Planta (2005) 222: 575–585
DOI 10.1007/s00425-005-0001-6

ORIGINAL ARTICLE

**Caroline Gutjahr · Michael Riemann
Axel Müller · Petra Düchting · Elmar W. Weiler
Peter Nick**

Cholodny–Went revisited: a role for jasmonate in gravitropism of rice coleoptiles

Received: 17 December 2004 / Accepted: 26 April 2005 / Published online: 27 July 2005

Abstract Gravitropism is explained by the Cholodny–Went hypothesis: the basipetal flow of auxin is diverted laterally. The resulting lateral auxin gradient triggers asymmetric growth. However, the Cholodny–Went hypothesis has been questioned repeatedly because the internal auxin gradient is too small to account for the observed growth asymmetry. Therefore, an additional gradient in indolyl-3-acetic acid (IAA) sensitivity has been suggested (Brauner and Hager in Planta 51:115–147, 1958). We challenged the Cholodny–Went hypothesis for gravitropism of rice coleoptiles (*Oryza sativa* L.) and found it to be essentially true. However, we observed, additionally, that the two halves of gravitropically stimulated coleoptiles responded differentially to the same amount of exogenous auxin: the auxin response is reduced in the upper flank but normal in the lower flank. This indicates that the auxin-gradient is amplified by a gradient of auxin responsiveness. Hormone contents were measured across the coleoptile by a GC-MS/MS technique and a gradient of jasmonate was detected opposing the auxin gradient. Furthermore, the total content of jasmonate increased during the gravitropic response. Jasmonate gradient and increase persist even when the lateral IAA gradient is inhibited by 1-*N*-naphtylphtalamic acid. Flooding with jasmonate delays the onset of gravitropic bending. Moreover, a jasmonate-deficient rice mutant bends more slowly and later than the wild type. We discuss a role of jasmonate as modulator of auxin responsiveness in gravitropism.

C. Gutjahr · M. Riemann
Institut für Biologie II, Albert-Ludwigs Universität Freiburg,
Schänzlestr. 1, 79104 Freiburg i. Br., Germany

A. Müller · P. Düchting · E. W. Weiler
Lehrstuhl für Pflanzenphysiologie, Fakultät für Biologie,
Ruhr-Universität Bochum, 44780 Bochum, Germany

P. Nick (✉)
Botanisches Institut 1, Universität Karlsruhe,
Kaiserstr. 2, 76128 Karlsruhe, Germany
E-mail: peter.nick@bio.uni-karlsruhe.de
Tel.: +49-721-6082144
Fax: +49-721-6084193

Keywords Auxin-responsiveness · Cholodny–Went hypothesis · Gravitropism · IAA (indolyl-3-acetic acid) · JA (jasmonate) · Rice (*Oryza sativa* L.)

Abbreviations ABA: Abscicic acid · DMSO: Di-methyl-sulfoxide · FW: Fresh weight · GC-MS-MS: Gas chromatography linked bidimensional mass spectrometry · IAA: Indole-3-acetic-acid · JA: Jasmonate · Me-JA: Methyl jasmonate · 2,5-NBD: 2,5-Norbornadien · NPA: 1-*N*-naphtylphtalamic acid · OPDA: *o*-Phytodienoic acid · OPR: *o*-Phytodienoic acid reductase

Introduction

Gravitropism offers the opportunity to study the role of intracellular communication in signal controlled morphogenesis. It allows to ask how direction is recognized and transformed into a systemic response of the whole organ. Perceptive site and effector system are spatially separated, which means that some kind of spatial information must be conveyed over a considerable distance.

It was shown first by Darwin and Darwin (1880) for phototropism of coleoptiles that the tip transmits spatial information in basipetal direction towards the growing tissue. Cholodny (1927) for gravitropism and Went (1926) for phototropism discovered simultaneously that the transmitted signal must be a hormone. Later, this hormone was characterized as indolyl-3-acetic acid (IAA, Kögl et al. 1934; Thimann 1935).

The Cholodny–Went hypothesis that is traditionally used to explain gravi- and phototropic bending is based on the assumptions that a redistribution of auxin results in a gradient of auxin. This drives a gradient of differential growth which is the cause for tropistic bending. The theory has been under continuous debate (see also Trewavas 1992) mostly for phototropism where cell-autonomous inhibitor-models starting from the

Blaauw-theory (Blaauw 1915) or models based on gradients of anti-auxins rather than the growth promoting hormone auxin resurrect at regular intervals (e. g. Bruinsma and Hasegawa 1990). On the other hand, the gravitropic auxin gradient was well established starting from Dolk (1936) who used a bioassay and ending up with tracer experiments with radioactively labeled auxin (Goldsmith and Wilkins 1964; Parker and Briggs 1990; Iino 1991; Godbolé et al. 2000) or direct measurement of the distribution of endogenous auxin within the tip (Philippar et al. 1999). On the basis that the epidermis is more responsive to auxin than the subepidermal tissue, MacDonald and Hart (1987) suggested a refinement of the Cholodny–Went hypothesis. According to their model, auxin transport and auxin effect might be confined to the epidermis. Thus, the gradient would not emerge across the whole organ. Instead, it would be built up locally by auxin influx from the inner tissue into the epidermis of the lower flank and efflux from the epidermis of the upper flank into the inner tissue.

However, the difference in IAA-content generally found between the two flanks of a coleoptile was not more than about 1:2 (Goldsmith and Wilkins 1964; Parker and Briggs 1990; Iino 1991; Godbolé et al. 2000). By contrast, the difference in initial growth rate between the two halves of a gravitropically stimulated coleoptile has been found to be more than 12-fold (Digby and Firn 1976). Moreover, the increase in growth rate in the lower flank and the decrease in growth rate in the upper flank are roughly equivalent (Himmelspach and Nick 2001). Since elongation growth of rice coleoptile segments is more or less proportional to the logarithm of auxin concentration (Wang and Nick 1998), a doubling of auxin concentration would not suffice to cause these observed changes in growth rate. Furthermore, submersion of gravitropically stimulated coleoptiles in relatively high concentrations of auxin causes positive gravitropic bending (Rorabaugh and Salisbury 1989; Waller 2000; Edelmann 2001). This suggests that the effect of the lateral auxin-gradient must be amplified by additional gradients. We thus asked the following questions: Is there an additional gradient of auxin responsiveness or "sensitivity" as claimed by Rorabaugh and Salisbury (1989)? Is there a second factor modulating this sensitivity as postulated by Brauner and Hager (1958)? Is the auxin gradient necessary at all for the gravitropic response?

To answer the questions we used rice coleoptiles (*Oryza sativa* L. *japonica* cv. "Nihonmasari") as model system as their ability for gravitropism in submersion and the lack of a diffusion barrier to auxin (Godbolé et al. 2000) is ideal for testing the role of auxin gradients in gravitropism.

We show in the present work that the central statement of the Cholodny–Went hypothesis holds true. However, we demonstrate that the effect of the auxin gradient is enhanced by a parallel gradient of auxin responsiveness. We further show for the first time that during gravitropic bending a jasmonate gradient develops opposite to the gradient of auxin. This jasmonate gradient acts as a positive modulator of gravitropic curvature.

