for mesocotyls, the whole length from the node to the base of the primary root. Fluorescence images of those strips were recorded on a 400 ASA film (Tri X Pan; Kodak, Rochester, N.Y., U.S.A.) with an excitation wavelength of 365 nm, a beam splitter at 395 nm, and an emission wavelength of 397 nm at an epifluorescence microscope (Axioplan; Zeiss, Oberkochen, Federal Republic of Germany). The negatives were projected and cell lengths and numbers were measured after calibration with an object micrometer scale (photographed at the same magnification).

Visualization of actin microfilaments

Actin microfilaments were stained by fluorescent-labeled phalloidin as described in Waller and Nick (1997) and viewed under a confocal laser scanning microscope (DM RBE; Leitz, Bensheim, 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. Each experiment was repeated at least twice, typically four times, and each treatment included 10 to 20 plants.

Time course of gravitropic curvature

Seedlings of wild-type rice and mutant *Yin-Yang* were cultivated for five days in complete darkness and then selected under dim green safelight (λ_{max} = 550 nm) for straightness and length. They were then transferred to 1.2% (w/v) agar in plexiglass boxes (95 mm × 95 mm × 60 mm, 15 seedlings for each box). The caryopses were stuck into the agar such that the coleoptile was parallel to the surface of the agar. The boxes were then positioned such that the coleoptiles were oriented vertically. After cultivation in complete darkness at high humidity in a moist chamber for a further day the coleoptiles were perfectly straight and vertical. For gravitropic stimulation, the box was tilted by 90°, and the bending response was followed over time by taking photographs against a source of green safelight on a 400 ASA film (Tri X Pan, Kodak). The negatives were projected onto a wall and curvature was then determined as angle between coleoptile tip and the direction of the gravity vector. Each time course was constructed from at least three independent experiments comprising 15–20 individual seedlings. In some experiments (Fig. 1 B), a solution of 2 µM cytochalasin D was sprayed over the seedlings 30 min prior to the onset of gravitropic stimulation. The length increment was determined in parallel from these projections and the average growth rate over the course of the experiment was found to be very similar for wild type (154 ± 23.4 µm/h) and mutant (142 ± 19.8 µm/h).

Elongation assay

Coleoptile segments of 10 mm length were excised under green safelight from 2–12 mm below the coleoptile tip of etiolated wild-type and mutant seedlings that had been cultivated for 8 days in complete darkness. The sections were incubated, under continuous rotation, for 1 h in water to wash out endogenous auxin. Subsequently 3-indoleacetic acid was added in various concentrations (Fluka, Neu-Ulm, Federal Republic of Germany) for 4 h at either 27 °C or at 30 °C. In some experiments, cytochalasin D (Sigma, Neu-Ulm, Federal Republic of Germany) was added to the incubation medium at concentrations from 2 nM to 20 µM. In a set of control experiments, cytochalasin D was replaced by the antimicrotubular drugs EPC or colchicine at concentrations from 10 µM to 5 mM. The length increments after 4 h were determined under a stereomicroscope. Each experiment was repeated at least three times with 10 to 15 individual segments for each experiment.

Table 1. Response of coleoptile and mesocotyl growth to antimicrotubular drugs in rice wild type and *Yin-Yang*

Structure	Length (mm) 9 days after sowing		
	water	EPC	colchicine
Wild type			
coleoptile	29.2 ± 2.6	3.4 ± 2.3(11.6)[a]	2.6 ± 3.1(8.9)
mesocotyl	0.7 ± 0.06	0.09 ± 0.02(12.9)	0.12 ± 0.01(17.1)
Yin-Yang			
coleoptile	32.4 ± 1.98	17.33 ± 0.86(53.5)	14.3 ± 0.62(44.1)
mesocotyl	3.55 ± 0.61	0.59 ± 0.05(16.6)	0.43 ± 0.06(12.1)

Seedlings were cultivated in complete darkness either on water, on 1 mM EPC, or on 5 mM colchicine (n = 30)
[a]In parentheses, length in percent of water control

Table 2. Inheritance of the *Yin-Yang* phenotype

Cross nr.	Parental phenotype		Expected parental genotypl	Offspring phenotype[a]	
	pollen donor	pollen acceptor		wild type	mutant
1	YY	YY	YY/YY × YY/YY	0	49
2	YY	YY[b]	YY/YY × YY/YY	0	158
3	YY	WT	YY/YY × WT/WT	62	0
4	WT	YY	WT/WT × YY/YY	25	0
5	WT	YY	WT/YY × YY/YY	43	47
6	WT	WT	WT/YY × WT/WT	71	0
7	WT	WT	WT/YY × WT/YY	80	25

[a]Values are absolute frequencies of offspring exhibiting rice wild type or *Yin-Yang* phenotypes.
[b]Parental plant was selfed. The mutant plants in cross 3 are the offspring of cross 1, the mutant plants in cross 4 originate from the selfing experiment in cross 2, the plants used for the crosses 5–7 are offspring from crosses 4 and 2.

Results

Isolation of the mutant

The rice mutant *Yin-Yang* (*Oryza sativa* L. ssp. *japonica* cv. Nihonmasari) was isolated during a screen for resistance to antimicrotubular drugs (Nick et al. 1994). Cell elongation was found to be more resistant to EPC and to colchicine than in the wild type (Table 1). Pure lines for the mutation were selected, and the mutation was found to be inherited in a monogenic Mendelian way with the wild-type allele being completely dominant over the mutant allele (Table 2).

