Differential Downward Stream of Auxin Synthesized at the Tip Has a Key Role in Gravitropic Curvature via TIR1/AFBs-Mediated Auxin Signaling Pathways

Takeshi Nishimura1,*, Hitomi Nakano1, Ken-ichiro Hayashi2, Chiharu Niwa1 and Tomokazu Koshiba1
1Department of Biological Sciences, Tokyo Metropolitan University, 1-1 Minami-Osawa, Hachioji-shi, Tokyo, 192-0397 Japan
2Department of Biochemistry, Okayama University of Science, Okayama-shi, Okayama, 700-0005 Japan

Since the early days of Darwin, monocot coleoptiles have been used to investigate indole-3-acetic acid (IAA) production, polar transport and tropisms. Here, using maize coleoptiles, we first showed that polar transport of IAA synthesized at the tip region is regulated by ZmPIN(s). Then, the TIR/AFBs-mediated auxin signaling pathway corresponds to the asymmetric IAA flow after gravistimulus, which results in tropic curvature. When [13C11 15N2]Trp was applied to coleoptile tips, substantial amounts of the stable isotope were incorporated into IAA at the tip region, and the labeled IAA was transported in a polar manner at approximately 7 mm h⁻¹. Immunohistochemical analyses revealed that ZmPIN1(s) was present in almost all cells. ZmPIN1(s) showed a relatively non-polar distribution at the tip, but a basal cellular localization at lower regions. Application of the IAA transport inhibitors 1-N-naphthylphthalamic acid (NPA) and brefeldin A (BFA) at the very tip region almost completely inhibited IAA movement from the tip. These inhibitors also severely suppressed gravitropic bending. PEO-IAA, an auxin antagonist that binds to TIR1/AFBs, suppressed not only the expression of an auxin-responsive ZmSAUR2 gene, but also gravitropic curvature. Expression of ZmSAUR2 was up-regulated on the lower side and down-regulated on the upper side of the coleoptile elongation zone, corresponding to the asymmetric IAA distribution. These results indicate that the asymmetric downward streams of IAA control the differential growth rate of the cells by attenuating TIR1/AFBs-mediated auxin response genes, including ZmSAUR2, and therefore result in tropic curvature.

Keywords: Coleoptiles • IAA • Maize (Zea mays) • PEO-IAA • ZmPIN1 • ZmSAUR2.

Abbreviations: BFA, brefeldin A; BH-IAA, tert-butoxycarbonylaminoxyethyl-IAA; DAPI, 4’,6-diamidino-2-phenylindole; EGTA, ethylene glycol bis (aminoethylether)tetra-acetic acid; GC-SIM-MS, gas chromatography-selected ion monitoring-mass spectrometry; IgG, Immunoglobulin G; KPB, potassium phosphate buffer; MTSB, microtubule-stabilizing buffer; NPA, 1-N-naphthylphthalamic acid; PEO-IAA, α-(phenylethyl-2-one)-IAA; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); Trp, tryptophan; YFP, yellow fluorescent protein.

Introduction

Plant growth responses such as gravitropism and fototropism have fascinated plant biologists since the pioneering work of Charles and Francis Darwin (Darwin and Darwin, 1880) more than 125 years ago. Later, studies on growth and tropism using mainly grass coleoptiles resulted in the characterization of indole-3-acetic acid (IAA), the major native auxin. Since then, IAA has been shown to be involved in many physiological events (Woodward and Bartel 2005). In particular, the IAA produced at the tip region of grass coleoptiles was shown to be transported downwards, and to participate in the plant bending response. This tropic response, as explained by the Cholodny–Went hypothesis, is the result of differential growth on the two sides of the elongating shoot, which can be explained by the asymmetrical IAA distribution (Went and Thimann 1937).

There are several loss-of-function mutations in the auxin-regulated transcriptional activator ARF7 that disrupt tropic responses in Arabidopsis (Arabidopsis thaliana) hypocotyls. Characterization of these mutants led to the hypothesis that changes in gene expression represent a critical molecular
response to the redistribution of IAA that occurs in response to tropic stimuli (Harper et al. 2000, Guilfoyle and Hagen 2007). AUX/IAA, SAUR and GH3 gene families have been identified as genes whose transcripts are rapidly induced by auxin (Hagen and Guilfoyle 2002). It was reported that the msg2/iaa19 mutant was defective in phototropism and gravitropism in hypocotyls, and the msg2/iaa19 dominant allele was coupled to ARF7 activity in Arabidopsis (Tatematsu et al. 2004). ZmSAUR1 and ZmSAUR2 genes are expressed in the cell elongation zone of maize coleoptiles. The proteins encoded by these genes bind to calmodulin, suggesting that they are involved in calcium/calmodulin-mediated signaling in auxin-mediated cell elongation (Yang and Poovaiah 2000, Knauss et al. 2003). The evidence suggests that IAA-mediated gene expression plays an important role in differential growth responses to photo and gravity stimuli. However, it is still unknown whether these genes are truly regulated by endogenous asymmetrical IAA distribution as explained by ‘early IAA-inducible genes’ or if they are essential genes for controlling tropisms.