Materials and methods

Plant material, treatment of seedlings and coleoptiles

Seedlings of rice (*Oryza sativa* L. ssp. *japonica* cv. "Nihonmasari", kindly provided by Dr. Osamu Yatou, Japan) were raised for 5 days at 25°C in photobiological darkness (wrapped in black cloth and placed in light–tight black boxes). For curvature assays in the absence of JA the JA-deficient rice mutant *hebiba* (Riemann et al. 2003) was utilized for comparison to the isogenic wild type.

For curvature assays, five rice seeds were fixed with medical adhesive ("B401", Factor II Inc., Lakeside, AZ, USA), 5 mm below the edge of a microscopy slide taking care that the embryo was pointing upwards (Fig. 1). The slides were placed in conventional staining trays and these in plexiglass boxes (95×95×60 mm). The plexiglass boxes were filled with deionised water such that the seeds were only partially covered to ensure optimal germination in aerobic conditions.

For elongation assays, seeds were sawn on floating plastic meshes as described in Nick and Furuya (1993). All manipulations of seedlings described in the following paragraphs were performed in green safe-light ($\lambda_{max} = 550$ nm).

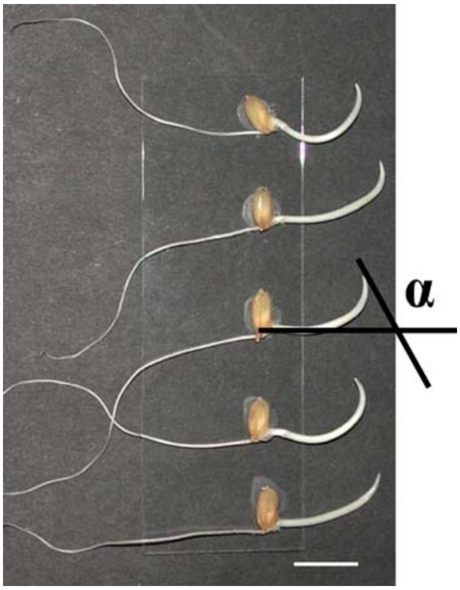

Fig. 1 Rice coleoptiles after 2 h of gravitropic stimulation at 90°: curvatures were defined by the tangential line along the most vertical part of the coleoptile with relation to the slide and the outer edge of the bent coleoptile tip. *Bar* = 1 cm

Gravitropic curvature, dose–response curves, bending kinetics

Seedlings fixed on microscopy slides were submerged in different solutions. For all solutions deionised water was used. For gravitropic stimulation, the whole staining tray was tilted by 90°. For determination of 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 was 25 ± 3°C. All points of a given graph were recorded at the same time.

For IAA dose–response curves, seedlings were gravitropically stimulated for 2 h in H_2O as control and in IAA solutions (Sigma-Aldrich, stock solution 100 mM in absolute ethanol) of the concentrations 0.03, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 µM. NPA (1-*N*-naphthylphthalamic acid) was synthesized (stock solution 10 mM in DMSO) according to Thompson et al. (1973). For NPA dose–response curves, seedlings were pre-incubated vertically for 1 h and then gravitropically stimulated for 2 h in H_2O as control and in NPA-solutions of the concentrations 0.1, 0.5, 1, 5, 10, 15, 20, 25 and 50 µM.

For bending kinetics in methyl-jasmonate (Me-JA), seedlings were pre-incubated vertically for 1 h in H_2O as control or in 10 and 50 µM Me-JA (Sigma-Aldrich, stock solution 10 mM in absolute ethanol) and then gravitropically stimulated. Samples were measured after 1 and 2 h of gravitropic stimulation. For all curvature assays all solutions were supplemented with the highest amount of the solvent added (absolute ethanol or DMSO) to equalize for its potential effects.

To assess gravitropic curvature in the absence of JA, the JA-deficient mutant *hebiba* (Riemann et al. 2003) was used and compared to the isogenic wild type. Samples were measured after 1 and 2 h of gravitropic stimulation, respectively.

To test the involvement of ethylene in the gravitropic response, coleoptiles were stimulated for 3 h in a moist chamber which consisted of a Plexiglass box (30×20×16 cm) with water-soaked tissue paper. The roots of coleoptiles were allowed to touch the moist tissue. For the treatment a petri dish with 10 µl of the volatile ethylene synthesis inhibitor 2,5-norbornadiene (2,5-NBD, 97%, Sigma-Aldrich) was placed in the chamber which was closed airtight with adhesive tape.

Elongation assays

From seedlings grown on floating plastic meshes coleoptile segments of 10 mm length were excised 3–13 mm below the tip. The segments were incubated under continuous rotation on a topover shaker in complete darkness for 1 h in H_2O to wash out endogenous auxin. Subsequently, they were incubated for 4 h in 1 µM NPA (Fig. 2b) or 10 mM succinate buffer pH 4.0 supplemented with 10 µM and 50 µM methyl-jasmonate (Me-JA) (Fig. 4c). The controls were supplemented with 0.01% DMSO (Fig. 2b) or 0.5% ethanol (Fig. 5c), to equalize all samples for the solvent of NPA and Me-JA, respectively. For Fig. 4c, 10 mM succinate buffer pH = 4.0 served as control for Me-JA treatment and10 mM succinate buffer pH 6.0 as control for acid growth. The length increment was determined under a stereomicroscope.

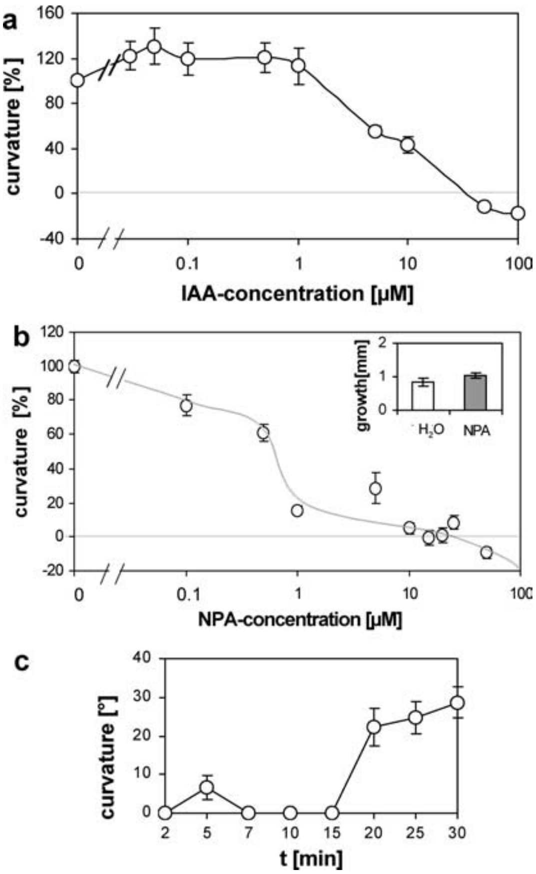

Fig. 2 **a** Concentration dependence of IAA on gravitropic curvature of rice coleoptiles stimulated for 2 h at 90°: values of five independent experiments are averaged. Since the amplitude of the control response differed slightly between days of experimentation, values are plotted as percent of the control. Per experiment and point 20 coleoptiles were used. Results are the mean ± SE $n = 5$, invisible error bars fall into symbols. **b** Concentration dependence of NPA on gravitropic curvature of rice coleoptiles stimulated for 2 h at 90°. *Inset* growth increment of 10 mm coleoptile segments after 4 h of incubation in 1 µM NPA. All results are the mean ± SE, $n = 20$ shown as percent bending of the control. **c** Decapitation kinetics: seedlings were submerged in water and gravitropically stimulated. After periods indicated on the abscissa, they were decapitated by 3 mm and returned for further stimulation up to a total period of 2 h. Results are the mean ± SE, $n = 20$