Specific alteration of gravitropic response in Yin-Yang coleoptiles

During a physiological characterization of various growth responses in the mutant *Yin-Yang*, characteristic changes were observed for the gravitropic response (Fig. 1). When wild-type coleoptiles were subjected to gravitropic stimulation by tilting the seedling axis by 90° vigorous gravitropic bending initiated from 30 min after the onset of stimulation and the seedlings had reached the vertical within 5–6 h later (Fig. 1 A). In contrast, the gravitropic response of *Yin-Yang* coleoptiles initiated virtually instantaneously (Fig. 1 C, inset), but proceeded at a somewhat slower pace as compared to the wild type. Interestingly, the mutant continued to curve even after it had reached the vertical resulting in significant overbend-

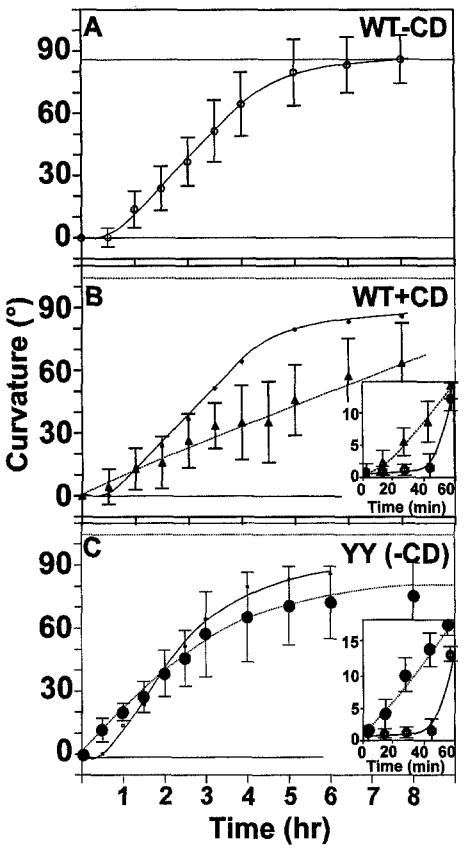

Fig. 1 A–C. Gravitropic response of rice wild type (*WT*) and mutant *Yin-Yang* (*YY*). Seedlings cultivated for six days in complete darkness where placed horizontally at time 0 and the gravitropic bending was followed over time. Each point represents the mean of 45–60 individual seedlings pooled from at least three independent sets of experiments. Gravitropic response of the wild type: **A** without pretreatment with cytochalasin D and **B** after pretreatment with 2 μMcytochalasin D 30 min prior to the onset of gravitropic stimulation. Solid curve represents the response without cytochalasin D pretreatment, dotted line the final curvature for the sample treated with cytochalasin D. **C** Gravitropic response of the mutant *Yin-Yang* without pretreatment with cytochalasin D. Dotted line represents the final curvature for the mutant. **Insets** Details of initial phases of gravitropic bending

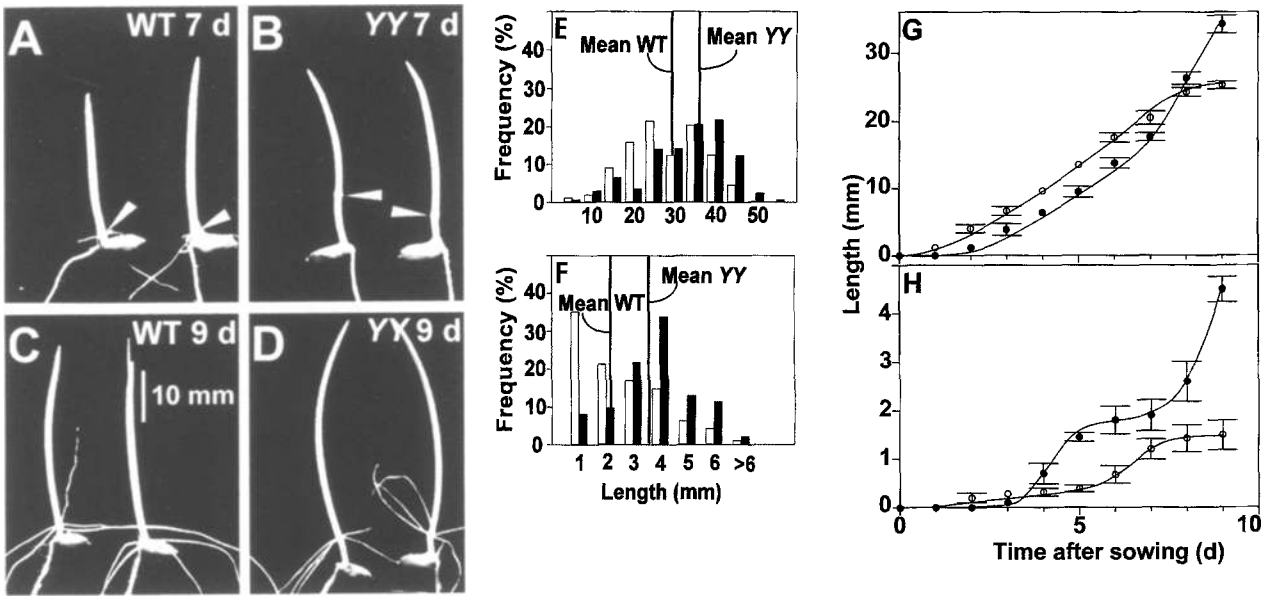

Fig. 2 A–H. Phenotype and growth in etiolated seedlings of rice wild type (*WT*) and mutant *Yin-Yang* (*YY*). **A–D** Etiolated seedlings of the wild type (*WT*) and the mutant (*YY*) at day 7 and at day 9 after sowing. **E** and **F** Distribution of final length for the coleoptile (**E**) and the mesocotyl (**F**) of etiolated seedlings constructed from 100 individual seedlings for each distribution. □ Wild type, ■ *Ying-Yang*. **G** and **H** Time course of elongation for etiolated coleoptiles (**G**) and mesocotyls (**H**). ○ Wild type, ● *Ying-Yang*. Error bars indicate standard errors. Each data point represents the mean of at least 30 individual seedlings

ing (Fig. 1 C). Both aspects of the mutant phenotype, precocious onset of curvature and overbending could be mimicked in the wild type by a pretreatment with 2 μM cytochalasin D administered 30 min prior to the gravitropic stimulus (Fig. 1 B, inset).

