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Basipetal Auxin Transport Is Required for Gravitropism in Roots of Arabidopsis[1]

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Auxin transport has been reported to occur in two distinct polarities, acropetally and basipetally, in two different root tissues. The goals of this study were to determine whether both polarities of indole-3-acetic acid (IAA) transport occur in roots of Arabidopsis and to determine which polarity controls the gravity response. Global application of the auxin transport inhibitor naphthylphthalamic acid (NPA) to roots blocked the gravity response, root waving, and root elongation. Immediately after the application of NPA, the root gravity response was completely blocked, as measured by an automated video digitizer. Basipetal [^{3}H]IAA transport in Arabidopsis roots was inhibited by NPA, whereas the movement of [^{14}C]benzoic acid was not affected. Inhibition of basipetal IAA transport by local application of NPA blocked the gravity response. Inhibition of acropetal IAA transport by application of NPA at the root-shoot junction only partially reduced the gravity response at high NPA concentrations. Excised root tips, which do not receive auxin from the shoot, exhibited a normal response to gravity. The Arabidopsis mutant *eir1*, which has agravitropic roots, exhibited reduced basipetal IAA transport but wild-type levels of acropetal IAA transport. These results support the hypothesis that basipetally transported IAA controls root gravitropism in Arabidopsis.

Polar auxin transport in higher plants is a directional and regulated process. In stems, auxin is transported from cell to cell and moves from the shoot apex toward the base (Lomax et al., 1995). Auxin transport is believed to control a variety of important growth and developmental processes, including the gravity response. The Cholodny-Went hypothesis, originally proposed in 1937, suggests that lateral transport of auxin across gravity-stimulated shoots may cause differential gravitropic growth (Evans, 1991; Trewavas, 1992). Lateral redistribution of radiolabeled indole-3-acetic acid (IAA) has been measured in both shoots (Parker and Briggs, 1990) and roots (Young et al., 1990), and the redistribution of IAA has been shown to precede differential growth and the gravity response (Parker and Briggs, 1990). Additionally, inhibition of auxin transport blocks root gravitropism (Muday and Haworth, 1994).

Although the validity of the Cholodny-Went hypothesis has been debated, recent molecular and genetic evidence has provided additional support for it (Trewavas, 1992; Chen et al., 1999). The construction of transgenic plants with an auxin-responsive promoter driving the expression of β-glucuronidase demonstrated the redistribution of auxin-induced gene expression across a gravity-stimulated shoot (Li et al., 1991). The ability of auxin transport inhibitors to block both differential auxin-regulated gene expression and gravitropic bending suggests that lateral auxin transport is the mechanism that leads to differential gene expression. Additionally, the isolation of mutants such as the synonymous mutants *agr1/eir1/pin2/wav6*, which are altered in the gravity response and have mutations in genes encoding proteins that appear to function in auxin transport, further supports the hypothesis that auxin transport is required for the plant gravity response (Chen et al., 1999).

In roots, the gravity response has also been linked to lateral auxin transport (Young et al., 1990); however, the complexity of auxin movement throughout the root has made this link more difficult to establish (Lomax et al., 1995). Analysis of the distribution of exogenously applied radiolabeled IAA to plants indicates that auxin is transported acropetally (from the base of the root toward the root tip) in the central cylinder of the root (Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978). However, there is also evidence for basipetal auxin transport (from the tip toward the base) near the root apex in *Phaseolus coccineus* (Mitchell and Davies, 1975), and microautoradiography suggests that basipetal auxin transport occurs in the peripheral layers of cells, primarily the epidermal and cortical cells (Tsurumi and Ohwaki, 1978). Both polarities of auxin transport were reduced by application of the auxin transport inhibitor 2,3,5-triiodo-benzoic acid (Tsurumi and Ohwaki, 1978), suggesting that a similar mechanism may control the transport of auxin in each polarity. It is not clear which of these two polarities of auxin transport controls the root gravity response.

Several lines of evidence in the literature suggest that basipetal IAA movements may control root elongation and the gravity response. Alteration of growth or tropisms in roots due to localized applications of IAA occurs only if they are applied at a position apical to the elongation zone, suggesting that IAA must reach the elongation zone by basipetal transport (Davies et al., 1976). Removal of a ring of epidermal and cortical cells around a maize (*Zea mays*)

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root blocked gravitropism, but only when the ring was apical to the elongation zone (Yang et al., 1990). As these tissues have been implicated in basipetal IAA transport (Tsurumi and Ohwaki, 1978; Muller et al., 1998b), this result is consistent with the basipetal movement of IAA controlling the gravity response. Finally, pretreatment of maize roots with auxin transport inhibitors reduced both basipetal auxin transport and gravitropic bending (Young and Evans, 1996).