Decapitation kinetics

Seedlings glued on microscope slides were gravitropically stimulated in H_2O. Samples were taken after 2, 5, 7, 10, 15, 20, 25 and 30 min and decapitated by 3 mm. After decapitation, seedlings were returned for further gravitropic stimulation to a total period of 2 h.

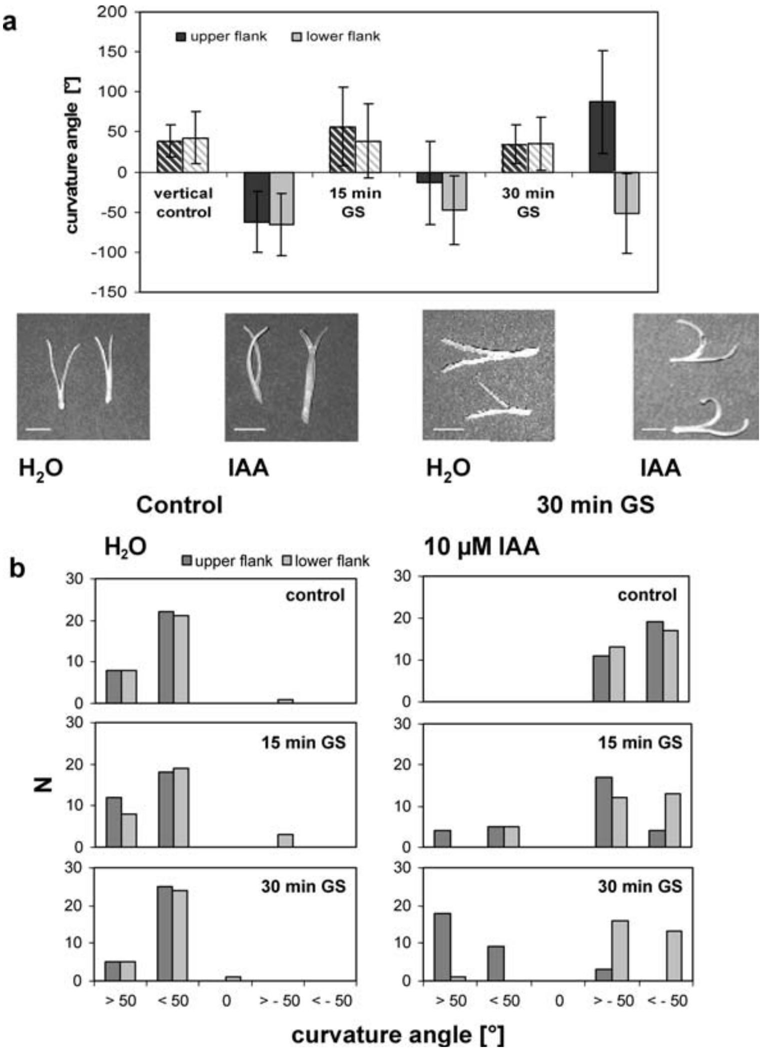

Fig. 3 **a** Effect of the gravitropic stimulus (GS) on IAA-responsiveness of the upper and lower halves of rice coleoptiles: coleoptiles were gravitropically stimulated for 15 and 30 min, cut into segments and split. Split segments were incubated in 10 µM IAA (*filled bars*) and water (*striped bars*) for 2 h. Outward bending is represented as positive value, inward bending as negative. Data were collected cumulatively on different days. Results are the mean ± standard deviation $n = 30$. Photos show representative samples of split segments that were incubated in water or 10 µM IAA: vertical control (*left*), after 30 min of gravitropic stimulation (*right*). *White bar* = 5 mm. **b** Frequency distribution of curvature angles. In the water control all halves bend outwards irrespective of pre-treatment. In 10 µM IAA, halves of the vertical control bend inwards. With increasing time of gravitropic stimulation (GS) as pre-treatment more and more upper halves bend outwards upon incubation in 10 µM IAA. *Each box* shows the distribution of 30 replicates. n = number of coleoptile segment halves

Split-test for IAA-responsiveness

As assay for IAA-responsiveness the split pea-hypocotyl test of Went and Thimann (1937) was modified. Rice seedlings were gravitropically stimulated in H_2O for 15 and 30 min, i.e. in the period before the onset of bending. As control, seedlings were incubated vertically in H_2O for 30 min. Coleoptile segments of 10–15 mm length were excised from 3 mm below the tip. They were split longitudinally in upper and lower halves (relative to the gravity vector) leaving a connection at the basal 3–5 mm (see Fig. 3a). To avoid differences in curvature caused by difference in thickness between the two halves, only strictly symmetrically cut segments were used. The segments were split by stabbing the edge of a razor blade into the lower part of the segment and pushing it towards the top. In a perfectly symmetric cut, the primary leaf is pushed out at the top of the segment. Subsequently the segments were incubated in water or 10 µM IAA for 2 h under continuous rotation on a topover shaker in complete darkness. The segments were then xerocopied and curvature angles of the two halves measured with a protractor. Images of split segments were recorded with a digital camera (Nikon, Coolpix 990).

Hormone extraction

Seedlings were stimulated gravitropically for 15, 30, 60 and 120 min in a moist chamber (not airtight) as described above in darkness. Microscopy slides with attached seedling were placed on the cloth such that the roots touched the water source. After gravitropic stimulation, coleoptiles were split in to upper and lower halves and after careful removal of the primary leaf immediately frozen in liquid nitrogen.

For each extraction 40 coleoptile halves were used. Plant material from liquid nitrogen was immediately covered with 1 ml of hormone standard containing 30 pmol ^{2}H-labelled IAA, 30 pmol ^{2}H-labelled ABA, 30 pmol ^{13}C-labelled JA, and 10 pmol ^{2}H-labelled OPDA in methanol. After adding 1 ml diethylether the samples were heated for 20 min at 50°C in a water bath. The remaining extract was completely evaporated under vacuum.