Altered cell elongation in Yin-Yang seedlings

Germination in *Yin-Yang* was retarded by about one day compared to the wild type, and up to one week the mutant coleoptiles are shorter than the wild type (Fig. 2 A, B). Between day 2 and day 7, the growth rate was not significantly different for wild-type and mutant coleoptiles. However, after day 7, the growth rate declined in the wild type, but increased in the mutant. This resulted in a final length of mutant coleoptiles about one third longer than the wild type (Fig. 2 C, D, E, G). In addition, the emergence of crown roots was delayed in the mutant (Fig. 2 A, B), although they did emerge eventually (Fig. 2 C, D). Moreover, the elongation of the mesocotyl was strikingly enhanced over that of the wild type (Fig. 2 F, H).

In order to test whether these changes in growth are caused by changes in cell length or by changes in cell number, epidermal strips were obtained over the whole length of the seedlings. For this analysis, a time point was chosen, where wild-type and mutant coleoptiles were of comparable length, but elongated at different growth rates (8 days after sowing). Surprisingly, the cells in the coleoptiles of *Yin-Yang* seedlings were not longer, but considerably shorter than those of the wild type, but this was compensated by an increase in cell number (Table 3). We were not able to detect any indications for postgerminative cell divisions in the coleoptile, suggesting that in *Yin-Yang* the cell number is increased already during embryogenesis. In contrast to the situation in the coleoptile, there was a strong increase of cell elongation in the mesocotyl of the mutant as compared to the wild type (Table 3).

Table 3. Length of epidermal cells in seedlings of wild type and *Yin-Yang*

Phenotype	Coleoptile cells		Mesocotyl cells	
	length (μm)	number	length (μm)	number
Wild type	512 ± 18.8	36.8 ± 0.34	202 ± 1.68	7.0 ± 0.08
Ying-Yang	432 ± 13.7	43.6 ± 0.22	271 ± 1.94	8.4 ± 0.1

Epidermal strips were obtained over the entire length of the coleoptile 8 days after sowing, when the coleoptiles of wild type and mutant were comparable in length. Values are the mean and the standard errors of the mean from at least 30 individual plants and scores of at least 50 cells per individual plant.

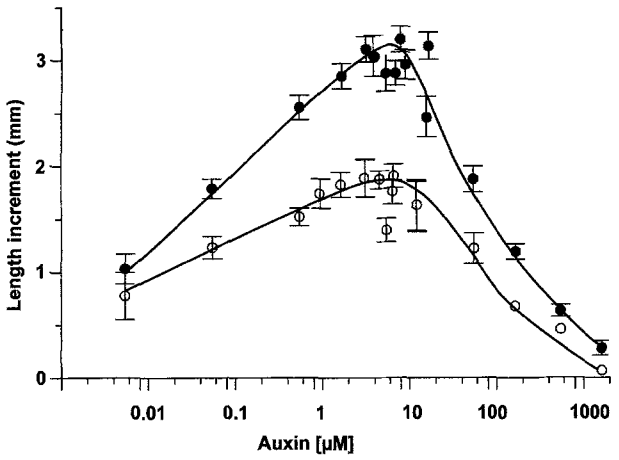

Fig. 3. Dose–response curve for auxin-dependent coleoptile elongation. 10 mm long segments were depleted from endogenous auxin by incubation in water for 1 h and then incubated for 4 h at 27 °C in various auxin solutions. ○ Wild type (n = 267), ● *Yin-Yang* (n = 249)

Cytochalasin D sensitivity of auxin-dependent cell elongation in Yin-Yang coleoptiles

The reduced cell length in the epidermis of *Yin-Yang* coleoptiles might be caused by a reduced sensitivity to auxin that controls coleoptile elongation in rice (Furuya et al. 1969). To test this possibility, a dose–response curve was measured for the auxin-dependent elongation of coleoptile segments (Fig. 3). In the wild type, a flat, bell-shaped curve was obtained with a threshold between 0.01 and 0.1 μM 3-indoleacetic acid, a broad peak between 1 and 10 μM, and an inhibition of growth for concentrations exceeding 100 μM. The curve for *Yin-Yang* was not shifted with respect to auxin concentration, i.e., the mutant was not altered in terms of auxin sensitivity. However, the response was enlarged in amplitude (Fig. 3), possibly reflecting the increased number of epidermal cells in the segment (Table 3).

The optimal auxin concentration of 5 μM was then used to assay the effect of cytoskeletal drugs on elongation. Pretreatment of the segments with the anti-microtubular drugs EPC or colchicine did inhibit growth, but with an identical dose–response relation when wild type and mutant were compared (data not shown). In order to check a potential influence of actin microfilaments, we repeated this assay with cytochalasin D, a drug that specifically eliminates actin (Cooper 1987, Miyata and Kinosita 1994). For wild-type coleoptile segments, growth inhibition became detectable at 0.1 μM cytochalasin D (Fig.

