

# Auxin-dependent microtubule responses and seedling development are affected in a rice mutant resistant to EPC

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## Summary

Mutants in rice (*Oryza sativa* L. cv. *japonica*) were used to study the role of the cytoskeleton in signal-dependent morphogenesis. Mutants obtained by gamma ray irradiation were selected that failed to show inhibition of coleoptile elongation by the anti-microtubular drug ethyl-*N*-phenylcarbamate (EPC). The mutation *EPC-Resistant 31* (ER31), isolated from such a screen, caused lethality in putatively homozygous embryos. Heterozygotes exhibited drug resistance, impaired development of crown roots, and characteristic changes in the pattern of cell elongation: cell elongation was enhanced in mesocotyls and leaf sheaths, but inhibited in coleoptiles. The orientation of cortical microtubules changed correspondingly: for etiolated seedlings, compared with the wild-type, they were more transverse with respect to the long cell axis in mesocotyls and leaf sheaths, but more longitudinal in coleoptiles. In mutant coleoptiles, in contrast to wild-type, microtubules did not reorient in response to auxin, and their response to microtubule-eliminating and microtubule-stabilizing drugs was conspicuously reduced. In contrast, they responded normally to other stimuli such as gibberellins or red light. Auxin sensitivity as assayed by the dose-response for callus induction did not show any significant differences between wild-type and mutant. The

mutant phenotype is interpreted in terms of an interrupted link between auxin-triggered signal transduction and microtubule reorientation.

## Introduction

Plants can tune their growth and development with their environment. This adjustment implies a link between signal transduction and morphogenesis. Form, polarity, and state of individual cells are often under the control of environmental signals, a phenomenon that is still far from being understood (Nick and Furuya, 1992). The cytoskeleton might be expected to control this phenomenon, since it can respond to external signals and mediate specific changes in cell shape. For instance, actin microfilaments appear to respond to signals transduced by G-proteins in animal cells (Ridley and Hall, 1992) or by elements of the phosphoinositide cycle in both animal and plant cells (Aderem, 1992; Drøbak, 1993).

In plants, it may well be the cortical microtubules, that connect signal transduction to cellular morphogenesis: they reorient readily in response to a range of various stimuli such as plant hormones (Shibaoka, 1991), blue light (Nick *et al.*, 1990), red light (Zandomeni and Schopfer, 1993), and gravity (Blancaflor and Hasenstein, 1993). They can control cellular morphogenesis by defining the mechanical properties of the cell wall (Green, 1969). Reorientation of cortical microtubules seems to be an essential step in the induction of a new cell polarity (Green, 1969; Hush *et al.*, 1990; Williamson, 1991) and both microtubule alignment and change in polarity have been extensively discussed with respect to auxin transport (Sachs, 1991). In Graminean coleoptiles, a correlation between blue light as environmental signal, redistribution of auxin transport (Nick *et al.*, 1992), reorientation of microtubules (Nick *et al.*, 1990), and the induction of a new polarity (Nick and Schäfer, 1991) has been observed. In order to address a possible causal relationship, it is necessary to interrupt this chain of events and study the consequences of this interruption.

A mutant approach has been chosen to achieve this goal. The search for an appropriate mutant in a Japanese rice cultivar was directed by the following considerations about possible phenotypes of such a mutant.

- (i) The mutation is expected to cause pleiotropic alterations of development, possibly leading to lethality or sublethality of homozygous plants.

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- (ii) The signal-dependent plasticity of the cytoskeleton should affect those responses that are under the control of auxin. In rice, a very reliable and conspicuous trait falling into this category is coleoptile elongation, a process that might be used as a selectable marker (Furuya *et al.*, 1969).
- (iii) In such responses, the changed plasticity of the cytoskeleton can lead to a changed sensitivity against appropriate drugs. A screen for resistance against such drugs should yield—amongst others—mutants with changed cytoskeletal plasticity in response to auxin.

A selection system was designed based upon these deliberations, with the resistance of coleoptile elongation to the antimicrotubular drug ethyl-*N*-phenylcarbamate (EPC) taken as a selectable marker. This drug prevents polymerization of plant tubulin *in vitro*, but does not disrupt preformed microtubules polymerized without the addition of taxol (Mizuno and Suzaki, 1990). This inhibition causes elimination of microtubules *in vivo* at a rate depending on the kinetics of depolymerization and repolymerization (Shibaoka and Hogetsu, 1977). As for other drugs with the same mode of action, for instance colchicine (Bergfeld *et al.*, 1988), EPC blocks coleoptile elongation and gravitropism (Nick *et al.*, 1991). Since the mutation was assumed to infringe on plant viability, the mutants were selected in a lethality screen, i.e. the population was not pooled, but each mutant line kept strictly apart to maintain lethal mutations by segregation from the offspring of heterozygous plants.

## Results

### *Mutant embryos and seedlings exhibit altered development*

The mutant *EPC-Resistant 31* (ER31) was detected by its ability to maintain normal coleoptile elongation and gravitropism in the presence of the antimicrotubular drug ethyl-*N*-phenylcarbamate (EPC). The plants recovered from this selection scheme were selfed and their offspring raised on water in total darkness for 6 days to ensure maximal elongation of the coleoptile (Pjon and Furuya, 1967).

The population segregated into three fairly distinct subpopulations (Figure 1). About a quarter of the seedlings exhibited a phenotype, that was identical to the wild-type, with long coleoptiles and extremely short mesocotyls, separated by a node with well-developed crown roots. About half of the population consisted of seedlings with a striking mutant phenotype. The coleoptiles were shorter than in the wild-type by about a third, whereas the mesocotyl was five times longer than in the wild-type.

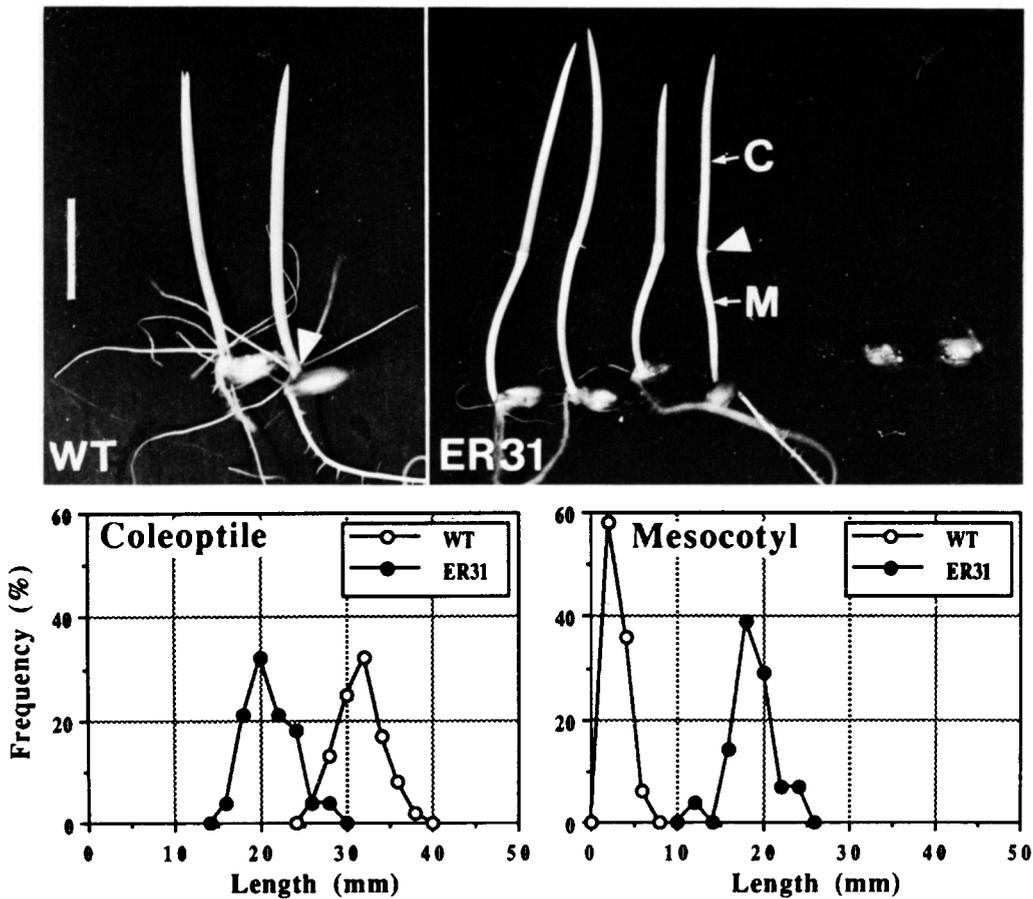
Moreover, crown-root development appeared to be delayed and impaired in those seedlings. The remaining quarter of the seeds did not germinate. They could not be made to continue development even by treatment with plant hormones and tissue-culture media (data not shown).