To see whether the JA-gradient develops independently of the IAA-gradient, gravitropically induced later IAA-transport was blocked with 5 μM NPA. Coleoptiles were pre-incubated in NPA-solution for 1 h to allow sufficient entry of the inhibitor and subsequently gravitopically stimulated for 1 h. The same was done for the control in water supplemented with 0.05% of the solvent DMSO. The vertical control was incubated vertically in water for 2 h. For harvest and hormone extraction, coleoptiles were treated as described above.

Hormone analysis

Hormone concentrations were analyzed as described in Müller et al. (2002).

Statistics

Data were processed with the program Microsoft Excel 2000. For statistical tests the program SPSS 11.5 was applied. *P* values for differences in total hormone content and segment growth were calculated by a Mann–Whitney *U* test. For paired data of hormone contents in upper and lower flanks the Wilcoxon test was used.

Results

Exogenous IAA and NPA inhibit gravitropic bending

Whether the auxin gradient plays a role in rice coleoptile gravitropism was tested by flooding the internal IAA-gradient with exogenous IAA and by inhibiting auxin transport with the phytotropin 1-*N*-naphtylphtalamic acid (NPA).

The dose–response curve for the effect of exogenous IAA on gravitropic bending (Fig. 2a) is characterized by a broad plateau around the internal IAA concentration of ca. 0.07–0.1 μM IAA (Riemann et al. 2003, see also Fig. 4a). For concentrations exceeding 1 μM IAA, gravitropic curvature gradually decreases. When the concentration of IAA is increased above 5 μM, an increasing number of individuals display positive gravicurvatures.

The dose–response curve of the NPA-effect on gravitropic curvature (Fig. 2b) is clearly sigmoidal. One micromole of NPA inhibits gravitropism by about 80%, whereas segment growth is not inhibited at this concentration (Fig. 2b, inset). For NPA concentrations exceeding 1 μM, an increasing number of individuals showed positive gravitropism.

The signal that conveys directional information moves at a rate similar to auxin

To test whether the operationally defined signal that originates in the tip and conveys the information on the direction of the stimulus to the more basal effector zone travels with a speed expected for IAA flux, intact coleoptiles were stimulated for various time intervals. Then the perceptive tissue was removed. Preliminary experiments had shown that gravitropic sensitivity was confined to the apical 3 mm (data not shown).

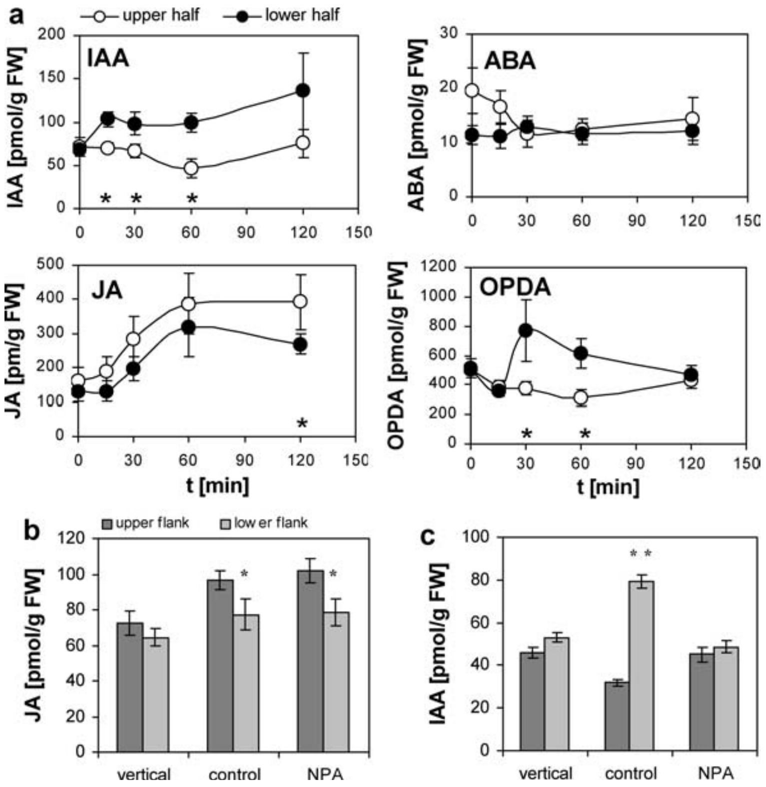

Fig. 4 **a** Kinetics of the lateral distribution of IAA, ABA, JA and OPDA during gravitropic stimulation: each value is the mean ± SE of data obtained for eight GC-MS/MS analyses from extracts of 40 coleoptile halves per sample. *FW* fresh weight. **b**Lateral distribution of JA and **c** IAA in coleoptiles that were gravitropically stimulated for 1 h either in water or in 5 μM NPA for elimination of the IAA-gradient. Each value is the mean ± SE of data obtained for nine GC-MS/MS analyses from extracts of 40 coleoptile halves per sample. *Asterisks* indicate significant differences as obtained by a Wilcoxon test for paired data $*P < 0.05$; $**P < 0.01$

Consequently, when the tip was removed immediately or briefly after the onset of stimulation, no significant curvatures were observed (Fig. 2c). However, a significant gravitropic curvature occured when coleoptiles were stimulated for at least 20 min in the non-decapitated state. Since the signal was initiated at the time point of tilting the coleoptiles to the horizontal, it needed 20 min to overcome 3 mm and thus traveled with a speed of 9 mm/h. IAA was found to travel with 10–12 mm/h in maize coleoptiles (Goldsmith 1967). Therefore, the signal responsible for gravitropic bending should be IAA.

Gravitropic stimulation induces a gradient of IAA-responsiveness

IAA responsiveness in the upper and lower half of gravitropically stimulated rice coleoptiles was tested with the *pea split test* of Went and Thimann (1937). It should be noted here that responsiveness is not necessarily the same as sensitivity. Sensitivity refers to the ability to perceive a signal whereas responsiveness is the ability to respond to a signal. In terms of Michaelis–Menten kinetics, sensitivity would correspond to K and responsiveness to $v$max. However, experimentally it is often difficult to discriminate changes of sensitivity from changes of responsiveness. Near the threshold of a dose–response curve an observed change in the amplitude of a response could be caused by a shift of the threshold, but it could also be caused by a regulation of the amplitude itself (responsiveness). Since our experimental set-up does not allow real statements about auxin sensitivity in sensu strictu versus responsiveness, we will use exclusively the latter term—which should include both—in the present work.

As shown in Fig. 3a, both segment halves bent outwards (represented in Fig. 3a as positive bending) when rice coleoptiles were kept in water, irrespective of whether or not they were pre-treated by 15 or 30 min of gravitropic stimulation. Upon incubation in 10 μM IAA, the segment halves of the un-stimulated control bent inwards (represented in Fig. 3a as negative bending). After gravitropic stimulation the lower half still bent inwards when incubated in auxin, but the upper half bent outwards. Comparing the reaction after 15 and 30 min of gravitropic stimulation it is evident that it was not the degree of individual curvatures that changed with the time of gravitropic stimulation; it was rather the frequency of individuals exhibiting this inverse response of the upper half that increased (Fig. 3b). Thus, the differential bending of the coleoptile halves seems to be an all-or-none response.