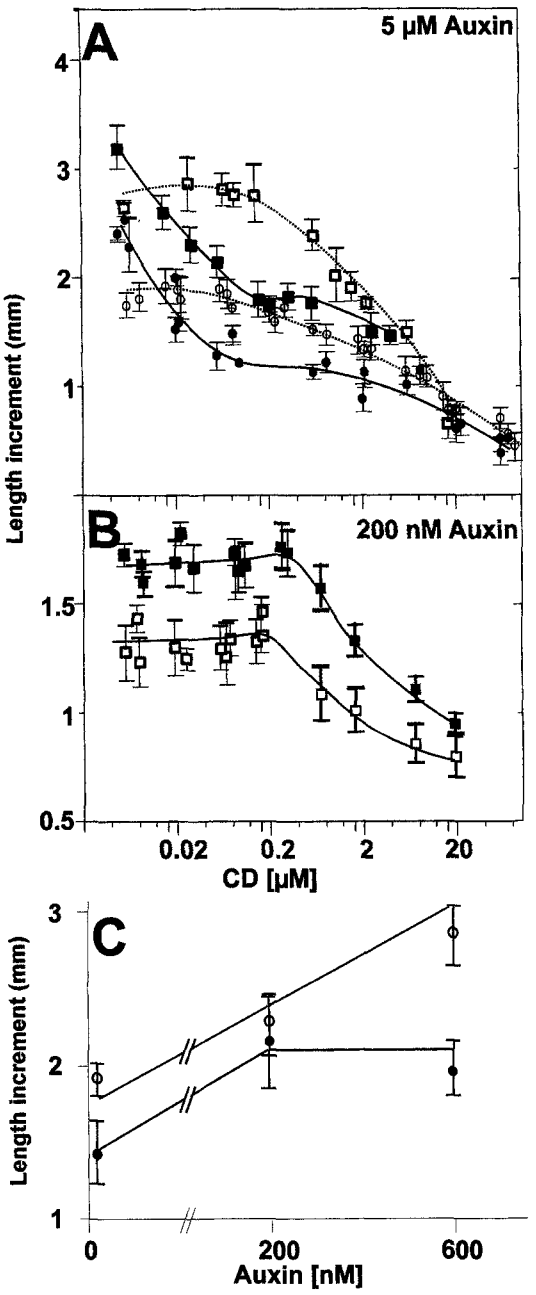

Fig. 4 A–C. Dose–response curve for the inhibition of auxin-dependent cell elongation by cytochalasin D. **A** and **B** 10 mm long segments were depleted from endogenous auxin and then incubated for 4 h at 27 °C in various solutions of cytochalasin D in the presence of 5 μM (**A**) or 200 nM (**B**) indole-acetic acid. ○, □ Wild type; ●, ■ *Yin-Yang*; ○, ● data for incubation at 27 °C; □, ■ data for incubation at 30 °C. For comparison, the curves obtained at 5 μM indole-acetic acid are plotted above the data obtained for 200 nM indole-acetic acid in **B** (wild type, n = 141; *Yin-Yang*, n = 193). **C** Length increment at a constant concentration of cytochalasin D (2 μM) for increasing concentrations of 3-indoleacetic acid. ○ Wild type (n = 78), ● *Yin-Yang* (n = 70)

4 A), whereas in *Yin-Yang* already 20 nM cytochalasin D produced a pronounced inhibition of growth (Fig. 4 A).

To test the possibility that the increased sensitivity of *Yin-Yang* coleoptiles against cytochalasin D is related to the increased growth rate observed in the mutant, the temperature was raised by 3 °C from 27 °C to 30 °C (Fig. 4 A). At the higher temperature, the dose–response curves for both wild type and *Yin-Yang* were enlarged in amplitude but they were not shifted with respect to drug sensitivity (Fig. 4 A). To mimick the elevated growth rate of the mutant at 27 °C, the dose–response curve for the wild type was determined for incubation at 30 °C. Under these conditions, the growth rate in absence of the drug was exactly the same. Nevertheless, the difference in the sensitivity to cytochalasin D between wild type and mutant remained. From the shift of the dose–response curves between wild type and mutant the sensitivity of *Yin-Yang* coleoptiles to cytochalasin D was estimated to be at least ten times higher as compared to the wild type.

Surprisingly, the sensitivity difference between wild type and mutant disappeared if the experiment was performed in the presence of low (200 nM) concentrations of 3-indoleacetic acid, just above the threshold of the dose–response curve for auxin-dependent elongation (Fig. 3). Whereas the curve for the wild type (Fig. 4 B) resembled that obtained for optimal auxin concentrations (Fig. 4 A), the data for *Yin-Yang* did not reveal any response to cytochalasin D that exceeded that of the wild type (Fig. 4 B). In order to test at which concentration of auxin the differential sensitivity of *Yin-Yang* coleoptiles becomes manifest, the concentration of cytochalasin D was kept constant at 2 μM, while the concentration of auxin in the assay was varied. Up to 200 nM of auxin the mutant responded with the same relative increase as the wild type (Fig. 4 C). If the concentration of auxin was raised further, growth in the wild type was stimulated, whereas it lagged behind in *Yin-Yang*.

These data suggest that growth of *Yin-Yang* coleoptiles is highly sensitive to cytochalasin D. However, to become manifest this sensitivity requires auxin in a concentration that exceeds 200 nM.

Disassembly of microfilaments in epidermal cells of Yin-Yang coleoptiles in response to auxins

To understand the difference in the sensitivity to cytochalasin D between mutant and wild type, actin microfilaments were visualized in epidermal cells of both wild-type and *Yin-Yang* seedlings.

In intact wild-type coleoptiles (data not shown) or in coleoptile segments that had been depleted from endogenous auxin by incubation in water, actin microfilaments were organized into condensed, longitudinal bundles (Fig. 5 A–C), such as typically found in epidermal cells of other gramineous species as well (Thimann and Biradivolu 1994, Waller and Nick 1997). When low (0.2 μM) concentrations of the actin depolymerizing drug cytochalasin D were added to the medium, the actin bundles collapsed into helicoidal structures in the cell center resembling a spring that had been stretched and attached to the cell poles and now returned to a relaxed state, after the attachment had been interrupted (Fig. 5 D). When the concentration of cytochalasin D was increased further, the longitudinal microfilaments disappeared progressively (Fig. 5 E, F). Simultaneously, a basket-like mesh of short filaments appeared around the nucleus and condensed with increasing concentration into a massive agglomeration of actin (Fig. 5 E, F). In presence of 5 μM auxin the actin bundles appeared to be finer (Fig. 5 G, H). The dense bundles characteristic for auxin-depleted cells (Fig. 5 A–C) seemed to be dissolved into finer strands of parallel filaments (Fig. 5 G, H, J). Occasionally, a very delicate meshwork of cortical filaments could be observed especially in the region near the nucleus (Fig. 5 H, I). The microfilaments disappeared already upon a very mild treatment with cytochalasin D and were replaced by the nuclear baskets mentioned above (Fig. 5 K–M).