Measurements of epidermal cell length (Figure 2) suggest that the observed inhibition of coleoptile elongation as well as the much increased mesocotyl growth in heterozygous plants is correlated to cell elongation. In other words, coleoptiles of mutant plants are short because their cells are short and the elevated elongation of the mesocotyl is based upon an elevated elongation of cells in this organ. However, in parallel, these changes in cell length are accompanied by less dramatic changes in cell number (from 167 in coleoptiles of the wild-type to 114 in mutant coleoptiles, and from 111 in mesocotyls of the wild-type to 196 in mutant mesocotyls). The time course for the increase in cell length reveals that elongation is complete at 6 days after sowing for both wild-type and mutant (Figure 2). Hence the shorter coleoptiles of the mutant cannot be explained in terms of a longer duration of growth at a slower rate.

Total seedling length is very similar in wild-type and mutant plants. Thus, the mutation appears to shift growth activity from the coleoptile to the mesocotyl. A similar enhancement of basal versus apical growth is observed later during the development of the leaves, when, in mutant plants, the elongation of the leaf sheath is stimulated over the elongation of the leaf blade (Table 1).

The inheritance of these traits was followed over several generations of selfing (Table 2). The offspring of the wild-type phenotype were found to be homogeneously wild-type, whereas the offspring of mutant plants segregated again in the fashion described above. These data are consistent with the assumption that the seedlings with the long mesocotyl, the short coleoptiles, and the impaired crown-root development mirror the heterozygous state of the mutation, whereas the non-germinating seeds represent its homozygous state. A  $\chi^2$  test revealed close congruence between the observed data and the hypothesis of monogenic Mendelian inheritance (Table 2). However, it cannot be excluded that the mutation (probably a deletion) spans several genes that belong to one linkage group. A more detailed genetic analysis involving backcrossing experiments is underway to clarify this question.

In order to understand the reason for the impeded germination of putatively homozygous mutant seeds, mature embryos were dissected immediately after soaking. The embryos of mutant seeds fell into three subsets (Figure 3d). About a quarter of them had dome-shaped, relatively stunted coleoptiles as found in the offspring of wild-type plants (Figure 3a). In about half of the embryo population, coleoptiles were found to be twice as long, but still dome-



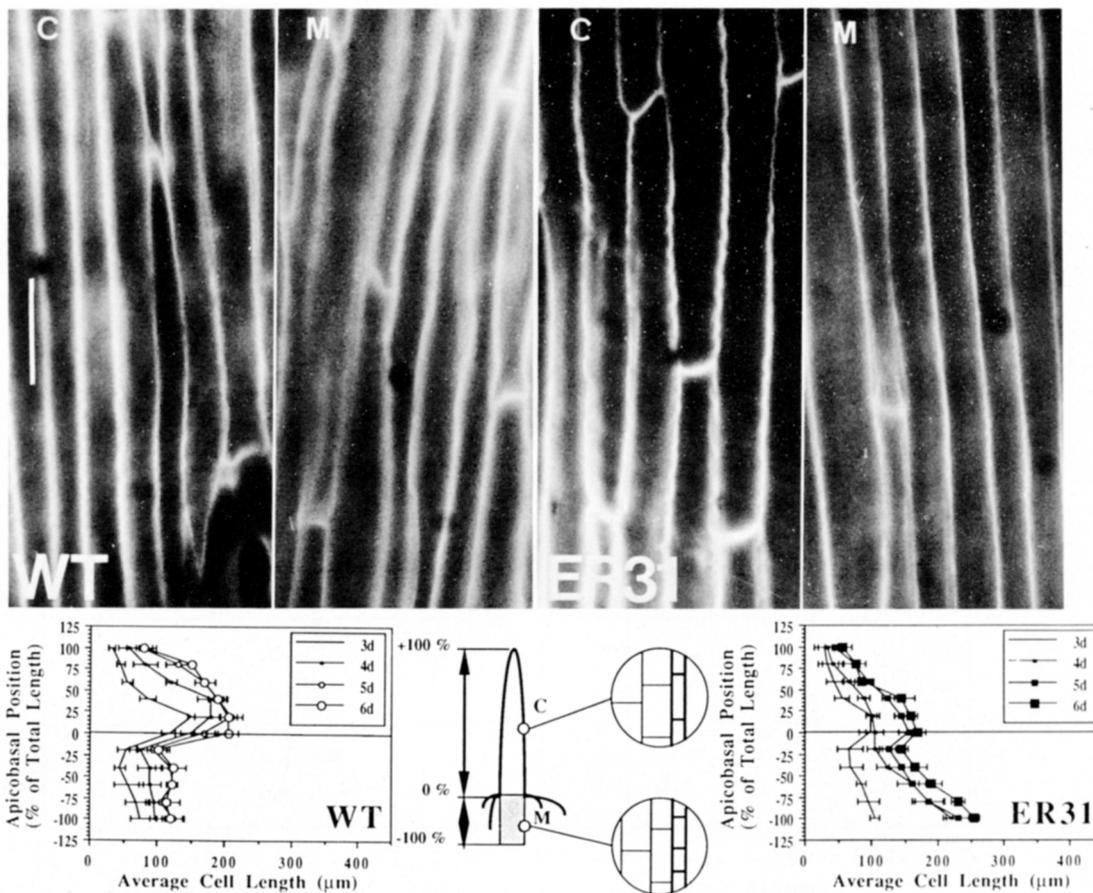
**Figure 1.** Phenotype of wild-type and *ER31* seedlings. Plants were grown for 6 days at 25°C in darkness to allow for maximal cell elongation. C, coleoptile; M, mesocotyl; separated by the first node (white arrow), from which the crown roots emerge. The offspring of mutant plants segregated into seedlings exhibiting the wild-type phenotype (WT), a majority of seedlings expressing the mutant phenotype (middle), and seeds with abortive development (right). The frequency distribution over length of coleoptiles and mesocotyls for seedlings of wild-type and *ER31* (exhibiting the mutant phenotype) is based upon the data from 100 individuals. The white bar corresponds to 10 mm.

**Table 1.** Enhanced elongation of leaf sheaths in heterozygous mutant plants—final length of sheath and blade of juvenile leaves after 4 weeks of cultivation in white light and probability *P* that the difference is significant

Leaf	Leaf sheath (mm)			Leaf blade (mm)		
	Wild-type	<i>ER31</i>	<i>P</i>	Wild-type	<i>ER31</i>	<i>P</i>
2	47 ± 14	54 ± 14	>0.90	67 ± 19	56 ± 7	<0.10
3	52 ± 9	86 ± 9	>0.95	68 ± 10	74 ± 4	<0.50
4	68 ± 13	104 ± 6	>0.99	109 ± 8	105 ± 17	<0.50
5	82 ± 7	134 ± 7	>0.99	123 ± 7	109 ± 20	<0.90

shaped (Figure 3b). Such embryos were not found in the offspring of wild-type plants and probably represent the heterozygous mutant phenotype. In the remaining quarter of seeds the coleoptiles were even longer and spindle-shaped in appearance (Figure 3c). In contrast to results obtained with the putatively heterozygous mutant embryos (see below), all attempts to induce callus from

this third type of embryo have failed so far. This suggests that these long embryos (Figure 3c) represent the lethal, homozygous state of the mutation. The comparison of coleoptile length in those embryos with the fully expanded coleoptile of etiolated seedlings indicates that coleoptile elongation during embryonic development is inversely correlated to post-embryonic coleoptile elongation.