We conclude from the differential reaction of the two halves of gravitropically stimulated coleoptiles to the same amount of exogenous IAA that gravitropic stimulation did not only evoke an IAA-gradient, but also a gradient of IAA-responsiveness.

Gravitropic stimulation induces a jasmonate gradient

The next question we asked was about the nature of the gradient in IAA-responsiveness. Could it result from a gradient of hormones other than auxin? We analysed the content of IAA, ABA, OPDA and JA by GC-MS-MS in the upper and lower half of gravitropically stimulated coleoptiles. Kinetics for the distributions of these hormones during the gravitropic response are shown in Fig. 4a. The IAA gradient (Fig. 4a) becomes detectable from 15 min with a ratio of 40:60 (upper:lower half) at the time points 15 and 30 min and 30:70 at 60 and 120 min. The difference between the upper and lower halves is significant at the 95%-level except for the 120 min point (Fig. 4a). ABA (Fig. 4a) displays a gradient at 15 min of gravitropic stimulation, but this gradient is already present before stimulation and completely disappears during gravitropic stimulation, which speaks against a direct involvement of ABA in the gravitropic response. The only hormone apart from IAA for which a consistent gradient emerged is JA (Fig. 4a). The total content of JA rises significantly (99.9% level after 120 min) during the time course of gravitropic stimulation. Additionally, the data show a gradient of 60:40 (upper:lower half) which is oriented opposite to the IAA gradient. However, only for the 120-min point, the gradient is significant at the 95%-level. To control for potential effects of the green safelight used for handling or drought stress coleoptiles were incubated for 120 min in the moist chamber but without gravitropic stimulation. No increase in JA-content could be detected (data not shown). Interestingly, OPDA, a precursor of JA shows a significant gradient from 30 min of stimulation that is reciprocally oriented to the JA gradient but diminishes for later time points (Fig. 4a). This gradient is mainly due to a rapid, but transient increase of this precursor in the lower flank. Another interesting point is that OPDA significantly decreases up to 15 min of stimulation (99% level). This might be explained by a rapid consumption for JA-synthesis which is followed by a delayed subsequent delivery of OPDA (30 min point, Fig. 4a).

Our next issue was to determine whether the JA-gradient is induced independently by gravitropic stimulation or just as a downstream effect of the IAA-gradient. Thus, we checked the JA-distribution after 1 h of gravitropic stimulation under concomitant inhibition of auxin transport by 5 μM NPA. Possibly as a consequence of submersion (necessary for the application of NPA), the overall content of JA was reduced as compared to stimulation under atmospheric conditions (Fig. 4a). Irrespective of this reduction of JA levels, a significant (95% level) JA-gradient develops in both controls and NPA-treated coleoptiles as shown in Fig. 4b. As NPA completely eliminated the IAA-gradient in the very same coleoptiles (Fig. 4c) we conclude that the JA-gradient develops independently of the IAA-gradient in response to gravitropic stimulation.

Additionally, the total JA content rises significantly (99% level) during 1 h of stimulation.

Elimination of the JA gradient delays the gravitropic response

To find out whether the JA-gradient has a function in gravitropism we tested what happens, when the JA gradient is eliminated: (1) by flooding the gradient with exogenous methyl-jasmonate (Me-JA) (2) by comparing the time course of bending for the JA-deficient rice mutant *hebiba* to the wild type.

Ten and fifty micromoles of Me-JA inhibit gravitropic bending (Fig. 5a). Whereas the higher Me-JA concentration of 50 µM mainly acts by dampening the amplitude of gravitropic curvature, 10 µM of Me-JA mainly causes a delay of gravitropic curvature up to about 1 h. Then bending proceeds with the same rate as in the control. To see whether the inhibition of curvature by Me-JA is specific for auxin-dependent growth responses such as the gravitropic reaction we tested whether Me-JA inhibits general elongation growth. As marker activity we used the acid growth response (Fig. 5c). In all three acid treatments (pH = 4.0) segment growth was significantly higher ($P = 0.000$) than in the control with pH 6.0. Acid induced growth was not significantly inhibited by 10 µM Me-JA ($P = 0.435$). However, it was slightly inhibited by 50 µM Me-JA ($P = 0.04$) but not as much as gravitropism was inhibited by this concentration (Fig. 5a). Moreover, the difference in growth between the coleoptile segments treated with 10 µm or 50 µM Me-JA is not significant ($P = 0.285$) (Fig. 5c).

Similar to flooding with Me-JA, the complete absence of JA affects the gravitropic response: It was clearly delayed in *hebiba* (Fig. 5b). However, after a strong initial delay, bending still occured, which means that JA is not absolutely necessary for gravitropic bending but mainly seems to accelerate the bending process. Elongation growth did not differ between segments excised from WT and *hb* coleoptiles (Fig. 5b, inset).

To test whether ethylene would act as regulator of gravitropic bending we measured the effect of the ethylene synthesis inhibitor 2,5-norbornadiene (2,5-NBD). Coleoptiles treated with 2,5-NBD bent more strongly than the control (significant at 99% level, Fig. 5d). Thus, ethylene apparently acts as negative modulator of the gravitropic response.

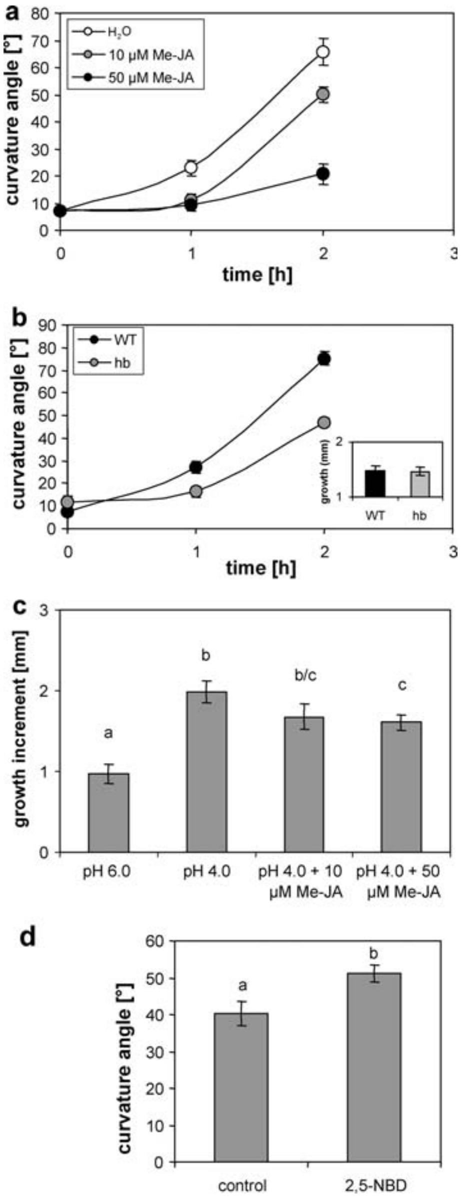

Fig. 5 Gravitropic curvature in absence of JA gradient: **a** Flooding of JA-gradient with two concentrations (10 µM and 50 µM) of external Me-JA. **b** Time course of gravitropic bending in the JA-deficient mutant *hebiba*. For **a** and **b** mean values from two independent experimental series are shown. Results are the mean ± SE, $n = 37$ for (**a**), $n = 35$ for (**b**). **c** Influence of 10 and 50 µM Me-JA on acid-induced growth increment of coleoptile segments within 4 h. Results are the mean ± SE, $n = 20$. **d** Mean curvature of coleoptiles gravitropically stimulated for 3 h upon inhibition of ethylene synthesis by 2,5-norbornadiene (2,5-NBD), $n = 23$. *Letters on top of the bars* indicate significant differences between samples as assessed by a Mann–Whitney *U* test