In epidermal cells of the *Yin-Yang* mutant, actin microfilaments were observed to be arranged into fine parallel strands that were arranged longitudinally in both, intact coleoptiles (data not shown) or in auxin-depleted coleoptile segments (Fig. 6 A, B). The fine strands of auxin-depleted mutant cells resemble the fine strands observed in wild-type cells that had been treated with 5 μM auxin (compare Fig. 5 G–J with Fig. 6 A–C). A surprising behavior of actin microfilaments was observed when the mutant was treated with 5 μM of auxin (Fig. 6 D, E): The longitudinal strands of actin disassembled partially (Fig. 6 D) and were replaced by the nuclear baskets (Fig. 6 E) that resembled the structures observed in the wild type after treatment with cytochalasin D (Fig. 5 F, L, M). This phenotype was even more pronounced, when auxin was accompanied by low concentrations of cytochalasin D (Fig. 6 F, G) that otherwise did not cause dramatic effects in the absence of auxin (Fig. 6 C). Thus,

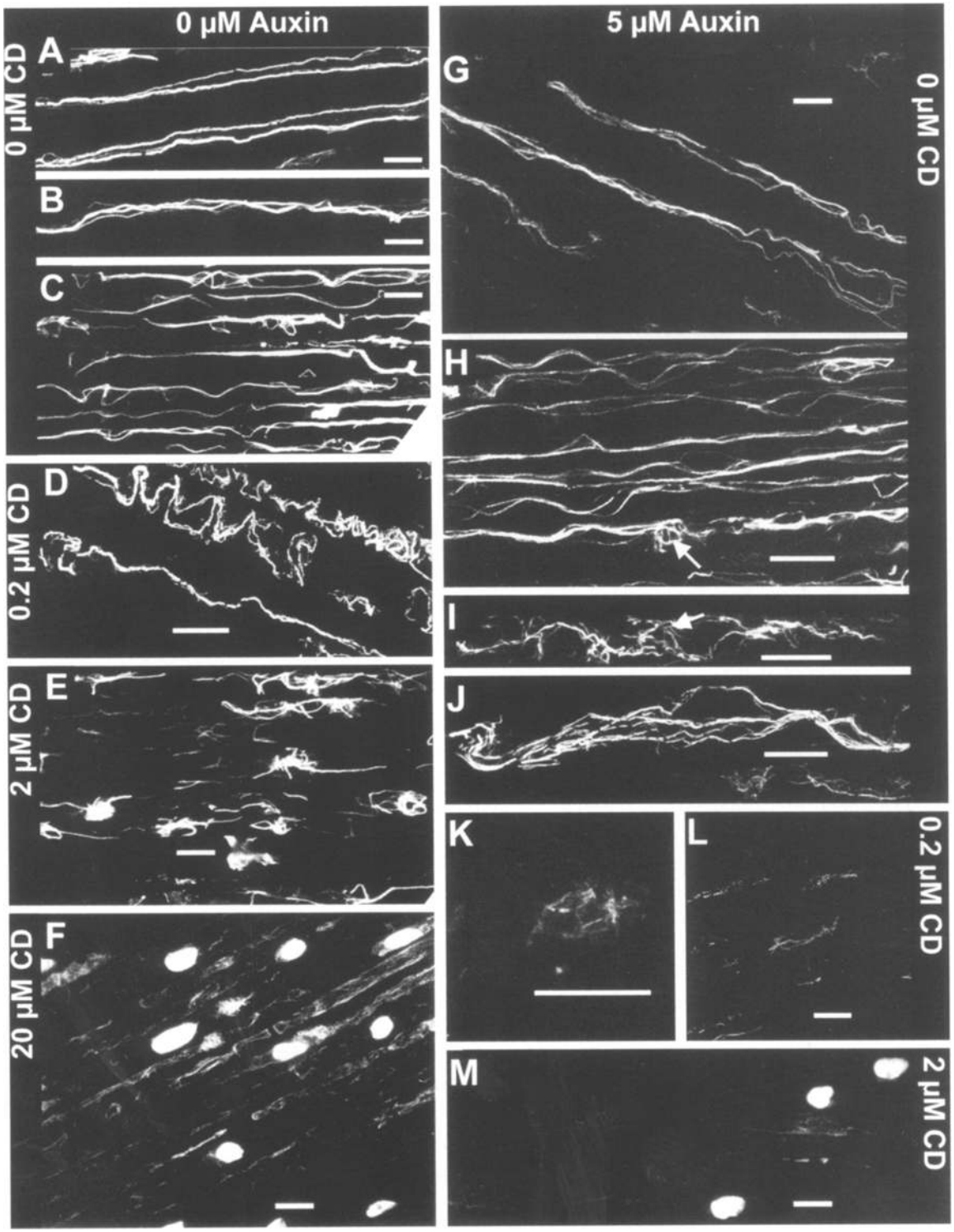

Fig. 5 A–M. Confocal laser-scanning micrographs of actin microfilaments in the epidermis coleoptite segments of the wild type after treatment with auxin and cytochalasin D. For details of the incubation protocol refer to the legend of Figs. 3 and 4. Bars: 10 µm. **A–F** Microfilaments upon depletion of auxin in the absence (**A–C**) and in the presence of 0.2 µM (**D**), 2 µM (**E**), and 20 µM (**F**) cytochalasin D. **G–M** Microfilaments in the presence of 5 µM auxin without (**G–J**) and with 0.2 µM (**K** and **L**) or 2 µM (**M**) cytochalasin D added. Arrows in **H** and **I** indicate the cortical meshwork of fine actin microfilaments