Monocot coleoptiles, particularly maize, provide an ideal model for researching the Cholodny–Went hypothesis. One advantage of the maize coleoptile system is that the tissue is relatively large, much larger than Arabidopsis roots, and thus enables direct measurement of endogenous IAA levels and detailed analysis of the movement of IAA molecules. Moreover, coleoptile tips have long been recognized as the site of IAA production (lino 1982, Koshiya et al. 1995, Philippar et al. 1999, Bennett et al. 2000, Mori et al. 2005, Nishimura et al. 2006). In tropic responses, it was believed that the distribution of IAA in the tissues is altered by lateral IAA transport. In phototropism, it is generally accepted that the tip region detects the direction of the photo-stimulus, and lateral IAA transport from the tip establishes the asymmetric IAA distribution that moves basipetally, causing the lower region to bend (Briggs et al. 1957, lino 1991, lino 1995, Fuchs et al. 2003). In gravitropism, the lateral IAA gradient throughout the length of the coleoptile is important (Hatfield and LaMotte 1985, Parker and Briggs 1990, lino 1991, lino 1995, Yoshihara and lino 2007). These physiological studies suggest that there is a specific transport system that relocates IAA, and that asymmetric IAA distribution is a key event in tropic curvature. However, neither the molecular components of this system nor the cellular mechanism of IAA redistribution have been reported for grass coleoptiles.

On the other hand, in Arabidopsis, recent powerful advances in molecular genetic analysis, mainly using gravitropic mutants, have led to the isolation of several key components of the gravitropic signal transduction pathway, including the auxin efflux carriers, the PIN family (Friml and Palme 2002, Moore 2002, Zažímalová et al. 2007). Localization of AtPIN1 is restricted to the basal side of IAA-transport-competent cells within root and shoot vascular tissues, indicating its involvement in IAA transport from shoot to root apexes (Gälweiler et al. 1998, Geldner et al. 2001, Wisniewska et al. 2006). AtPIN2 localized on the upper membrane of epidermal and lateral root cap cells mediates basipetal IAA transport in the root tip. AtPIN2 roots show an agravitropic phenotype, indicating that AtPIN2 has a role in creating the lateral distribution of IAA for root gravitropism (Abas et al. 2006, Jaillais et al. 2006). AtPIN3 is also a reliable candidate having a role in IAA redistribution in root tip cells in response to gravi-stimuli, although pin3 mutants exhibit only a weak agravitropic phenotype (Friml et al. 2002). There is no direct evidence that the polarity of PIN localization is correlated with the direction of IAA flow, but the experimental evidence from mutants clearly suggests that PIN must be localized correctly for ordinary IAA transport to occur. Unfortunately, because these studies have focused mainly on Arabidopsis root systems, the exact movement of IAA molecules could not be investigated in response to tropic stimuli. Recently, ZmPIN1a, 1b and 1c, novel putative orthologs of AtPIN1, were identified in maize (Carraro et al. 2006, Gallavotti et al. 2008). An anti-AtPIN1 antibody recognized at least ZmPIN1a and ZmPIN1b proteins in maize. Immunohistochemical studies revealed that the ZmPIN1 proteins were localized in the leaves, the primary root and the shoot apical meristem (Boutté et al. 2006, Carraro et al. 2006, Gallavotti et al. 2008). Analysis of the ZmPIN1a:ZmPIN1a:YFP expression pattern showed that ZmPIN1a:YFP expression was up-regulated in axial meristems and lateral organ primordia. These data suggested that directional IAA transport is necessary to form axial meristems and lateral primordia in maize, as in Arabidopsis. However, ZmPIN1 localization and functions in other tissues, including the coleoptile, have not been reported.

Here, using the maize coleoptile system, we show that (i) IAA is newly synthesized from Trp at the very tip region, and moves to basal parts via polar transport regulated by ZmPIN1(s); (ii) 1-N-naphthylphthalamic acid (NPA) and brefeldin A (BFA) treatment at the local tip region strongly inhibits IAA transport from the tip to the base and also inhibits lateral transport at the tip region after gravi-stimulus, resulting in an almost complete block of gravitropic curvature; and (iii) ZmSAUR2 gene expression, regulated via the auxin receptors TIR1/AFBs, may play an important role in the cell elongation response to IAA redistribution, resulting in tropic curvature. These results reveal the important role of the tip region of maize coleoptiles in perception of gravi-stimulus, and in transmitting the perceived information to the lower region using IAA as a messenger. For these processes, IAA production and transport from the tip region are key factors that rapidly affect IAA-inducible genes such as ZmSAUR2 in the elongation zone, which might regulate the rate of coleoptile growth and therefore tropic curvature. We have experimentally confirmed that ‘some influence’, first described...
by the Darwins more than 125 years ago in their book, is IAA: ‘some influence is transmitted from the upper to the lower part, causing the latter to bend’ (Darwin and Darwin, 1880).