**Figure 2.** Differences in cell length between wild-type (WT) and *ER31* seedlings.

The upper panel shows epidermal cells of an etiolated coleoptile (C) and mesocotyl (M) at the site indicated in the schematic drawing 6 days after sowing. Cell walls were visualized by their autofluorescence in UV-A light. The bar represents 10  $\mu\text{m}$ . The lower panels show the time course of cell elongation in the epidermis along the coleoptile (positive ordinate values) and mesocotyl (negative ordinate values) starting from the first node as indicated in the schematic drawing.

**Table 2.** Segregation in the offspring of mutant plants with respect to parental phenotype

No.	Phenotype	WT	Mutant	Lethal	$n$
1	Mutant	27	53	20	34
1-A	Mutant	26	52	22	31
1-B	WT	100	0	0	27
1-C	Mutant	21	47	32	26
2	WT	100	0	0	28
3	WT	100	0	0	28
4	Mutant	27	48	25	20
5	Mutant	33	33	34	24

The  $\chi^2$  test was based upon the model of monogenic Mendelian inheritance with incomplete dominance.

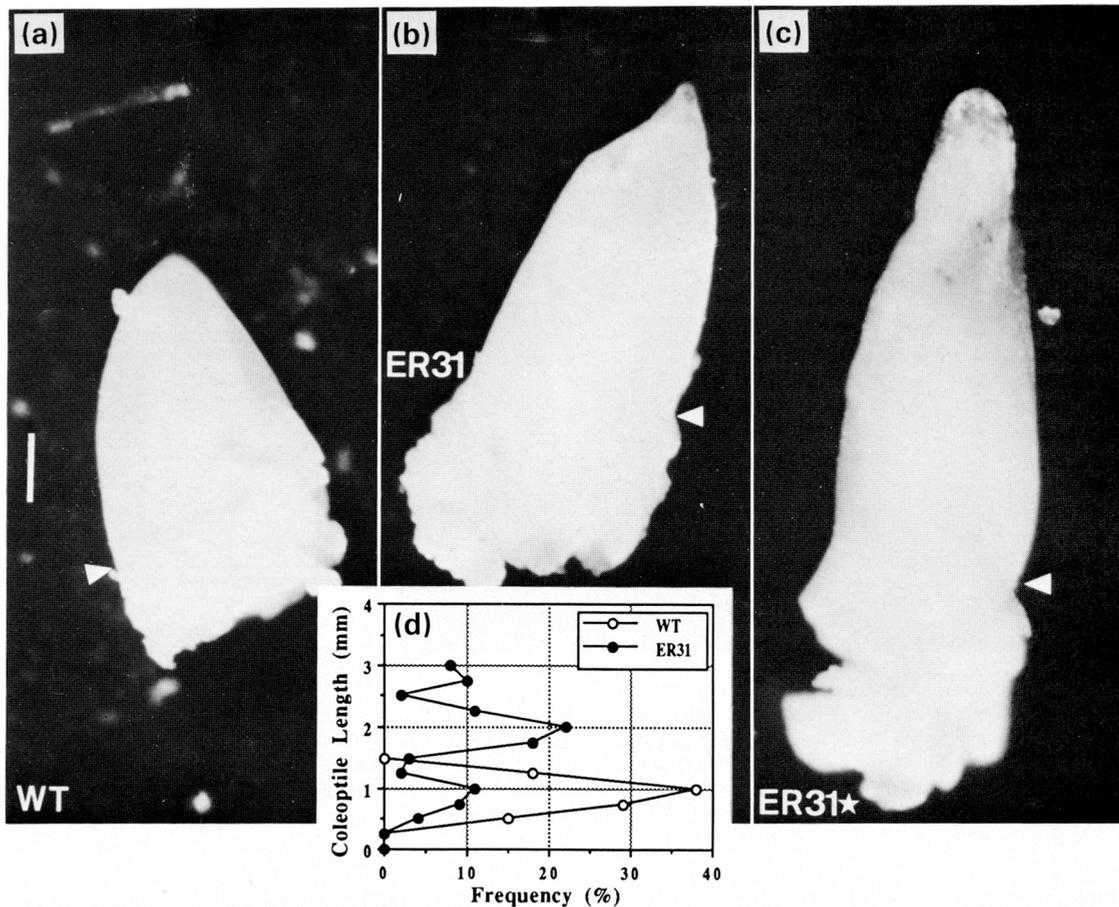
$\chi^2 = 4.5$  for 16 degrees of freedom ( $P_{0.99} = 32$ ,  $P_{0.5} = 15.3$ ,  $P_{0.01} = 5.8$ ).

*The mutation ER31 confers resistance against microtubule-eliminating and microtubule-stabilizing drugs*

If seedlings of the wild-type were grown in the presence of the microtubule-eliminating drug EPC, a severe reduction

of coleoptile and root elongation was the consequence (Figure 4). Moreover, coleoptiles apparently were not able to respond to gravity, resulting in a twisted and distorted growth habit. In contrast, shoots and roots of putatively heterozygous mutant seedlings were able to undergo normal growth in the presence of the drug and their morphology was comparable with that of mutant plants raised in the absence of the drug (Figures 1 and 4).

A dose–response curve of coleoptile and mesocotyl elongation versus drug concentration in the cultivation medium was obtained (Figure 4, right-hand panel). Compared with the wild-type, the mutant requires higher concentrations of the drug to show impaired elongation of coleoptiles and mesocotyls. For concentrations exceeding 0.1 mM EPC, this leads to a situation in which mutant coleoptiles are longer than coleoptiles from wild-type seedlings raised under the same conditions. It should be emphasized, however, that the concentrations indicated on the abscissa are those in the incubation medium—the actual concentration within the cells may well be much



**Figure 3.** Mature embryos of wild-type and *ER31*. Embryos were dissected from briefly soaked mature seeds. The positions of the first nodes are marked by the white arrows. White bars represent 250 μm. (a) Wild-type. (b) Most common embryo type in *ER31* seeds. (c) Severe mutant phenotype (*ER31\**), found in some of the *ER31* seeds. (d) Frequency distribution over coleoptile length, based upon 80 embryos dissected from the wild-type and *ER31* seeds.