Discussion

Cholodny–Went: auxin transport and auxin gradient are necessary

From our results that (1) flooding of coleoptiles with IAA and NPA (Fig. 2a, b) inhibits gravitropic curvature, (2) that the curvature inducing signal originates in the coleoptile tip and travels with the speed of auxin (Fig. 2c), (3) that a gradient of extractable auxin emerges during the gravitropic reaction (Fig. 4a), we conclude and confirm that auxin transport establishing a lateral auxin gradient is necessary for gravitropism of rice coleoptiles.

Interestingly, in rice coleoptiles, gravitropism is much more sensitive to exogenous IAA than elongation growth. Whereas the broad optimum for gravitropic curvature was at around 0.1 μM IAA and significant inhibition was observed from 1 μM IAA (Fig. 2a), elongation growth reached its maximum at 3 μM IAA and was significantly inhibited only from 10 μM IAA (Wang and Nick 1998; Waller 2000). Salisbury et al. (1988) found that the growth response to auxin was enhanced in gravitropically stimulated soybean hypocotyls when compared to controls that were kept vertically. Thus, gravitropic strimulation increased the auxin responsiveness of the whole organ. Functionally this might ensure that the response to a changing gravitropic stimulus remains fast and flexible and will not result in overbending.

The inhibition of gravitropic curvature by high concentrations of exogenous IAA shows that IAA does enter the coleoptile and can overrun the gravitropically induced gradient of endogenous IAA. However, this result is not as trivial as it might appear at first sight: a concentration difference by a factor of almost 100 was necessary to inhibit curvature by 50–70% of the optimum-curvature (Fig. 2a). The inhibition of gravitropic curvature at these very high concentrations of IAA cannot be a mere consequence of a general growth stimulation (which is optimal at 3 μM—Wang and Nick 1998; Waller 2000). Otherwise a much more substantial inhibition would be expected for IAA concentrations around 3 μM.

Moreover, starting from 5 μM IAA an increasing proportion of individuals with inversed gravitropism was observed. The same phenomenon occurred for NPA-concentrations that exceed 1 μM. Five micromoles of NPA has been shown to completely eliminate longitudinal and lateral auxin transport in rice coleoptiles (Godbolé et al. 2000).

The observation of positive gravitropic bending for very high concentrations of exogenous IAA or after complete inhibition of auxin transport are difficult to explain in the framework of the classical Cholodny–Went hypothesis. These findings call for an additional gradient of IAA-responsiveness as already postulated by Brauner and Hager (1958) and later shown in sunflower and soybean hypocotyls (Salisbury et al. 1988; Rorabaugh and Salisbury 1989). Thus, the Cholodny–Went hypothesis holds true, when the lateral gradient of auxin is complemented by a lateral gradient of auxin responsiveness.

We found a strong and significant gradient of extractable IAA in gravitropically stimulated coleoptiles already after 15 min when the first measurement was taken (Fig. 4a). This is consistent with results from maize coleoptiles (Iino 1991; Philippar et al. 1999) and contradicts an older assumption of Briggs (1963), who postulated that only diffusible, but not extractable auxin plays a role for the establishment of tropistic curvature. The ratio of the IAA-gradient of 40:60 (15 min) and 30:70 (60 min) is very similar to that measured with radioactively labeled IAA administered to the tip of gravitropically stimulated rice coleoptiles (Godbolé et al. 2000) and similar to published data for corn coleoptiles (Gillespie and Briggs 1961; Parker and Briggs 1990; Iino 1991). At the molecular level, Waller et al. (2002) showed that the auxin responsive gene *Os*ARF1 is differentially expressed in the upper and lower flank of gravistimulated rice coleoptiles reflecting the auxin gradient.

A gradient of IAA-responsiveness amplifies the effect of the IAA gradient

Applying the pea split test of Went and Thiemann 1937 we found that gravitropic stimulation induces a qualitatively different reaction of the upper and lower coleoptile halves to the same amount of exogenous auxin (Fig. 3a). The hormonal response of a plant depends on effective concentration of and sensitivity to the hormone within the responsive tissue (Trewavas 1982). If the hormone is supplied in excess, the sensitivity or in our case, the responsiveness becomes limiting for the response such that differences in responsiveness should become manifest.

One could argue that the differential response of the two halves of gravitropically stimulated coleoptiles does not result from differential IAA responsiveness. Instead it might be caused by differential growth that was already triggered upon gravitropic stimulation and just proceeded after the segments had been split. However, the coleoptiles are not yet curved after 30 min of stimulation (nor are they after 15 min, when the difference in the response to auxin is already laid down). Moreover, as shown by Fig. 2a, differential growth is already inhibited by the high IAA concentration of 10 μM. Thus, we conclude that additionally to the IAA gradient a gradient of IAA-responsiveness across the coleoptile is involved in the gravitropic reaction.

The gradient of auxin responsiveness that becomes manifest in the split-coleoptile assay can also explain the downward curvatures observed for superoptimal concentrations of exogenous IAA (Fig. 2a). Even in the absence of an auxin gradient, a gradient of auxin responsiveness should result in a growth differential between the two coleoptile flanks. It is the upper flank where auxin responsiveness becomes reduced during gravitropic stimulation (Fig. 3a). At superoptimal concentrations of auxin, the flank with the lower responsiveness should experience a stronger stimulation of growth as compared to that with the higher responsiveness that is now inhibited such that the coleoptile bends downwards. The positive gravi-curvatures observed for high concentrations of the auxin-transport inhibitor NPA (Fig. 2b) might be caused by a similar phenomenon, when endogenous auxin, due to impaired efflux, accumulates to supra-optimal levels.

How is the gradient of IAA-responsiveness brought about? Figure 3a shows that the lower flank reacts normally to auxin whereas the upper flank does not react. Thus, it must be assumed that the gradient of responsiveness is modulated by means of inhibition at the upper flank not by promotion of the auxin effect at the lower flank.

JA as modulator of IAA-responsiveness?