Results

Incorporation of stable isotope from $[^{13}\text{C}_{11}^{15}\text{N}_{2}]$Trp into IAA and polar transport of labeled IAA

When $[^{13}\text{C}_{11}^{15}\text{N}_{2}]$Trp at varying concentrations was applied locally to the tip surface, the heavy isotope label was incorporated into IAA in 3 mm tips. After incubation, the labeled IAA was transported from the tip into the agar block receiver (Supplementary Fig. S1). No significant difference in the amount of IAA was observed in the buffer control. The proportion of labeled molecules in IAA in the tip tissue increased until 60 min after application, when approximately 65% of the IAA was labeled with $^{13}\text{C}_{10}^{15}\text{N}_1$ (when $[^{13}\text{C}_{11}^{15}\text{N}_{2}]$Trp was supplied at 500 ng ml$^{-1}$). The ratio continued to increase up to 120 min incubation. A dose-dependent response was observed with labeled-Trp concentrations of 50, 250 and 500 ng ml$^{-1}$. The ratio of labeled molecules in transported IAA was relatively similar to that in IAA in the tip tissue. However, labeled IAA was only detected in the transported IAA from 30 to 60 min after application, whereas labeled IAA was detected in IAA at the tip tissue after 30 min of Trp treatment. This time-lag may represent the time taken for newly synthesized IAA at the tip to move into the agar block receiver. The efficiency of label incorporation at different regions within the tip was determined by applying labeled Trp directly to the top outer surface and inner region of the coleoptile dome (Table 1 and also see Supplementary Fig. S2). The results show that the apical region located approximately 0–1 mm from the tip is the site where labeled Trp is incorporated most effectively into IAA. This indicates that the very tip region is the primary site of IAA synthesis from Trp. To investigate labeled-IAA movement from the tip region, labeled Trp was pulse-fed for 15 min at the top surface of intact seedlings, which were incubated for 90 min (Fig. 1 and also see Supplementary Fig. S3). The amount of IAA and proportion of labeled IAA/total free IAA were quantified in each 1 mm section from the tip to 13 mm below the tip. No significant change was observed in the amounts of endogenous IAA before and after Trp feeding. The peak of labeled IAA moved down the tip after pulse feeding at approximately 7 mm h$^{-1}$, indicating that labeled IAA is transported at exactly the same rate as native IAA. These results clearly indicate that IAA is constantly synthesized from Trp in a rate-limited manner at the tip region, and the synthesized IAA is immediately transported to lower parts in a polar manner.

Presence and localization of ZmPIN1(s) in maize coleoptiles

A highly conserved 16 amino acid peptide sequence of the large intracytosolic loop of AtPIN1 was used to raise rabbit antiserum as described in Boutté et al. (2006). In the 4-day-old maize coleoptile protein fraction, the pre-purified antiserum detected a major polypeptide (approximately 65 kDa) and a minor polypeptide (approximately 45 kDa polypeptide), but the affinity-column-purified serum recognized only a 65 kDa polypeptide (Fig. 2A).

![Fig. 1 Basipetal transport of IAA from tip region of maize coleoptiles.](image)

(A) Endogenous free IAA levels in each section. (B) Proportion of IAA labeled with $[^{13}\text{C}_{11}^{15}\text{N}_{2}]$Trp from the top surface of coleoptiles for 15 min. After indicated time periods, IAA was extracted from each 1 mm section from top to basal parts and quantified by GC-SIM-MS. Data were obtained from three independent experiments and values are means ± SE.
size was consistent with the putative ZmPIN1a (65.2 kDa) and ZmPIN1b (64.5 kDa) polypeptides (Carraro et al. 2006). Expression of ZmPIN1 mRNA was analyzed by RT–PCR using RNA extracted from 0–3 mm tip and 3–6 mm subapical regions of the coleoptile. Specific primer combinations for ZmPIN1a, ZmPIN1b and ZmPIN1c were used to detect their corresponding mRNA expression. ZmPIN1a and ZmPIN1b transcripts were expressed at almost the same levels in both the apical and subapical regions, while ZmPIN1c expression was much lower in both regions (Fig. 2B). Thus it is postulated that both ZmPIN1a and ZmPIN1b proteins are present in maize coleoptile cells and the anti-AtPIN1 antibody recognized both proteins as reported by Carraro et al. (2006), though we could not discriminate between them.

We used an immunostaining assay in maize coleoptiles to investigate the localization of ZmPIN1 proteins. The purified antibody was used to detect ZmPIN1(s). The pre-immune antiserum did not recognize any protein specifically (data not shown). Fig. 2C shows three regions within a longitudinal section of the non-vascular plane of the coleoptile tip: I, the top 0.5 mm; II, 0.5–1.0 mm; III, 1.0–1.5 mm. In region I, cells are arranged irregularly, and there is a layer of outer epidermis (Fig. 2D). The ZmPIN1 signal was detected in almost all cells in this region, and appeared to be located along the entire plasma membrane of the cells (Fig. 2H). In region II, establishment of cell alignment was initiated (Fig. 2E). The ZmPIN1 signals were weaker at both lateral sides of cell membranes, but stronger at the cells’ basal membranes (Fig. 2I). In addition, the outer epidermis had no obvious signals at the basal membrane. In contrast, cells of the inner epidermis showed clear ladder-like staining with fluorescent signals localized on the basal membrane. In region III and the lower region, cells were further elongated (Figs. 2F, G), and ZmPIN1 was mainly localized on the cells’ basal membranes (Figs. 2J, K). To confirm intracellular localization more precisely, immunostained sections were observed under a laser scanning confocal microscope. Confocal images of region I showed non-polar cellular localization of ZmPIN1 (Fig. 2L), while ZmPIN1 was localized on basal membranes in region III cells (Fig. 2M). These results indicate that IAA movement at the IAA synthetic region in the tip is non-directional, whereas it is transported in a polar manner in the subapical region towards the lower parts of the plant.