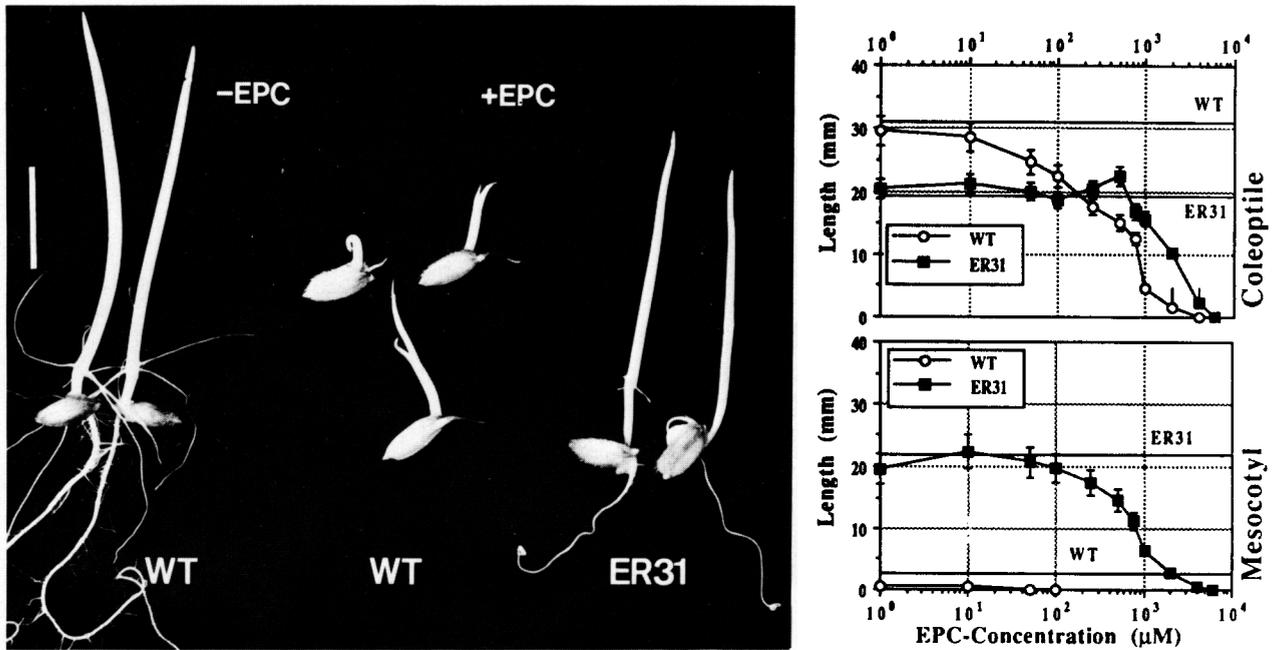
lower, since the drug has to pass the endodermis and vascular system on the way to the target tissue. It cannot be excluded that the 'resistance' of the mutant plants might be the consequence of a reduced drug translocation in the mutant. However, roots of mutant plants imbibed in the solution with the drug are still able to grow, whereas roots of wild-type plants cannot grow under those conditions (Figure 4). Moreover, non-embryonic calli obtained from mature embryos of wild-type and mutant show a differential response to the drug (Table 3). This does not suggest differences in EPC transport producing an apparent resistance *in planta*, but rather favours the view that the increased resistance of mutant seedlings is based upon differential drug responses of the target cells. However, it cannot be excluded that reduced uptake into the target cells takes place in the mutant. To exclude this possibility, this study is to be extended by uptake measurements using radioactively labelled EPC accompanied by experiments with microinjection of EPC.

In order to test this assumption, the responses of micro-

**Table 3.** Inhibition of callus growth by EPC

Time (weeks)	Callus fresh weight per culture vessel (g)			
	Wild-type		<i>ER31</i>	
	Control	+EPC	Control	+EPC
0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
1	1.9 ± 0.4	1.3 ± 0.6	1.5 ± 0.9	1.3 ± 0.3
2	3.7 ± 1.3	1.5 ± 0.4	2.7 ± 0.2	2.1 ± 0.9
3	7.0 ± 0.8	1.5 ± 0.9	5.4 ± 1.8	4.3 ± 0.7
4	15.3 ± 2.6	1.8 ± 1.0	10.6 ± 0.9	7.6 ± 2.1

tubules to EPC (as microtubule-eliminating drug) and taxol (as microtubule-stabilizing drug) were assayed in apical coleoptile segments. These segments were incubated in 0.1 mM IAA to ensure optimal elongation. In wild-type plants, the treatment with EPC caused the microtubules to disappear within 1 or 2 h (Figure 5), although fluorescent dots remained aligned along the lateral walls, possibly representing 'end-on' fluorescence



**Figure 4.** Resistance of mutant seedlings to EPC.

Seeds were germinated in darkness for 6 days at 25°C in the presence of 10<sup>-3</sup> M EPC. Left: appearance of wild-type (WT) seedlings grown without EPC, Middle: wild-type seedlings grown in the presence of EPC. Right: mutant seedlings (*ER31*) grown in the presence of EPC. The white bar indicates 5 mm. Right-hand panels: dose-dependence of growth on the concentration of EPC. Length of etiolated coleoptiles (upper panel) and mesocotyls (lower panel) after 6 days of cultivation on various concentrations of EPC is shown for wild-type (open symbols) and heterozygous *ER31* plants (closed symbols). Solid horizontal lines give the response of the water controls. Data represent the average of 20 plants.

of microtubules down the radial walls. Electron-microscopical studies of auxin-mediated microtubule reorientation in the epidermis of maize coleoptiles (Bergfeld *et al.*, 1988) demonstrate that cortical microtubules along the radial walls lack the dynamics observed for the microtubules adjacent to the outer epidermal wall. In contrast to the wild-type, in the mutant plants, microtubules did not appear to be any different from those in mutant cells, which were incubated in water. On the other hand, incubation with taxol, a drug stabilizing the polymerized form of tubulin (Morejohn, 1991), produced in the wild-type additional microtubules aligned in parallel to the pre-existing microtubules eventually covering the cell with a dense carpet of microtubules. The mutant did not show such a response.

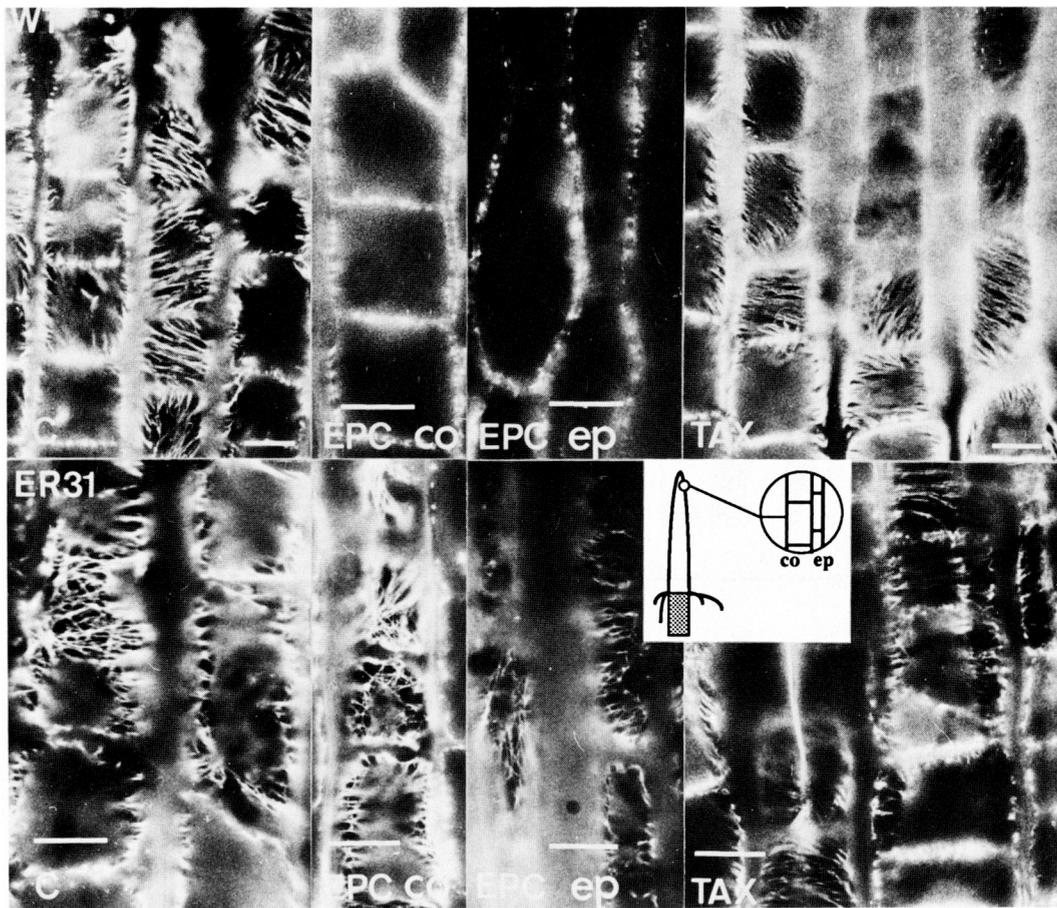
#### *Auxin-dependent microtubule reorientation is affected in mutant plants*

In etiolated coleoptiles of wild-type seedlings raised on water without EPC, the cells of the outer epidermis displayed oblique arrays of cortical microtubules with respect to the long cell axis (Figure 6, left-hand panel and white frequency distribution). In contrast, in epidermal cells of mutants raised in darkness on water without the drug, almost longitudinal arrays were more abundant

(Figure 6, right-hand panel and black frequency distribution). The microtubules in the cells of the subepidermal cortex were found to be transverse in the wild-type (Figure 6, left-hand panel and white frequency distribution), but oblique in the mutant (Figure 6, right-hand panel and black frequency distribution).