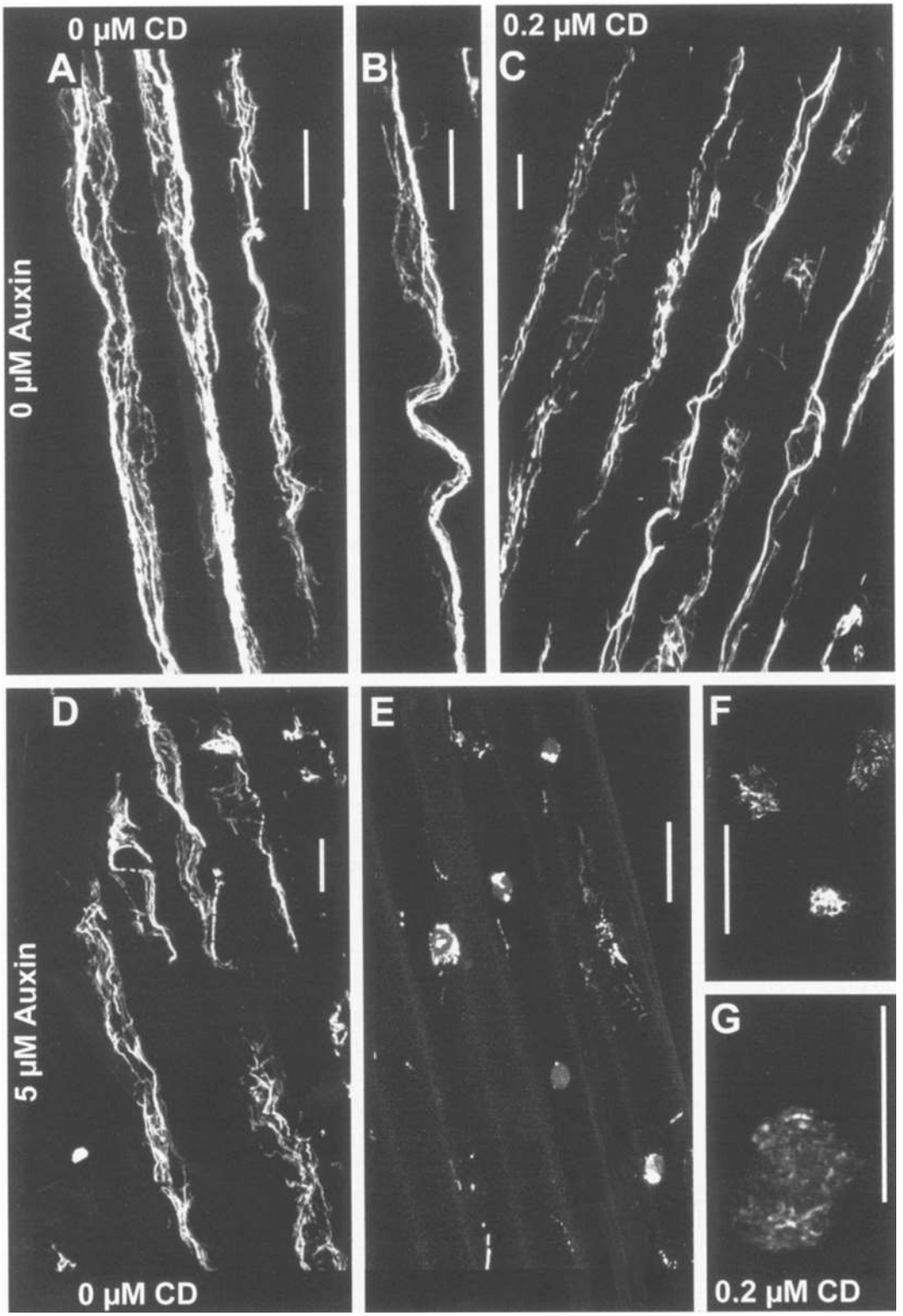

Fig. 6 A–C. Confocal laser-scanning micrographs of actin microfilaments in the epidermis of coleoptile segments of the mutant *Yin-Yang* after treatment with auxin and cytochalasin D. For details of the incubation protocol refer to the legend of Figs. 3 and 4. Bars: 10 µm. **A–C** Microfilaments upon depletion of auxin in the absence (**A** and **B**) and in the presence of 0.2 µM (**C**) cytochalasin D. **D–G** Microfilaments in the presence of 5 µM auxin without (**D** and **E**) and with 0.2 µM (**F** and **G**) cytochalasin D added. Note the formation of nuclear baskets in the presence of auxin (**D** and **E**)

the auxin response of actin microfilaments in the mutant could be mimicked in the wild type by treatment with cytochalasin D.

Discussion

Correlation between changed growth pattern of Yin-Yang coleoptiles and change in cell number

Coleoptiles of young *Yin-Yang* seedlings are shorter than those of wild-type seedlings of the same age (Fig. 2 A, B, G). This difference in length is related to a decrease in the length of individual cells (Table 3). There are no indications for cell divisions taking place in wild type or mutant after germination (data not shown) consistent with the situation found in coleoptiles of other gramineous species (Waller and Nick 1997). This means that coleoptile growth has to be explained in terms of cell elongation. After a certain delay of elongation observed during the early development of *Yin-Yang* coleoptiles (Fig. 2 G), the capacity for elongation seems to recover and eventually to exceed that of wild-type coleoptiles (Fig. 2 C, D, E, G). From one week after sowing the growth rate is conspicuously increased in coleoptiles of *Yin-Yang* as compared to the wild type (Fig. 2 G), and this difference is not only observed in intact plants, but as well in coleoptile segments that are incubated with auxin (Figs. 3 and 4 A, B). Most of this surplus of growth can be attributed to the increased number of cells that is found in mutant coleoptiles (Table 3). Thus, the growth patterns observed in intact coleoptiles (Fig. 2) and coleoptile segments (Figs. 3 and 4) of the mutant seem to originate primarily from a higher number of (shorter) cells that otherwise exhibit a capacity for elongation that is comparable to that of the wild type.

The cause for the increased cell number has to be sought in late embryogenesis, when the coleoptile is laid down. There might be a shift from cell expansion towards cell division in the mutant resulting in an increased number of shorter cells. Auxin is known to regulate several aspects during the development of gramineous embryos, such as the induction of apical meristems (Fischer and Neuhaus 1996). The altered response of actin microfilaments that is discussed below is expected to interfere with the transition from cell division towards cell elongation. This transition takes place during the establishment of the young coleoptile. If this transition is delayed as consequence of an altered auxin response of actin microfilaments, the cells of coleoptile are expected to be smaller and more numerous as observed in the mutant *Yin-Yang*. In this context it should be mentioned that overexpression of a potential auxin receptor (expected to promote this transition) results in plants with fewer, but larger cells, i.e., in a phenotype that is opposite to that observed for the mutant *Yin-Yang* (A. Jones, University of North Carolina, Chapel Hill, N.C., pers. comm.).