Function of ZmPIN1(s) in IAA polar transport in maize coleoptiles

Our previous studies showed that basipetal transport of IAA was inhibited by NPA, causing accumulation of IAA in the tip (Mori et al. 2005, Nishimura et al. 2006), but we did not study the time-dependent change in amounts of IAA transported from the tip to the lower regions. We determined the change in amount of IAA in the 3 mm tip, and the amount of IAA that transported from the 3 mm tip into the...
agar receiver before and after NPA and BFA treatment (see Supplementary Fig. S4). The results are shown in Fig. 3A, B. NPA applied at 50–100 μM maximally inhibited IAA transport, and resulted in a decrease in the amount of transported IAA. BFA applied at 300–500 μM strongly inhibited IAA transport. After 90 min of NPA treatment and 60 min of BFA treatment, IAA transport was inhibited by approximately 90% and 80%, respectively, compared with the maximum rate of IAA transport (Fig. 3A). Since about 160 pg 20 min⁻¹ IAA was produced constitutively from non-treated sections, IAA accumulated at the tip region after application of the inhibitor(s) (Fig. 3B). Comparing the levels of transported IAA with tip IAA revealed that the decrease in the amount of transported IAA after application of inhibitors corresponded to the level of IAA accumulation in the tip. Our data show that application of BFA and NPA largely suppresses the movement of IAA from the biosynthetic region at the tip to the lower regions. In our previous studies, we showed that 10 μM NPA affected the accumulation of IAA at the tip region in abraded coleoptiles (Mori et al. 2005, Nishimura et al. 2006). In the present work, we also confirmed that 20 μM NPA and 25 μM BFA affected IAA accumulation (see Supplementary Table S1).

To analyze the effects of NPA and BFA on intracellular ZmPIN1 distribution, we investigated the localization of ZmPIN1(s) in NPA- and BFA-treated coleoptiles (Fig. 3C–E). In the section treated with 300 μM BFA for 90 min, some ZmPIN1 signals were concentrated in both the tip and basal regions of cells, whereas no alteration was detected in the section treated with 100 μM NPA, the same as the non-treated control. We observed the intracellular localization of ZmPIN1(s) more precisely using confocal fluorescence microscopy (Fig. 3F–H). At the inner side of the coleoptile tip region, a perinuclear aggregation of ZmPIN1(s) was observed after 90 min treatment with 300 μM BFA. This was not observed in the untreated control. The signal from the plasma membrane became weaker when cells were treated with BFA. An endocytic tracer marker, FM4-64, also accumulated in these cells. FM4-64 colocalized with ZmPIN, indicating that the aggregation body originated from endocytic recycling of ZmPIN1(s) (Fig. 3H).

Effect of local NPA and BFA treatment on gravi-curvature

A gravitropic response occurs in intact etiolated maize seedlings, but we found that even detached coleoptile segments (1–3 cm long) exhibited this response. We observed that shoots bent most effectively when the coleoptile was set vertically along the plane of the two vascular tissues, with the

---

Fig. 3 Effects of NPA and BFA on polar IAA transport and cellular ZmPIN1 localization in maize coleoptiles. (A) Effect of NPA and BFA on amounts of IAA transported into the agar block. (B) Effect of NPA and BFA on endogenous IAA levels in tips. Inhibitors at indicated concentrations were locally applied to the inside of the top 2 mm region of 3 mm coleoptile tip, and tips were placed on an agar block (see Supplementary Fig. S4). Data were obtained from six or eight independent experiments and values are means ± SE. (C)–(E) Effect of NPA and BFA on localization of ZmPIN1. (C) Region approximately 0.5 mm from tip. (D) Magnified images of (C). (E) Region 1.5–2.0 mm from top. (F)–(H) Formation of aggregation body after BFA treatment. (F) Cell membrane stained with FM4-64 fixable analog. (G) Immunodetection of ZmPIN1. (H) Merged images of ZmPIN1 (green) and FM4-64 (red) signals. Immunolocalization images were obtained by confocal fluorescence microscopy. FM4-64 signals are red, and blue signals are DAPI-stained nuclei. KP8, 50 μM NPA or 300 μM BFA (red bar in (A) and (B)) were applied locally to top 2 mm region of the coleoptile for 90 min (see Supplementary Fig. S4). FM4-64 was applied for 1 h before BFA treatment. Arrows show colocalized vesicle of ZmPIN1 and FM4-64. Bar 20 μm.
thinner side down (see Supplementary Fig. S5). Using this system with 2-cm coleoptile segments, we investigated effects of NPA and BFA treatment on the tip region (Fig. 4A). The inhibitors were applied locally to the top 2-mm region (at the inside of the coleoptile; see Supplementary Fig. S4). In the buffer control (Mock), gravitropic curvature occurred after 60 min of gravistimulus. Curvature was almost completely inhibited when the coleoptile was treated with 100 µM NPA or 500 µM BFA (Fig. 4A). This was consistent with the markedly decreased IAA diffusion rate (see Fig. 3A, B). To further clarify the importance of IAA supplied from the tip region for bending at lower parts, NPA was applied locally at 8–10 mm below the tip (Fig. 4A). Parts above the NPA-treated region exhibited bending after gravistimulus, but parts below the treated region remained straight. This result clearly confirms that polar-transported IAA from the tip region is responsive to gravi-curvature of coleoptiles.