Coleoptile elongation in rice is under the control of auxin (Furuya *et al.*, 1969) and in epidermal cells the orientation of microtubules can be influenced by this hormone (Bergfeld *et al.*, 1988). Therefore, an experiment was designed to induce transverse microtubule arrays in mutant coleoptiles by the addition of indolyl-3-acetic acid. Subapical segments of etiolated coleoptiles of the wild-type were depleted of endogenous auxin by incubation in water for 2 h. This treatment yielded steeply oblique microtubule arrays, imitating the situation in etiolated, intact mutant coleoptiles (Figure 7). By the addition of 0.1 mM IAA they were rearranged into transverse arrays within 1 h of addition of the hormone. On the other hand, even 3 h of incubation in 0.1 mM IAA was not able to induce transverse microtubule arrays in mutant coleoptiles.

It might be that the mutation confers insensitivity to auxin causing the observed failure of this hormone to elicit microtubule reorientation. Therefore, a dose-response relationship for the auxin-dependent switch of mature embryos from germination to callus formation was deter-



**Figure 5.** Effect of EPC and taxol on microtubules in apical coleoptile segments. Apical segments were incubated in 0.1 mM IAA (left, control C), with 0.1 mM IAA and 1 mM EPC (middle, EPC), or with 0.1 mM IAA and 20  $\mu$ M taxol (right, TAX) for 1 h. Immunofluorescence images show cells of the cortex (co) and for the EPC treatment also of the epidermis (ep). Note the elimination of cortical microtubules by EPC in the wild-type (focus on cell surface) and the 'end-on' fluorescence along the cell walls. The schematic drawing indicates location and tissue layers (bold lines) used for this experiment. The white bars represent 10  $\mu$ m.

mined (Table 4). It did not reveal any significant differences between wild-type and mutant, suggesting that, at least for callus induction, auxin perception and transduction are not altered in the mutant. To assess whether reorientation of microtubular arrays in the mutant occurs at all, the orientation of microtubules was followed in sub-epidermal cells of the cortex after treatment of excised coleoptile segments with gibberellic acid (Nick and Furuya, 1993). In both wild-type (data not shown) and mutant, microtubules were found to be aligned in more transverse arrays compared with the oblique orientation in untreated controls 2 h after addition of 10  $\mu$ M GA<sub>3</sub> to the culture medium (Figure 8).

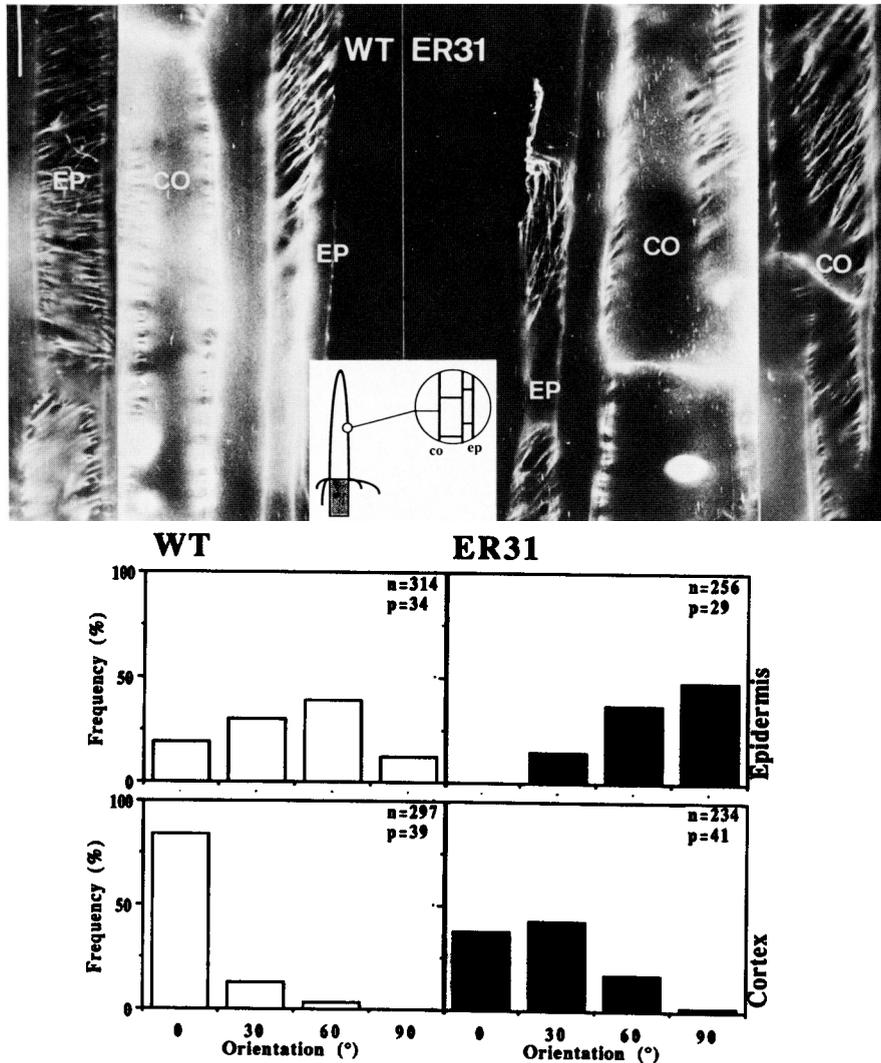
Alternatively, intact coleoptiles were subjected to a pulse irradiation with red light as described in Nick and Furuya (1993). This time, in both wild-type (data not shown) and mutant, more longitudinal arrays of microtubules were found 2 h after a pulse of 1  $\mu$ mol m<sup>-2</sup> of red light.

Thus, microtubules can change their orientation in mutant plants in response to gibberellic acid and red light. Moreover, even in epidermal cells of mutant plants transverse or oblique microtubules can be found: mesocotyl elongation, which seems to be under the control of gibberellin rather than auxin (Nick and Furuya, 1993), is enhanced in the mutant compared with the wild-type (Figure 1), and microtubules in the epidermis of such etiolated mutant mesocotyls are more transverse with respect to the long cell axis, in contrast to the fairly longitudinal arrays found in the wild-type (Figure 9).

## Discussion

### *The mutation ER31 disrupts the link between auxin transduction and reorientation of microtubular arrays*

The mutant phenotype is characterized by a block in auxin-dependent microtubule reorientation (Figure 7).



**Figure 6.** Orientation of microtubules in etiolated coleoptiles of wild-type and *ER31*. Immunofluorescence images of cortical microtubules in epidermal (EP) and cortical (CO) cells of etiolated coleoptiles 5 days after sowing. Left-hand panel, wild-type; right-hand panel, mutant. The white bar represents 10  $\mu$ m. The schematic drawing indicates location and tissue layers (bold lines) used in this experiment. Lower panel: frequency distribution for microtubule orientation defined as the angle between the axes of the microtubule array and the short cell axis with 0° indicating transverse and 90° longitudinal microtubules. n, Number of cells; p, number of plants used for the frequency distribution.