What could be the inhibiting factor for IAA responsiveness? Analysing the distribution of the four hormones IAA, ABA, OPDA and JA in the two flanks of rice coleoptiles during the gravitropic reaction we found that the total JA content rises significantly during the time course of stimulation and that JA is distributed in a gradient reciprocally oriented to the IAA-gradient (Fig. 4a). The correlation between gravitropic reaction and rise in JA level is supported by findings of Moseyko et al. (2002) in *Arabidopsis* and Kramer et al. (2003) in wheat seedlings showing that the expression of lipoxygenase, which is one of the first enzymes in JA-biosynthesis (León and Sánchez-Serrano 1999), is up-regulated upon gravitropic stimulation. Furthermore, the transcript level of the JA-responsive gene GER1 in the two coleoptile flanks mirrors the pattern of JA-levels observed in this study (data not shown). Interestingly, the JA gradient is accompanied by a gradient of the JA-precursor OPDA in opposite direction. This gradient is mainly due to a rapid and transient increase of this precursor in the lower coleoptile flank (Fig. 4a). Since JA is generally increasing in both flanks during gravitropic stimulation, the early steps of JA synthesis might be triggered ubiquitously by gravitropic stimulation. However, there might be a second level of regulation in a late step establishing the gradient, when OPDA is converted to the final product JA. This late step seems to be delayed or impaired in the lower flank, but seems to work efficiently in the upper flank. Thus, the observed gradient of JA might be caused by a gradient in the activity of a late step of JA synthesis (downstream of OPDA), not by a differential induction of the pathway as such. In this ontext it might be most relevant to investigate transcript levels of *Os*OPR, the enzyme that metabolizes OPDA. However, it is important to identify those isoforms of this enzyme that might be involved in the gravitropic response.

Investigating whether the JA-gradient is induced independently or as downstream effect of the IAA-gradient we found that 5 μM NPA efficiently suppressed the establishment of an IAA-gradient (Fig. 4c) but not the JA-gradient (Fig. 4b). Thus, the JA-gradient is not induced via the IAA-gradient. This result shows also that the JA-gradient is not sufficient to induce bending since in the presence of 5 μM NPA the gravitropically induced JA-gradient is not accompanied by significant curvature (Figs. 2b, 4b).

What could be the functional relevance of JA in gravitropism? Overrunning the JA-gradient by submerging coleoptiles in Me-JA and observing the bending kinetics of the JA-deficient mutant *hebiba* (Riemann et al. 2003) we found that in both cases the gravitropic response was delayed (Fig. 5a, b). This cannot be due to a general inhibition of the growth machinery as such, because segment elongation assays show that IAA-independent, acid induced growth is not inhibited by 10 μM Me-JA (Fig. 5c). Fifty micromoles of Me-JA inhibits acid induced elongation growth only slightly and much less compared to gravi-curvature (Fig. 5a, c). Thus, we conclude that it is not the total amount of JA but its spatial distribution that is relevant for gravitropic bending. Moreover, the JA-deficient mutant *hebiba* is not impaired in growth as compared to the wild type (Fig. 5b, inset) and auxin-induced growth is even more pronounced in this mutant (Riemann et al. 2003). However, as *hebiba* still shows a gravitropic reaction, JA is not absolutely necessary for gravitropic bending but it seems to accelerate the gravitropic response.

Could the JA-gradient be the cause of the gradient of IAA-responsiveness? IAA induced growth is inhibited by JA in monocotyledons (Miyamoto et al. 1997). This is attributed to inhibition of cell wall polysaccharide synthesis (Ueda et al. 1994, 1995) and pH increase (Irving et al. 1999). For both processes IAA has been shown to act antagonistically (Kutschera and Briggs 1987; Brummer et al. 1985). Furthermore, jasmonate might act by modulating potassium channels (Evans 2003) that are involved in auxin-induced growth and even differentially expressed in gravitropism of maize coleoptiles (Philippar et al. 1999). On the molecular level jasmonate might reduce auxin responsiveness by recruiting signalling factors such as AXR1 that are limiting for auxin signalling (Schwechheimer et al. 2001). This mutual interaction of hormonal signaling might provide the conceptual framework to link the observed jasmonate gradient with the observed gradient of auxin responsiveness.

Upon submersion rice coleoptiles progressively switch from auxin towards ethylene control of cell elongation. In contrast to other plants, where ethylene acts as inhibitor of elongation diverting cell expansion in favour of lateral growth (Lang et al. 1982), ethylene acts as stimulator of cell elongation in the case of rice coleoptiles (Imaseki and Pjon 1970). We therefore tested, whether ethylene under the conditions of the JA-experiment described above, would act as positive modulator of gravitropism. This experiment was also stimulated by experiments in rye, where the coleoptile had been removed and the agravitropic primary leaves could be rendered competent for gravitropism by treatment with ethylene (Kramer et al. 2003). However, since in rice coleoptiles the ethylene synthesis inhibitor 2,5-NBD promoted gravitropic bending (Fig. 5d), ethylene appears to act as negative modulator of the gravitropic response and is therefore probably not involved in the action of jasmonate, at least in our system.

The findings listed above suggest that the growth differential underlying gravitropic curvature depends on an IAA-gradient that, in turn, is differentially inhibited by a gradient of JA. An inhibitor would be most efficient as amplifier of an activator gradient if it were distributed in a gradient that is reciprocally oriented. In other words, where growth is already badly promoted by IAA, it would become additionally inhibited. Thus, although JA is not absolutely necessary (Fig. 5b) nor sufficient for gravitropic bending, it could be at least part of a machinery that is responsible for the gradient of IAA-responsiveness.

Acknowledgements NPA was synthesized and kindly provided by Dr. Wolfgang Michalke (Albert-Ludwigs-Universität Freiburg). The authors would like to thank Dr. Christina Süßlin for experimental support, Dr. Osamu Yatou (Hokuriku National Agricultural Experiment Station, Niigata, Japan) for providing seed material and Dr. Gerd Leubner (Albert-Ludwigs-Universtiät Freiburg) for a kind gift of 2,5-NBD. The study was supported partially by grants from the Forschungsschwerpunkt "Molekulare Analyse der Phytohormonwirkung" of the DFG to E.W. and P.N, a grant from the Volkswagen-Foundation Nachwuchsgruppen-Programme to P.N., and a partial grant of the Studienstiftung des deutschen Volkes to C.G..