Fig. 4 Effect of NPA and BFA on gravitropic curvature of 20-mm coleoptile segments, and redistribution of IAA in coleoptiles after gravistimulus. (A) Effect of NPA and BFA on gravitropic curvature of 20-mm coleoptile segments. (B) Redistribution of IAA in coleoptiles after gravistimulus and local NPA treatment (0–2 mm). (C) Redistribution of IAA in coleoptiles after gravistimulus and local NPA treatment (8–10 mm). We applied 100 µM NPA and 500 µM BFA locally to the inside of the top 2 mm and 8–10 mm coleoptile regions (see Supplementary Fig. S4). Inhibitors were applied for 30 min before initiation of gravity stimulus (‘0 min’). The bases of the coleoptiles were fixed in agar, then the coleoptiles were tilted horizontally. Photos were taken at indicated times. Broken lines represent region of NPA and BFA treatment. Gray bar, upper side of coleoptiles; black bar, lower side of coleoptiles. Data were obtained from three independent experiments and values are means ± SE.
Effect of NPA treatment on IAA redistribution after grav-stimulus

To investigate the distribution and movement of IAA in detail, IAA levels in each upper and lower half of 0–3, 3–7, 7–11 and 11–15 mm sections of the coleoptile were determined before and after grav-stimulus (Fig. 4B). Before exposure to grav-stimulus (0 min), IAA was evenly distributed between the upper and lower half (right or left half), and there were >2.5-fold greater levels of IAA in the top 0–3 mm compared with that in the lower regions. After 10 min grav-stimulus, the distribution was similar to that at time 0, although there was a small increase in IAA in the 0–3 mm region. After 30 min, IAA was clearly redistributed throughout the coleoptile, and IAA levels on the lower side were greater than those on the upper side. Lateral movement appeared to occur between 10 and 30 min after grav-stimulus in these regions. After 60 min, the difference became more significant. Because IAA is transported at approximately 7 mm h⁻¹ as shown above, it is likely that the lateral redistribution occurs mainly at the 0–3 mm region, and the different concentrations are transported into lower parts. It appears that some IAA is laterally transported in the 7–15 mm region.

NPA strongly inhibited polar IAA transport, resulting in IAA accumulation in the tip region (Fig. 3B). As shown in Fig. 4A, we used local application of the inhibitor NPA to determine changes in IAA distribution after grav-stimulus (Fig. 4B, C). NPA (100 µM) was applied to the 0–2 mm tip region, and the coleoptile was kept vertical for 30 min. In these conditions, IAA accumulated in the 0–3 mm tip region, while it significantly decreased in the lower regions (0 min in Fig. 4B). The coleoptile was then shifted to a horizontal position. After 10, 30 and 60 min of grav-stimulus, IAA distribution resembled that at time 0, although there was increased accumulation of IAA at the tip region and decreased IAA level in the lower regions over time. When NPA was applied locally at 8–10 mm positions, parts above the NPA-treated region exhibited clear IAA redistribution after grav-stimulus, but parts below the treated region did not (Fig. 4C).

Effect of PEO-IAA on ZmSAUR2 expression and grav-curvature

Recently, tert-butoxycarbonylaminoethyl-IAA (BH-IAA), an α-alkyl-IAA, was reported to be a new auxin antagonist that binds to TIR1/AFBs receptors (Hayashi et al. 2008). This antagonist specifically binds to the auxin binding site of TIR1/AFBs auxin receptors and blocks TIR1/AFBs functions in Arabidopsis, rice (Oryza sativa) and moss (Physcomitrella patens). α-(Pheny lethyl-2-one)-IAA (PEO-IAA) is also an α-alkyl-IAA, and shows more potent anti-auxin activity in auxin-responsive gene expression and in the cell division and elongation pathway that is mediated via SCF TIR1/AFBs (Hayashi et al. unpublished data). To investigate the effect of PEO-IAA on gravitropic curvature, we observed coleoptiles after PEO-IAA treatment at several concentrations (Fig. 5A). When PEO-IAA was applied to the top 0–15 mm of coleoptiles (including the elongation zone; equivalent to approximately 3–20 mm from the top region in this study), gravitropic curvature was slightly inhibited at 250 µM and strongly inhibited at 500 µM. We also measured the effects of lower concentrations of PEO-IAA (2, 20 and 200 µM) on abraded coleoptiles, and found that concentrations as low as 2 µM PEO-IAA significantly inhibited gravitropic curvature. The coleoptile was then shifted to a horizontal position. After 10, 30 and 60 min of grav-stimulus, IAA distribution resembled that at time 0, although there was increased accumulation of IAA at the tip region and decreased IAA level in the lower regions over time. When NPA was applied locally at 8–10 mm positions, parts above the NPA-treated region exhibited clear IAA redistribution after grav-stimulus, but parts below the treated region did not (Fig. 4C).
as 20μM PEO-IAA significantly inhibited gravi-stimulus (see Supplementary Fig. S5). Expression of ZmSAUR2 in these tissues was analyzed by RT–PCR. ZmSAUR2 mRNA was detected constitutively in coleoptiles. However, ZmSAUR2 mRNA was not detectable when IAA decreased in the elongation zone of coleoptiles after NPA treatment at the 0–2 mm region, or as a result of decapitation of the 0–3 mm tip region. Furthermore, PEO-IAA treatment at 0–15 mm also suppressed expression of this gene (Fig. 5B). These results indicate that endogenous IAA levels directly regulate the IAA signaling pathway that includes the auxin F-Box receptors TIR1/AFBs. Our results show that IAA redistribution was detectable after 30 min gravi-stimulus, when higher IAA levels were detected in lower tissues than in upper tissues (see Fig. 4B). The expression of ZmSAUR2 was affected by the differential IAA redistribution—it was less induced at the upper side, but slightly increased at the lower side (Fig. 5C). Thus ZmSAUR2 rapidly responded to endogenous IAA. These results demonstrate that the SCF{TIR1/AFB} pathway regulates coleoptile elongation leading to gravitropic curvature in response to endogenous asymmetric IAA distribution.