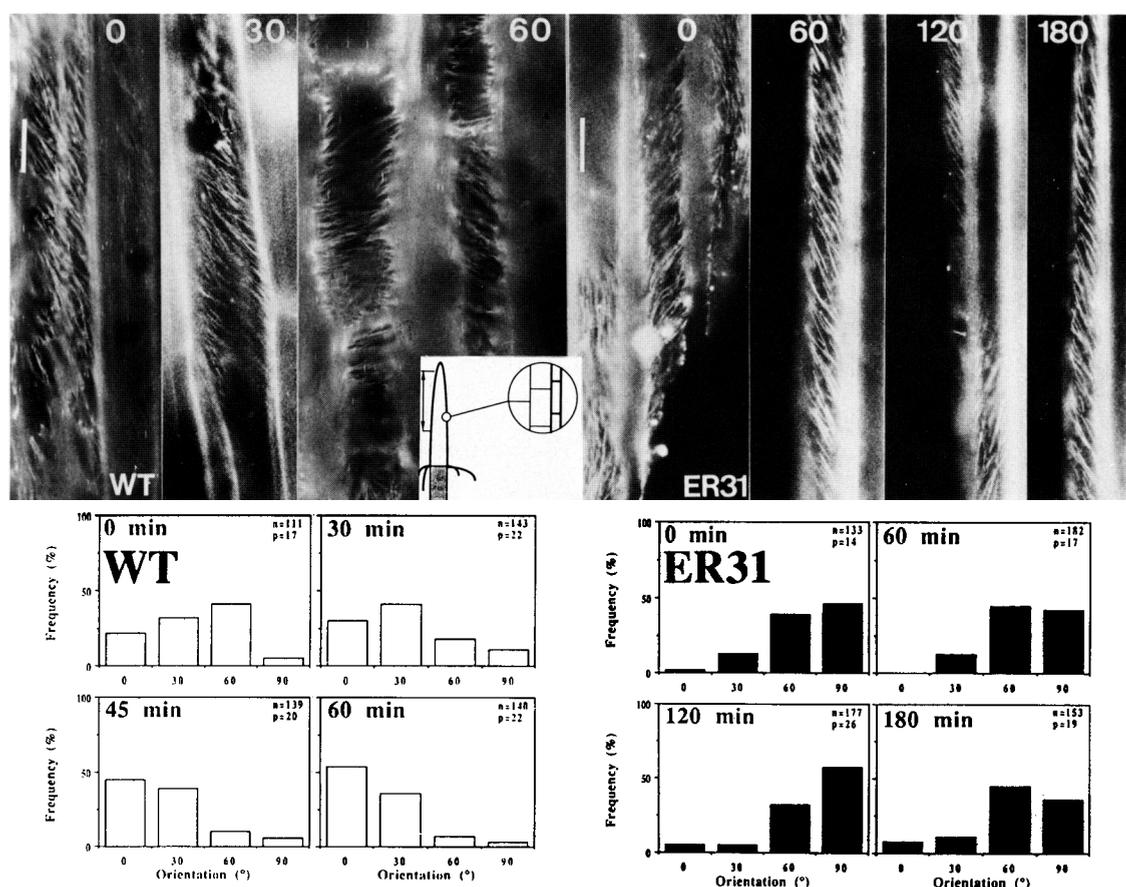
Further, auxin-dependent growth responses such as cell elongation in the coleoptile (Furuya *et al.*, 1969) or the formation of crown roots (Thimann, 1936) are hampered by this mutation (Figures 1 and 2). There are three ways to interpret this phenomenon.

- (i) Auxin-dependent signal transduction might be altered. However, responses (Table 4) exhibiting a normal auxin dose-response relation exist. This implies that at least one auxin-dependent signal chain in the mutant is reasonably intact.
- (ii) Microtubule arrays have lost their ability to reorient. However, a reorientation can be induced by other stimuli such as red light or gibberellic acid (Figure 8), or in tissues (Figure 9) whose growth appears to be

controlled by factors other than auxin (Nick and Furuya, 1993). Hence, the machinery necessary for

**Table 4.** Dose-response relation for callus induction by auxin

IAA (p.p.m.)	Number of calli per 10 embryos induced in 3 weeks			
	Wild-type		<i>ER31</i>	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
0.0	0	0	0	0
0.1	1	0	2	0
0.3	1	3	2	1
1.0	9	7	5	10
3.0	9	10	10	10
10	10	8	9	8



**Figure 7.** Failure to induce transverse microtubular arrays in *ER31* by auxin.

Subapical segments of etiolated coleoptiles (location indicated in the schematic drawing) were depleted of endogenous auxins by incubation in water for 2 h. At time 0,  $10^{-4}$  M IAA were added and microtubule orientation was assayed at 0, 30, and 60 min for the wild-type (left-hand panel), and at 0, 60, 120, and 180 min for heterozygous *ET31* coleoptiles (right-hand panel). The white bars indicate 10  $\mu$ m. Lower panel: frequency distribution for microtubule orientation. For details refer to the legend of Figure 6.

the reorientation of microtubular arrays is essentially intact in the mutant.

- (iii) The link between auxin-dependent signal transduction and the processes leading to microtubule reorientation is interrupted. The hypothesis could explain why other auxin-triggered responses are not affected in the mutant (Table 4) as well as the observation that microtubule orientation can still respond to other stimuli, such as gibberellic acid and red light (Figure 8).

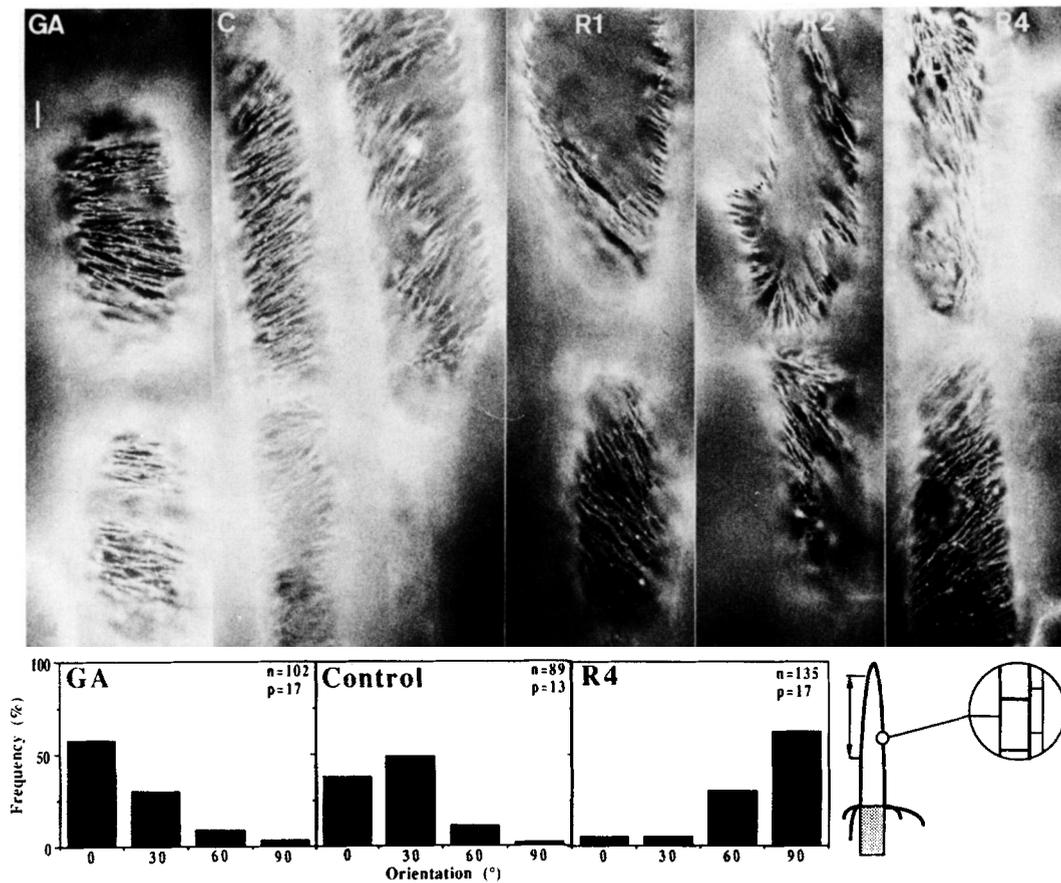
This third possibility is the only one that can be reconciled with the data reported here.