The stimulation of mesocotyl elongation in the mutant (Fig. 2 F, H) is probably a secondary effect of the reduced coleoptile growth, because it occurs during the time, when the coleoptile is shorter than that of the wild type (Fig. 2 G, H). The elongated mesocotyl of *Yin-Yang* seedlings originates from hypertrophic elongation of individual cells rather than from dramatic changes in cell number (Table 3). A similar stimulation of cell elongation in the mesocotyl was observed, when coleoptile elongation was inhibited by aberrant microtubule orientation (Nick et al. 1994) or by treatments that impede coleoptile growth such as abscisic acid, fusicoccin or KCl (Takahashi 1972). These observations could be easily explained by assuming that mesocotyl and coleoptile mutually compete for a factor that limits growth. By treatment with exogenous gibberellins this limitation seems to be released allowing for stimulated mesocotyl growth (Toyomasu et al. 1994). The stimulation of mesocotyl elongation is therefore more likely a pleiotropic consequence of the *Yin-Yang* mutation rather than being an event located near the primary target.

Aspects of the Yin-Yang phenotype mimicked in the wild type by cytochalasin D treatment

The mutant displays characteristic aberrations of the gravitropic response, such as a precocious initiation of bending, along with a reduced rate of bending and overbending resulting in final curvatures that exceed 90° (Fig. 1 C). All three aspects can be mimicked, at least qualitatively, in the wild type by a pretreatment with the actin-polymerisation blocker cytochalasin D (Fig. 1 B). These observations implicate two conclusions: (1) The mutant behaves, in terms of gravitropism, like a cytochalasin D-treated wild type, and (2) intact actin microfilaments seem to be required for efficient gravitropism (although the gravitropic response can proceed, to a reduced efficiency, in the presence of cytochalasin D). The exact role of actin microfilaments in gravitropic perception is presently under investigation (R. Godbolé et al. unpubl.), but it has already been shown for rice coleoptiles that grav-

itropic stimulation causes a rapid reorientation of cortical microtubules in the gravity-sensing cells of the bundle sheath (Nick et al. 1997). Pretreatment with cytochalasin D can induce this reorientation in the absence of gravitropic stimulation what might be the cause for the precocious initiation of bending in cytochalasin-treated coleoptiles (Fig. 1 B). Interestingly, microtubules in the bundle-sheath of *Yin-Yang* coleoptiles tend to loose the strictly transverse array characteristic for the wild type and to become longitudinal (Q.-Y. Wang unpubl. results).

In epidermal cells of the mutant, actin microfilaments depolymerize in response to auxin and reorganize into a nuclear basket (Fig. 6 D, E), a response that is not observed in the wild type (Fig. 5 G–J). However, nuclear baskets can be induced in the wild type, when the coleoptiles are treated with cytochalasin D (Fig. 5 E, F, K–M). On the level of growth, the segments of *Yin-Yang* coleoptiles are characterized by an increased sensitivity to cytochalasin D (Fig. 4 A), but this sensitivity requires auxin above a threshold of 200 nM to become manifest (Fig. 4 B, C).

Thus, characteristic aspects of the mutant phenotype can be phenocopied, at least qualitatively, when the wild type is treated with an actin-polymerisation blocker.

Increased sensitivity of coleoptile elongation in the mutant is not a trivial consequence of stimulated growth

In the presence of auxin, coleoptile segments excised from the mutant grow faster, and they are more sensitive to cytochalasin D (Fig. 4 A). This might indicate a common imbalance of the actin system under conditions of hypertrophic growth that render it more sensitive to the effect of the drug. To test this possibility, the dose–response curves at 30 °C were determined, a temperature that allows for more rapid growth in both wild type and mutant (Fig. 4 A). For two reasons the results of these experiments speak against a relationship between increased growth rate and increased sensitivity of growth to cytochalasin D. (1) Although the growth rate in the mutant is conspicuously higher than that of the wild type at 27 °C it can be increased to even higher levels when the temperature is raised to 30 °C (Fig. 4 A). (2) By incubation at 30 °C the growth rate of the wild type can be increased such that it reaches the level of the mutant at 27 °C (Fig. 4 A). If the sensitivity of growth to cytochalasin D were a consequence of the increased growth rate in the mutant one would expect that at 30 °C the wild type as well should be more sensitive to the drug. This was not observed.

Concluding, the increased sensitivity of the mutant to cytochalasin D is not caused by the increased growth rate, but must be explained in terms of altered properties of the actin cytoskeleton itself.

Yin-Yang mutation interferes with dynamics of the actin cytoskeleton

The sensitivity of cell elongation to the actin-eliminating drug cytochalasin D is strikingly increased in coleoptile segments of the mutant in presence of auxin concentrations that are optimal for growth (Fig. 4 A). Actin microfilaments were found to be less tightly bundled in epidermal cells of *Yin-Yang* than in the wild type (compare Fig. 5 with Fig. 6). Whereas auxin causes a loosening of these microfilament bundles in the wild type (Fig. 5), it produces a partial depolymerization of actin microfilaments in the mutant (Fig. 6). This depolymerization induced by auxin is accompanied by an elevated growth response (Figs. 3 and 4 A, C) indicating that in the mutant elongation was limited by the longitudinal bundles of actin microfilaments. In cultured cells of soy bean the stiffness of actin microfilament bundles is reduced upon addition of auxin (Grabski and Schindler 1996). A similar mechanism might be active during the auxin-triggered elongation response of the *Yin-Yang* mutant.

Cytochalasin D requires a certain turnover of actin microfilaments to be effective. The drug blocks actin polymerisation and eliminates microfilaments due to the ongoing depolymerisation on their shrinking end (Cooper 1987). The sensitivity of a given microfilament is therefore expected to reflect the level of subunit exchange. The increased drug sensitivity in the mutant (Fig. 4 A) might thus indicate that an elevated level of actin polymerisation is needed to maintain elongation under these conditions. This is consistent with previous findings observed for auxin-induced elongation of oat coleoptiles (Thimann et al. 1992, Thimann and Biradivolu 1994).