Discussion

Maize coleoptiles as ideal tissues for studying mechanisms of tropic curvature

Grass coleoptiles, mainly maize, are an ideal model for plant tropism research, because the tissue is relatively large. Using this tissue, we can investigate in detail the levels and movement of endogenous IAA. Usually, bending is visible within 60 min, and an asymmetric distribution of IAA occurs <30 min after tropic stimulus (Iino 1991, Philippar et al. 1999 and Fig. 4 in this study). In many cases where exogenous inhibitors are applied to plant stems or coleoptiles, thick cuticles prevent chemicals from entering the plant tissues, and these cuticles must be disrupted using abrasives, e.g. fine aluminum oxide powders (Theologis et al. 1985). In the present work, we found that NPA and BFA inhibited IAA transport and PEO-IAA suppressed ZmSAUR2 gene expression when they were applied to the inside of maize coleoptiles. This allowed us to examine the effects of NPA, BFA and PEO-IAA without the wound effects of abrasion (see Figs. 3, 4, 5 also refer to Supplementary Figs. S2, S4). Also, this method enabled application of chemicals to localized, targeted positions on the coleoptiles.

It is important to consider the source of the IAA produced at the tip region. Conjugated IAA is transported in maize seedlings from kernels to shoots, and is hydrolyzed there to produce free IAA (Cohen and Bandurski 1982). However, it is also known that IAA is synthesized at the tip region from Trp and the synthesized IAA is transported to lower parts (Iino and Carr 1982, Weiler and Wischnewski 1984, Koshiba et al. 1995, Mori et al. 2005). Based on the results of recent genetic research (Woodward and Bartel 2005, Gallavotti et al. 2008, Sugawara et al. 2009) and molecular structural analysis of IAA using gas chromatography–mass spectrometry (GC-MS) (Glawischnig et al. 2000), it is now widely accepted that IAA is synthesized from Trp. However, the relevant genes, enzymes and intermediates in the pathway are not yet fully defined, and the site of de novo IAA synthesis remains unknown, even in Arabidopsis (Sugawara et al. 2009).

In the present work, we used an IAA-labeling method to determine whether IAA is synthesized at the tip region, and to analyze movement of IAA to basal parts. Incorporation of the stable isotope from [13C11 15N2]Trp into IAA mainly occurred within the top 0–1 mm region (Table 1). The synthesized IAA was immediately transported to lower regions via polar transport (Fig. 1). These results, as well as our previous GC-MS results, show that conjugated IAA does not participate in these events (Mori et al. 2005, Nishimura et al. 2006). Our results indicate that the tip region is the site of IAA biosynthesis from Trp, and also that the rate of IAA synthesis is tightly controlled in response to physiological and/or environmental conditions (Nishimura et al. 2006).

ZmPIN1(s) is a reliable component for IAA polar transport in maize coleoptiles

In previous studies, IAA transport rate was examined in maize coleoptiles using [14C]IAA and [3H]IAA as tracers. However, because the IAA was externally applied to coleoptile sections or the coleoptile surface, the amount of endogenous IAA could not be determined accurately, nor could the biosynthetic site of IAA in intact coleoptiles be determined. In the present study, we used GC-MS to measure IAA synthesized and transported from the tip. Our results clearly and accurately showed movement of IAA in these regions (see Fig. 1). It has long been postulated that IAA efflux and influx carriers are involved in IAA transport within maize coleoptiles (Rubery and Sheldrake 1973). More recent reports describe ZmPIN1(s) and its functional role in IAA transport in maize roots and meristems (Carraro et al. 2006). We showed here that ZmPIN1a and ZmPIN1b mRNAs are expressed relatively evenly in the coleoptile tip region (at least in the top 6 mm) (Fig. 2), and their proteins are detected throughout the coleoptile (Fig. 2). ZmPIN1 immunohistochemical analysis suggested that IAA is transported in all directions in the top 0–0.5 mm region, and it is transported basipetally in the lower regions. This strongly suggested that ZmPIN1 is responsible for IAA transport from the tip to lower regions, though a possibility of the contribution of other PIN members is not excluded. Gravi-curve was almost completely inhibited by NPA and BFA treatment at the tip region (Fig. 4). NPA and BFA treatment at lower parts resulted in bending of upper parts, while lower parts remained straight. These results indicate that PIN proteins
are responsible for NPA- and BFA-sensitive polar transport of IAA in maize coleoptiles, and the intracellular polarity of ZmPIN1 correlates with the direction of IAA flow.