#### *The mutation ER31 slows down microtubular turnover*

Mutant seedlings are able to elongate normally in the presence of the microtubule-eliminating drug EPC (Figure 4). One explanation for this phenotype assumes a disturbed translocation of the drug in mutant plants.

However, a differential drug response is found in non-embryogenic calli (Table 3), in cells of apical coleoptile segments (Figure 5), and in roots (Figure 4), i.e. in situations where translocation of the drug should not be limiting. These results suggest a 'true' drug resistance of the target cells rather than an impaired distribution of the drug to those cells. This resistance includes both cell elongation (coleoptiles) and cell division (roots, see Figure 4).

EPC has been suggested to eliminate microtubules by preventing the polymerization of tubulin (Mizuno and Suzaki, 1990). Microtubules with a high rate of assembly and disassembly are expected to be especially sensitive to the drug, whereas drug-resistant microtubules might be produced by decreasing the rate of turnover. The counter-acting drug taxol stabilizes polymerized microtubules (Morejohn, 1991) and its effects should be most dramatic in a situation of high assembly activity. In apical segments obtained from mutant coleoptiles neither EPC nor taxol was able to cause the effects seen in the wild-type (Figure 5). The effects of taxol could be explained in terms



**Figure 8.** Induction of microtubule reorientation by gibberellin and red light in *ER31*.

Subapical segments of etiolated coleoptiles of heterozygous seedlings of *ER31* (location as indicated in the schematic drawing) were incubated for 2 h in  $10^{-5}$  M  $GA_3$  (GA). Alternatively, intact plants were subjected to a pulse irradiation with  $1 \mu\text{mol m}^{-2}$  red light and microtubules assayed at 0 (control C), 1 (R1), 2 (R2), or 4 (R4) h after induction. The white bars indicate 10  $\mu\text{m}$ . Lower panel: frequency distribution over microtubule orientation. For details refer to the legend of Figure 6.

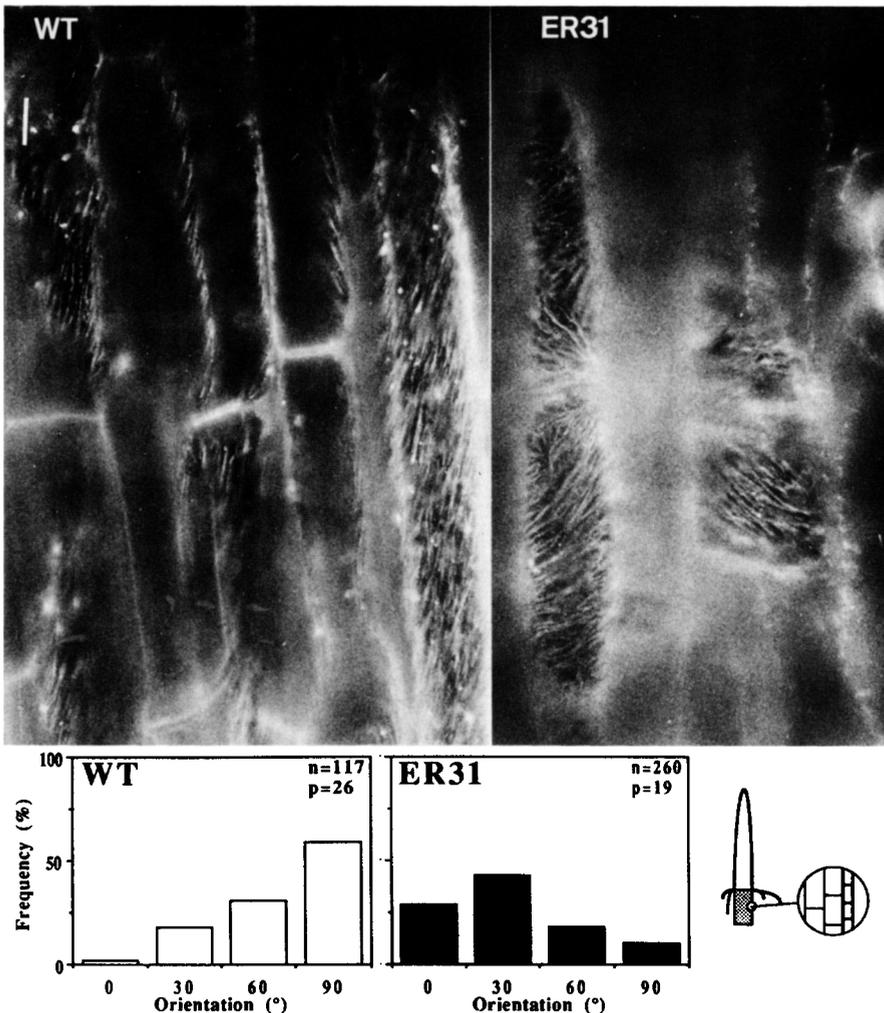
of a smaller pool of soluble tubulin in the mutant, such that the formation of microtubule carpets would be limited. However, a smaller pool of soluble tubulin cannot explain at all the observed stability of microtubules in the presence of EPC. Thus, it seems more likely that microtubules in mutant coleoptiles exhibit a lowered rate of assembly and disassembly. This could possibly be either the consequence of changed tubulin isotypes (Murphy, 1991) or alterations in the pattern of microtubule-associated proteins (Schellenboum *et al.*, 1992). Two-dimensional gel electrophoresis of wild-type and mutant tubulin should allow future discrimination between these possibilities.

#### *Pleiotropic effects of the mutation ER31*

In its homozygous state, the mutation *ER31* is lethal (Figure 1, Table 2). Mature, putatively heterozygous mutant embryos are longer than wild-type embryos (Figure 3). Putatively homozygous mutant embryos are even longer than the putatively heterozygous ones, and it is possible

that this abnormal elongation is related to the observed lethality. In homozygotes, neither treatment with plant hormones nor tissue-culture media (data not shown) were able to induce germination of callus formation suggesting that those embryos are already dead. Preliminary data using tetrazolium staining (data not shown) confirm this interpretation. One may speculate whether the precocious elongation of mutant cells interferes with the developmental switch towards anabiosis in the late phase of seedling development.

In contrast to coleoptile growth before the desiccation phase, cell elongation after germination appears to be inhibited in mutant coleoptiles (Figure 2). This inhibition might be caused by the lack of a microtubule response to auxin (Figures 6 and 7). It is not known which factors control growth during late embryogenesis (Sachs, 1991), but the long mutant embryos suggest that hormones other than auxin might be involved. Alternatively, embryonic coleoptile growth, predominantly based upon cell division, might be controlled by an auxin signal transduction path-



**Figure 9.** Orientation of microtubules in etiolated mesocotyls of wild-type and *ER31*. Immunofluorescence images of microtubules in the epidermis of etiolated mesocotyls 5 days after sowing (location and tissue layers indicated in the schematic drawing) of wild-type (WT) and heterozygous mutant (*ER31*) seedlings. White bars indicate 10 μm. Lower panel: frequency distribution over microtubule orientation. For details refer to the legend of Figure 6.

way that is different from that triggering postgerminative cell elongation. The existence of such differing pathways has been inferred from experiments with tobacco plants, that had been transformed by the *rol* gene from *Agrobacterium rhizogenes* (Maurel *et al.*, 1991).

The growth inhibition observed in heterozygous mutant coleoptiles is accompanied by a growth stimulation in the mesocotyl, such that total seedling length in mutants is comparable with wild-type plants (Figure 1). Thus, in mutant plants, the partitioning of growth between coleoptiles and mesocotyls seems to be shifted in favour of the mesocotyl. This shift points towards competition between coleoptile and mesocotyl for limiting factors essential for growth—the stimulation of mesocotyl elongation in the mutant might therefore be a pleiotropic consequence of the blocked cell elongation in the coleoptile. This interpretation is strengthened by the observation that inhibitors of coleoptile growth, such as for instance abscisic acid, are able to stimulate mesocotyl growth (Takahashi, 1972). A similar repartitioning of growth towards the basal regions

is observed later in development when leaf sheath elongation is elevated, whereas elongation of the leaf blade is not (Table 1).