In the wild type, the condensed actin bundles that are characteristic for auxin-depleted cells (Fig. 5 A–C) seem to be less sensitive to low concentrations of cytochalasin D as compared to the fine, loose filaments that are observed in the presence of auxin (compare Fig. 5 D with K and L). In the mutant, auxin results in the disintegration of the long microfila-

ment bundles (Fig. 6 D). Concomitantly, short microfilaments appear that form a basket-like structure around the nucleus (Fig. 6 E). Similar nuclear baskets can be induced in the wild type by treatment with cytochalasin D (Fig. 5 F, L, M). Apparently, the excess of monomeric actin produced by the drug in the wild type or by auxin in the mutant is reassembled into short perinuclear filaments of reduced dynamics. In this context it should be mentioned that actin microfilaments are attached to the nuclear envelope in dividing cells (Traas et al. 1987, Lloyd 1991), but in the elongating cells of the coleoptile these nuclear attachment sites seem to be either masked or their affinity to actin might be too low to compete with those sites that organize the longitudinal bundles of actin. However, if the pool of monomeric actin is increased (e.g., by cytochalasin D in the wild type or by auxin in the mutant), the situation might be reversed.

Yin-Yang gene product possibly limiting for actin polymerisation

The increased sensitivity of *Yin-Yang* coleoptile segments to cytochalasin D disappeared at low concentrations of auxin (Fig. 4 B). On the other hand, even in the wild type, the sensitivity of actin microfilaments to cytochalasin D was more pronounced in the presence of 5 μM auxin (compare Fig. 5 D and E to K–M). Since the sensitivity of a given actin microfilament to cytochalasin D is expected to depend on its turnover, the increased sensitivity of microfilaments to cytochalasin D observed in the presence of auxin (Fig. 5 K–M) can be interpreted in terms of an auxin-dependent increase of actin depolymerisation that is counterbalanced by an increased polymerisation. This counterbalance seems to work in the wild type, but not in the mutant leading to an increased pool of G-actin (in a way similar to wild-type cells that have been treated by cytochalasin D) and the formation of nuclear baskets. A similar increase of actin polymerisation has been suggested to be a prerequisite for auxin-triggered growth of oat coleoptiles (Thimann and Biradivolu 1994). Thus, the gene product affected in the mutant *Yin-Yang* might be essential for the auxin-triggered stimulation of actin polymerisation.

Several actin populations with different functions?

When the relation between actin and elongation growth is analyzed, a seemingly paradoxical situation emerges: The longitudinal bundles of actin characteristic for most elongating cells appear to limit growth. This is demonstrated by the observation that the stimulation of growth by auxin is accompanied by a loosening of these longitudinal bundles and by the appearance of fine cortical filaments (Fig. 5 G–J). This is consistent with previous results on phytochrome-dependent cell elongation in maize (Waller and Nick 1997), and the observation that auxin can reduce the rigor of the actin cytoskeleton in soy-bean cells (Grabski and Schindler 1996). From these data one would expect that the elimination of the longitudinal actin bundles by cytochalasin D should produce a stimulation of cell growth. However, the opposite is observed (Fig. 4) consistent with results published for oat coleoptiles (Thimann et al. 1992). This indicates the existence of a second subpopulation of actin microfilaments that is required to support elongation growth and that is more sensitive to cytochalasin (which means that they are probably more dynamic). In maize coleoptiles, a polar meshwork of delicate filaments is observed in cells that undergo elevated elongation (Waller and Nick 1997), and the observation of the collapsed, but otherwise intact helicoidal arrays of actin that are observed for a mild treatment with cytochalasin D (Fig. 5 D) suggests that the dense actin bundles are tethered to the cell poles by a very sensitive meshwork of microfilaments. In addition to the longitudinal actin bundles, there exists a cortical meshwork of extremely delicate actin filaments that can be observed in some of the auxin-treated cells (Fig. 5 H, I). A similar cortical array of actin that coexists with the longitudinal bundles had been described earlier for cells that were pretreated with protein cross-linkers (Sonobe and Shibaoka 1989).

These observations could be explained by the following working hypothesis: There exist two subpopulations of actin microfilaments with different functions. (1) The longitudinal, condensed bundles of microfilaments limit cell elongation, perhaps even physically as indicated by the experiments by Grabski and Schindler (1995, 1996). These bundles are characterized by a reduced exchange of monomers and thus a relatively low sensitivity to cytochalasin D. These bundles are loosened by auxin (possibly accompanied by an increased rate of monomer exchange) relieving the (mechanical) constraint put upon cell elongation. (2) The cortical meshwork of delicate microfilaments is required to support cell elongation (Thimann et al. 1992). This meshwork is characterized by an elevated rate of monomer exchange and, consequently, an increased sensitivity to cytochalasin D. Auxin stimu-

lates the dynamics of these microfilaments even further, and the *Yin-Yang* gene product is expected to be involved, directly or indirectly, in this stimulation. The function of this cortical meshwork is still a matter of speculation – it could be related to vesicle transport towards the poles of the cell. Alternatively, it might be important for the correct organization of the microtubular cytoskeleton (Seagull 1990).

The coexistence of different actin arrays with possibly different functions poses an intriguing logical problem: Actin is a relatively conservative molecule. Although it cannot be excluded that cortical meshwork and longitudinal bundles of actin consist of different actin isotypes, it appears more likely that the difference is extrinsic to the actin molecule itself, but caused by decoration with a different set of binding proteins. Among the binding proteins, the myosins seem to be the most attractive candidates, because the rapid spatial control of cell expansion is expected to involve a force-producing mechanism. In this context the recent finding has to be mentioned that a fusion of the green fluorescent protein to the head domain of an unconventional myosin, Mya2, decorates specifically the cortical actin meshwork of elongating tobacco cells in vivo, but not the longitudinal bundles of microfilaments (Freudenreich and Nick unpubl.). Future studies will therefore focus on the auxin response of different myosins in the mutant *Yin-Yang*.

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