Mechanism of directional changes of IAA transport after gravi-stimulus

In Arabidopsis, it is postulated that AUX1, PIN2 and PIN3 are involved in relocating IAA in response to tropic stimuli (Friml and Palme 2002, Moore 2002). However, there are many reports that other factors also control the change in the direction of IAA flow, such as the members of the PGP family in Arabidopsis and LAZY1 in rice (Noh et al. 2003, Li et al. 2007, Nagashima et al. 2008). In the present study, IAA redistribution along coleoptiles was observed within 30 min of gravity stimulus (Fig. 4), but intracellular distribution of ZmPIN1 did not change after the stimulus (data not shown). Polar IAA transport is thought to maintain its direction even after the orientation of coleoptile or shoots is reversed (Little and Goldsmith 1967). Our result showing that ZmPIN1(s) was not relocated in the cells after the change in orientation can explain how polar transport is maintained even after the tissues are placed upside down. These facts support the hypothesis that ZmPIN1(s) is responsible for IAA polar transport, and are consistent with its localization (Fig. 2) and with the results of the inhibitor experiments (Figs. 3, 4). Our present results clearly showed that NPA inhibits differential IAA redistribution in response to a gravity stimulus. This indicates that some component(s) other than ZmPIN1 is important in the directional change of IAA flow in maize coleoptiles, as in Arabidopsis root tissues. Although the component(s) that regulates the direction of IAA flow is yet to be determined, it is clear that IAA is redistributed in coleoptiles after gravi-stimulus. Lateral IAA transport may occur throughout the length of the coleoptile; however, differential IAA distribution is predominantly observed in the tip region (Fig. 4). Thus the lateral IAA transport responsible for IAA redistribution might occur mainly at the coleoptile tip region. Furthermore, local treatment with NPA at the tip resulted in not only inhibition of IAA redistribution, but also complete inhibition of gravity bending at lower parts. These results suggest that key component(s) specific for lateral IAA transport must exist at the tip region, where IAA is synthesized.

TIR1/AFBs-mediated auxin signaling pathways play an important role for coleoptile growth in the elongation zone

ZmSAUR1 and ZmSAUR2 encode calmodulin-binding proteins, and are involved in cell elongation in maize coleoptiles (Yang and Poovaiah 2000, Knauss et al. 2003). When a calmodulin antagonist and NAA were applied to coleoptiles, NAA-induced cell elongation was inhibited. In the present work, we showed that IAA redistribution in coleoptiles immediately regulated ZmSAUR2 gene expression within 30 min of gravity stimulus. Furthermore, when the IAA antagonist PEO-IAA was applied to the coleoptile elongation zone, both the expression of ZmSAUR2 and the gravitropic curvature were inhibited. These results suggest that ZmSAUR2 may be one of the important factors in the cell’s elongation response to IAA redistribution, which results in tropic curvature. This result suggests that differential growth is controlled by regulation of gene expression via TIR1/F-Box proteins. However, we cannot exclude the possibility that some other TIR1/AFBs-dependent IAA-inducible genes, such as the AUX/IAA and GH3 gene families, are also involved in the response. In addition, TIR1/F-Box proteins have not yet been characterized in maize. The IAA antagonists BH-IAA and PEO-IAA blocked the auxin response in Arabidopsis, rice, moss (Hayashi et al. 2008) and maize in this work. This suggests that the auxin SCF–TIR1 pathway is universally conserved in plants.

Concluding remarks

IAA is now recognized as a master signaling substance for plant embryogenesis, organ development and plant body formation (Zažímalová and Napier 2003), and is the ‘morphogen’ or ‘positional signaling factor’ as proposed by Wolpert (Wolpert 1969). Polar IAA transport is one of the most important mechanisms to create the dynamic gradient or flow networks of IAA that are involved in whole plant development (Schlicht et al. 2006, De Smet and Jürgens 2007, Grieneisen et al. 2007). The source of the IAA is, therefore, a key factor in considering the formation of the IAA flow network. However, neither the specific IAA synthetic cell(s) nor the IAA biosynthetic gene(s) have been identified, even in Arabidopsis. As described above, the maize coleoptile tip region is a strong candidate as the specific IAA biosynthetic site. Within that region, there must be a highly regulated system controlling the rate of IAA synthesis and the direction of IAA flow, and this system must be tightly connected to the mechanisms that sense environmental stimuli. In this study, we showed that the movement of the signaling substance IAA is connected to cell elongation via regulation of IAA-inducible gene expression at the elongation zone.

Materials and Methods

Plant materials and growth conditions

Seeds of maize (Zea mays L. cv. Golden Cross Bantam 70) were germinated at 25°C under red light for 2 days and then in darkness for 1 day as described previously (Mori et al. 2005). We used intact coleoptile tissues to investigate labeled IAA movement (Fig. 1), for ZmPIN1 western blot and mRNA expression experiments (Figs. 2, 5), and for immunohistochemical observation of ZmPIN1 (Fig. 2). Coleoptile tip sections (3 mm) were used for determination of endogenous...
IAA and transported IAA after NPA and BFA treatments (Fig. 3). Coleoptile segments 2 cm in length were used for local treatments with NPA and BFA (Fig. 4) and for PEO-IAA treatment experiments (Fig. 5).