Alternatively, microtubular proteins of the cells in the coleoptile could be different from those in the mesocotyl and this difference could cause changes in microtubular dynamics. To approach this question, an analysis of tubulin isotypes by two-dimensional gel electrophoresis in coleoptiles and mesocotyls from wild-type and mutant is presently being prepared.

#### Outlook

The mutation *ER31* seems to block an event linking auxin-triggered signal transduction with the reorientation of microtubular arrays—possibly by reducing microtubular turnover (Figure 5). Possible candidates for such a mutation might be those kinases that regulate the activity of microtubule-associated proteins (Ma 1993; Shiina *et al.*, 1992). The mutant *ER31* may well open approaches for

the study of such proteins. Alternatively, this mutant could provide a tool for the analysis of signal-triggered polarity induction in plants.

## Experimental procedures

### *Induction and selection of rice mutants*

The mutants were obtained by  $\gamma$ -ray irradiation from a  $^{60}\text{Co}$  source at the Institute of Radiation Breeding (National Institute of Agrobiological Resources, Ohmiya-machi, Japan) administered to seeds of the *japonica* type cultivar 'Nihonmasari'. A screen for resistance to ethyl-*N*-phenylcarbamate (EPC) was performed with the progenies of those seeds produced after two generations of selfing. The dose of irradiation (200 Gy) was chosen such that the likelihood of one mutation per panicle was maximized. The panicles were harvested separately and treated as putatively independent mutant lines.

Random samples of 20 seeds from each mutant line were used for selection. Seeds were cultivated for 6 days on selection medium on a floating plastic mesh in complete darkness at 25°C as described earlier (Nick and Furuya, 1993) to allow for maximal coleoptile elongation (Pjon and Furuya, 1967). Various antimicrotubular drugs such as colchicine, propyzamide, and oryzalin were tested in preliminary dose–response studies (data not shown). However, even in the wild-type population the response was heterogeneous, possibly the result of problems with drug solubility and uptake. Since the samples of mutant seeds assayed in the screen were expected to show genetic segregation, this heterogeneity would have obscured the expression of the mutant phenotype and could therefore not be tolerated.

The herbicide ethyl-*N*-phenylcarbamate (EPC) was chosen for its homogeneous response, for its high solubility in water and for its low cost. A saturating concentration of  $3 \times 10^{-3}$  M (Figure 5) was used in selection medium. This is about 10 times the saturation level for the inhibition of microtubule assembly *in vitro* (Mizuno and Suzuki, 1990), but the actual drug concentration in the coleoptile may well be much lower, because the drug has to cross several barriers on its way to the target cells.

From 6593 mutagenized lines, 203 putatively drug-resistant lines were recovered in a first screen. Among those, a second screen confirmed the drug resistance in 39 lines. These lines were maintained by transplanting the resistant seedlings to pots containing a mixture of clay and peat (1:1 v/v) under 8000 lux  $\text{m}^{-2}$  of continuous white light at 25°C and 80% humidity in a phytotron (Koitoiron, Tokyo, Japan). Flowering was then induced by 30 short days (10 h darkness, 14 h light) and seeds were obtained after selfing. Five of the 39 mutant lines segregated into subpopulations of EPC-resistance mutants with altered morphology, plants resembling the wild-type, and non-developing seeds. After a preliminary phenotypic characterization (data not shown), *EPC-Resistant 31* (ER31) was chosen for a more detailed analysis. If not specified otherwise, the term 'mutant' signifies plants that are heterozygous for the trait *ER31*, because homozygotes were lethal.

### *Physiological treatments*

Seedlings were grown on a floating plastic mesh in complete darkness for 6 days to ensure maximal elongation growth (Nick and Furuya, 1993). Frequency distributions over coleoptile and mesocotyl length were determined with a population of 100 wild-

type and heterozygous mutant seedlings, respectively. The heterozygotes could be recognized by their characteristic morphology (Figure 1). Cell length was determined in epidermal strips at different positions along the seedling axis using 15 cells per spot in five plants for each time point. The measurements of leaf sheath and leaf blade growth were done after 4 weeks of cultivation under 6500 lux  $\text{m}^{-2}$  of white light at 25°C and high humidity. Mature embryos were dissected from briefly (1 h) pre-soaked mature seeds from wild-type and heterozygous mutant plants. A frequency distribution over coleoptile length was constructed from 80 individuals. For the determination of the dose–response relation (Figure 5), seeds were cultivated on various concentrations of EPC prepared from a 0.5 M ethanolic stock solution. All solutions were made up to 1% of ethanol to equalize possible effects of the solvent.

### *Induction of non-embryogenic calli from mature embryos*

Calli were induced from the scutellum of mature embryos as described by Hartke and Lörz (1989). After 3 weeks growth in darkness, shoots, roots, and endosperm were removed and the callus was transferred to the same medium solidified with 1.2% agar. Every 4 weeks the callus was transferred to new induction medium with agar. Four months after induction, a liquid culture was initiated by omitting the agar, and the microcalli were subcultured weekly. Soft, white, and apparently non-embryogenic callus was obtained for the wild-type, whereas the mutant callus was friable and yellowish in colour. After a further month of liquid culture, 1 g of callus was subcultured on medium with 0 or 1 mM EPC. Callus weight was followed over 4 weeks with weekly transfers to fresh culture medium. For the dose–response curve of callus formation (Table 4), the 2,4-D in the induction medium was replaced by various concentrations of indolyl-3-acetic acid (IAA).

### *Evaluation of microtubule responses*

Cortical microtubules were stained by means of immunofluorescence. After prefixation for 45 min at 'room temperature' in 3.7% (w/v) paraformaldehyde in microtubule-stabilizing buffer (0.1 M piperazine-diethanolsulphonic acid, 1 mM  $\text{MgCl}_2$  5 mM ethylene glycol-bis-(3-aminomethyl-ether)-*N,N,N',N'*-tetraacetic acid, 0.2% v/v Triton X100, 1% w/v glycerol, pH 6.9), tangential sections were cut with a fresh razor blade and collected in the fixation medium. This was followed by 40 min of postfixation in the same solution and three washes in the fixation medium without paraformaldehyde. The sections were then incubated for 20 min at 'room temperature' with sheep normal serum (Nordic Immunology, Tilbury, Netherlands) diluted 1:20 in phosphate-buffered saline with 0.1% v/v Triton X100. Then they were treated for 1 h at 37°C with a monoclonal antibody from mouse raised against  $\beta$ -tubulin (Amersham, Little Chalfont, UK) diluted 1:1000 in phosphate-buffered saline containing 0.1% v/v Triton X100. After three washes in this buffer without antiserum, sections were re-incubated overnight at 4°C with a secondary antibody from sheep against mouse IgG and labelled with fluorescein-isothiocyanate (Nordic Immunology, Tilbury, Netherlands) diluted 1:50 in phosphate-buffered saline supplemented with 0.1% v/v Triton X100. After three washes in the same buffer they were mounted in antifading solution (Citifluor, London, UK) and viewed under an epifluorescence microscope (Leitz, Wetzlar, Germany). The orientation of microtubular arrays was classified with respect to the angle they formed with the short cell axis with 0° indicating transverse microtubules, 30° slightly oblique microtubules, 60° indicat-

ing steeply oblique microtubules, and 90° indicating longitudinal microtubules. For each experiment, frequency distributions were constructed using the data for 89–314 cells from 15–41 individual seedlings. The gibberellin and the red-light treatments to change the orientation of microtubules in the subepidermal cells of the cortex (Figure 9) are described in detail in Nick and Furuya (1993).

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