References

Blaauw AH (1915) Licht und Wachstum II. Bot Zent 7:465–532

Brauner L, Hager A (1958) Beiträge zur Analyse der geotropischen Perzeption. Planta 51:115–147

Briggs WR (1963) The phototropic response of higher plants. Annu Rev Plant Physiol 14:311–352

Bruinsma J, Hasegawa K (1990) A new theory of phototropism—its regulation by a light induced gradient of auxin-inhibiting substances. Physiol Plant 79:700–704

Brummer B, Bertl A, Portykus I, Felle H, Parish RW (1985) Evidence that fusicoccin and indole-3-acetic acid induce acidification of *Zea mays* cells. FEBS Lett 189:109–114

Cholodny N (1927) Wuchshormone und Tropismen bei den Pflanzen. Biol Zent 47:604–626

Darwin C, Darwin F (1880) Sensitiveness of plants to light: it's transmitted effect. The power of movement in plants. John Murray, London, pp 574–592

Digby J, Firn RD (1976) A critical assessment of the Cholodny–Went Theory of shoot gravitropism. Curr Adv Plant Sci 8:953–960

Dolk HE (1936) Geotropism and the growth substance. Recl Trav Bot Neerl 33:509–585

Edelmann H (2001) Lateral redistribution of auxin is not the means for gravitropic differential growth of coleoptiles: a new model. Physiol Plant 112:119–126

Evans NH (2003) Modulation of guard cell plasma membrane potassium currents by methyl-jasmonate. Plant Physiol 131:8–11

Gillespie B, Briggs WR (1961) Mediation of the geotropic response by lateral transport of auxin. Plant Physiol 36:364–368

Godbolé R, Michalke W, Nick P, Hertel R (2000) Cytoskeletal drugs and gravity-induced lateral auxin transport in rice coleoptiles. Plant Biol 2:176–181

Goldsmith MHM (1967) Movement of pulses of labeled auxin in corn coleoptiles. Plant Physiol 31:51–82

Goldsmith MHM, Wilkins MB (1964) Movement of auxin in coleoptiles of *Zea mays* L. during geotropic stimulation. Plant Physiol 39:151–162

Himmelspach R, Nick P (2001) Gravitropic microtubule orientation can be uncoupled from growth. Planta 212:184–189

Iino M (1991) Mediation of tropisms by lateral translocation of endogenous indole-3-acetic acid in maize coleoptiles. Plant Cell Environ 14:279–286

Imaseki H, Pjon CJ (1970) The effect of ethylene on auxin-induced growth of excised rice coleoptile segments. Plant Cell Physiol 11:827–829

Irving HR, Dyson G, McConchie R, Parish RW, Gehring CA (1999) Effects of exogenously applied jasmonates on growth and intracellular pH in maize coleoptile segments. J Plant Growth Regul 18:93–100

Kögl F, Hagen-Smit J, Erxleben H (1934) Über ein neues Auxin („Hetero-Auxin") aus Harn. Hoppe-Seyler's Z Physiol Chem 228:104–112

Kramer S, Piotrowski M, Kühnemann F, Edelmann HG (2003) Physiological and biochemical characterization of ethylene-generated gravicompetence in primary shoots of coleoptile-less gravi-incompetent rye seedlings. J Exp Bot 54:2723–2732

Kutschera U, Briggs WR (1987) Rapid auxin-induced stimulation of cell wall synthesis in pea internodes. Proc Natl Acad Sci USA 84:2747–2751

Lang JM, Eisinger WR, Green PB (1982) Effects of ethylene on the orientation of microtubules and cellulose microfibrils of pea epicotyl cells with polylamellate cell walls. Protoplasma 110:5–14

León J, Sánchez-Serrano JJ (1999) Molecular biology of jasmonate biosynthesis in plants. Plant Physiol Biochem 37:373–380

MacDonald IR, Hart JW (1987) New light on the Cholodny-Went theory. Plant Physiol 84:568–570

Miyamoto K, Oka M, Ueda J (1997) Update on the possible mode of action of jasmonates: focus on the metabolism of cell wall polysaccharides in relation to growth and development. Physiol Plant 100:631–638

Moseyko N, Zhu T, Chang HS, Wang Z, Feldman LJ (2002) Transcription profiling of the early gravitropic response in Arabidopsis using high-density oligonucleotide probe microarrays. Plant Physiol 130:720–728

Müller A, Düchting P, Weiler EW (2002) A multiplex GC-MS/MS technique for the sensitive and quantitative single-run analysis of acidic phytohormones and related compounds, and its application to *Arabidopsis thaliana*. Planta 216:44–56

Nick P, Furuya M (1993) Phytochrome-dependent decrease of gibberellin sensitivity to auxin. J Plant Growth Regul 12:195–206

Parker KE, Briggs WR (1990) Transport of indole-3-acetic acid during gravitopism of intact maize coleoptiles. Plant Physiol 94:1763–1768

Philippar K, Fuchs I, Lüthen H, Hoth S, Bauer CS, Haga K, Thiel G, Ljung K, Sandberg G, Böttger M, Becker D, Hedrich R (1999) Auxin-induced K^+ channel expression respresents an essential step in coleoptile growth and gravitropism. Proc Natl Acad Sci USA 96:12186–12191

Riemann M, Müller A, Korte A, Furuya M, Weiler E, Nick P (2003) Impaired induction of the jasmonate pathway in the rice mutant *hebiba*. Plant Physiol 133:1820–1830

Rorabaugh PA, Salisbury FB (1989) Gravitropism in higher plant shoots VI. Changing sensitivity to auxin in gravistimulated soybean hypocotyls. Plant Physiol 91:1329–1338

Salisbury FB, Gillespie L, Rorabaugh P (1988) Gravitropism in higher plant shoots. V. Changing sensitivity to auxin. Plant Physiol 88: 1186–1194

Schwechheimer C, Serino G, Callis J, Crosby WL, Lyapina S, Deshaies RJ, Gray WM, Estelle M, Deng XW (2001) Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCF^{TIR1} in mediating auxin response. Science 292:1379–1382

Thimann KV (1935) On the plant growth hormone produced by *Rhizopus suinus*. J Biol Chem 109:279–291

Thompson KS, Hertel R, Müller S, Tavares JE (1973) 1-*N*-naphtylphthalamic and 2,3,5-triiodobenzoic acids. In vitro binding to particulate cell fractions and action on auxin transport in corn coleoptiles. Planta 109:337–352

Trewavas AJ (1982) Growth substance sensitivity: the limiting factor in plant development. Physiol Plant 55:60–72

Trewavas AJ (1992) Forum: What remains of the Cholodny–Went theory? Plant Cell Environ 15:759–794

Ueda J, Miyamoto K, Aoki M (1994) Jasmonate inhibits the IAA-induced elongation of oat coleoptile segments: a possible mechanism involving the metabolism of cell wall polysaccharides. Plant Cell Physiol 35:1065–1070

Ueda J, Miyamoto K, Kamisaka S (1995) Inhibition of the synthesis of cell wall polysaccharides in oat coleoptile segments by jasmonate. Relevance to its growth inhibition. J Plant Growth Regul 14:69–76

Waller F (2000) *Os*ARF1, ein Auxin Response Factor aus Reis (*Oryza sativa* L.), der selbst auxinreguliert ist. PhD Thesis, Albert-Ludwigs-Universität Freiburg

Waller F, Furuya M, Nick P (2002) *Os*ARF, an auxin response factor from rice, is auxin-regulated and classifies as primary auxin responsive gene. Plant Mol Biol 50:415–425

Wang QY, Nick P (1998) The auxin response of actin is altered in the rice mutant *Yin-Yang* . Protoplasma 204:22–33

Went F (1926) On growth accelerating substances in the coleoptile of *Avena sativa*. Proc Kon Ned Akad Weten 30:10–19

Went FW, Thimann KV (1937) Phytohormones. Macmillan, New York