**Labeling of IAA with heavy isotopes from \[^{13}\text{C}_{11}^{15}\text{N}_2\] Trp, and tracing labeled IAA from tip to base**

Incorporation of labeled isotopes from Trp to IAA was monitored as follows: 2 µL \[^{13}\text{C}_{11}^{15}\text{N}_2\]Trp (200 ng µL⁻¹ in 10 mM KPB, pH 6.7) was locally applied to the top 2 mm of the coleoptile, and to positions at 0–1, 1–2, 2–3 and 9–11 mm along the 2-cm long coleoptile segments as explained in Supplementary Fig. S2. In the pulse–chase experiment, an agar block containing \[^{13}\text{C}_{11}^{15}\text{N}_2\]Trp (200 ng µL⁻¹) was placed at the 0–1 mm region on intact seedling coleoptiles (see Supplementary Fig. S3). After 15 min incubation, the agar block was replaced by a cold \[^{12}\text{C}_{14}\text{N}\]Trp-containing agar block and the incubation was continued. After 0, 30, 60, and 90 min incubation, the coleoptiles were cut into 1 mm thick sections from the top towards the node. IAA and incorporation of labeled isotopes were determined in each section by GC-MS.

**RNA extraction and semi-quantitative RT–PCR**

Total RNA was extracted from maize coleoptile tissues using the Micro-to-Midi Total RNA Purification System (Invitrogen). Total RNA (1 µg) was subjected to cDNA synthesis using oligo(dT) primers and M-MLV reverse transcriptase (Promega) according to the manufacturer’s protocol. ZmPIN1a, ZmPIN1b, ZmPIN1c, ZmSAUR2 and ubiquitin-specific primer combinations (specific primer sequences are shown in Supplementary Table S2) were used to amplify these transcripts. PCR was performed with the HotStarTaq Master Mix Kit (QIAGEN) as follows: 28 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min.

**Protein extraction and western blot analysis**

Approximately 30 mg of maize coleoptile tips (0–5 mm) was pulverized, and crude proteins were extracted in 600 µL extraction buffer (200 mM Tris–HCl, pH 7.4 containing complete protease inhibitor) with a small amount of glass beads (1.0 diameter, approximately 200 mg) at 4°C in a bead cell disruptor (Micro-smash, Tomy). After disruption, the crude protein extracts were mixed with 200 µL SDS sample buffer and incubated for 30 min at room temperature (without heat treatment). After centrifugation at 15 000 rpm for 20 min at room temperature, 20 µL supernatant was separated by SDS–PAGE (7.5% w/v polyacrylamide). Separated peptides were electrophoretically onto PVDF membrane. The blots were blocked overnight at 4°C with Tris-buffered saline, pH 7.2, containing 0.05% Tween 20 and 2% skim milk and then incubated with the anti-AtPIN1 antibody (details are described in Boutté et al. 2006) at a 2000-fold dilution for 1 h at 4°C. Signals were detected using a peroxidase-conjugated secondary antibody (Vector Laboratories) at a 5000-fold dilution, and by chemiluminescence (ECL kit; Pierce) followed by X-ray film exposure.

**Immunolocalization of ZmPIN1(s)**

Coleoptile sections were excised from the seedlings and immediately fixed in 4% paraformaldehyde in 0.5-fold MTSB [50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) pH 7.2, 5 mM MgSO₄, 5 mM EGTA] for 3 h at room temperature, then washed twice in MTSB for 30 min. The fixed tissues were transversely sectioned by hand, using two layered razor blades. Sections were approximately 100 µm thick. The sample sections were treated with detergent solution (10% DMSO and 3% Nonidet P-40 in MTSB) for 30 min and washed three times in MTSB for 10 min. The sample sections were incubated overnight at 4°C with an anti-AtPIN1 antibody at a 2000-fold dilution in 3% skim milk. Sections were rinsed three times for 10 min with MTSB containing 0.1% Tween-20, and were then incubated with Alexa488-conjugated goat anti-rabbit IgG (Molecular Probes) at a 200-fold dilution for 3 h at room temperature. After washing three times for 10 min, the sample sections were sealed with 50% glycerol. The prepared samples were observed with an epifluorescence microscope (model BX51; Olympus) or a laser scanning confocal microscope (model LSM5; Zeiss).

**Measurement of IAA distribution after gravistimulus and NPA treatment, and observation of gravi-curvature of coleoptile segments**

Coleoptile segments approximately 20 mm long were excised from the seedlings. We applied 10 mM potassium phosphate buffer (KPB) pH 6.7, or NPA or BFA or PEO-IAA in KPB to the inner surface of the coleoptile tip, at regions 0–2, 8–10, 11–13 and 3–15 mm from the tip (see Supplementary Figs. S2, S4). Then, the base of each coleoptile was clamped in 1% agar and tilted horizontally. After 0, 10, 30 and 60 min incubation, 0–3, 3–7, 7–11 and 11–15 mm sections of the coleoptiles were collected and the amount of free IAA was determined (Fig. 4). To observe gravity bending, photos of coleoptiles were taken after incubation for 0, 60, 120 and 180 min (Figs. 4, 5).

**Acknowledgments**

We are grateful to Drs T. Okamoto and T. Komano, and Ms A. Sudo from our laboratory for helpful discussions and technical assistance. We also thank Drs A. Asada and S. Hisanaga for their help with the confocal microscope analysis. This work was supported in part by the ’Initiatives for Attractive Education in Graduate School’ (IAGE) grant.
awarded to the Department of Biological Sciences, Tokyo Metropolitan University, from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), by Grants-in-Aid for Scientific Research in Priority Areas from MEXT to T. K. (15031222, 17027022, 20061025 and 21027030), and by a Grant-in-Aid from the Japan Society for the Promotion of Science (JSPS) to T. N. (19 7171).

References


(Received August 4, 2009; Accepted September 15, 2009)