The hypothesis that the two polarities of auxin movement control distinct growth and developmental processes was supported by a recent report examining the polarity of auxin movement controlling lateral root development (Reed et al., 1998). Reed et al. (1998) presented evidence linking acropetal IAA transport from the shoot into the root with the control of lateral root development in Arabidopsis. Several different approaches were used to block transport of shoot-derived auxin into the root, and these treatments resulted in reduction of lateral roots. These treatments included: localized application of the auxin transport inhibitor naphthylphthalamic acid (NPA) to the root-shoot junction, removal of the shoot, and growth of plants in the dark. Lateral root inhibition by all of these treatments was reversible by the application of IAA. These experiments indicated that acropetal auxin transport controls lateral root development.

The goal of this study was to assess whether basipetal auxin transport from the root tip toward the base is responsible for root gravitropism. First, it was necessary to determine if there is measurable basipetal IAA transport in Arabidopsis roots. Second, basipetal auxin transport had to be separated from acropetal auxin transport in order to determine which IAA transport polarity controls the gravity response. This was done using three approaches: chemical inhibition of auxin movement with NPA, physical separation of root tips from the rest of the root, and genetic lesions in Arabidopsis plants that result in reductions in one polarity of auxin transport. These experiments indicated that in Arabidopsis, basipetal auxin transport is sufficient to control root elongation and gravitropism.

MATERIALS AND METHODS

Chemicals

NPA was purchased from Chemical Services (West Chester, PA). Triton X-100 and Suc were from Fisher Scientific (Pittsburgh). Absolute ethanol was purchased from McCormick Distilling (Weston, MO), 3-[5(n)-^{3}H]IAA (27 Ci/mmol) and [ring-U-^{14}C]benzoic acid ([ring-U-^{14}C]-BA) (126 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). All other chemicals were purchased from Sigma (St. Louis).

Seed Germination and Plant Growth

Wild-type Arabidopsis seeds (ecotype *Landsberg erecta*) were purchased from Lehle Seeds (Round Rock, TX); Arabidopsis ecotype Columbia seeds were from Dr. Mark Estelle; and *eir1* seeds were obtained from the Arabidopsis Biological Resource Center at Ohio State University (Columbus, OH). Seeds were soaked in distilled water for 30 min and surface-sterilized with 95% (v/v) ethanol for 5 min, followed by 10% (v/v) bleach with 0.01% (v/v) Triton X-100 detergent for 5 min. After five washes in sterile distilled water, seeds were germinated and grown on sterile control medium (0.8% [w/v] agar [Sigma type M, plant tissue culture]; 1× Murashige and Skoog salts, pH 6.0; 1.5% [w/v] Suc; 1 μg mL^{-1} thiamine; 1 μg mL^{-1} pyridoxine HCl; 0.5 μg mL^{-1} nicotinic acid; and 50 μg mL^{-1} sterile-filtered ampicillin). Seeds were grown in vertically oriented Petri dishes in continuous 94 μmol s^{-1} m^{-2} fluorescent light at room temperature (22°C) for 4 to 5 d, until cotyledons had emerged and roots reached the length of 1 to 1.5 cm. Ten seedlings were transferred to new plates containing control agar or agar plus compounds at the indicated final concentrations, followed by the indicated treatments.

Application of NPA

Control agar (0.8%, w/v), as described above, was supplemented with NPA at 10^{-4} M or at the indicated range of concentrations. Compounds were added to (50°C) molten control agar and either poured into plates or allowed to harden in a sterile Pasteur pipette for global and local application, respectively. The agar could be dispensed directly from the pipette with gentle pressure for localized application. NPA was dissolved in dimethylsulfoxide (DMSO) at a range of concentrations and was added to agar with a final DMSO concentration of 0.1% (v/v). All controls contained agar with 0.1% (v/v) DMSO. All agars were stored at 4°C. The supplemented agar was stored in the dark. Agar-containing NPA was made at least every 10 d to minimize effects due to the breakdown of this compound.

In plants treated with locally applied compounds, agar was applied to 4-d-old plants in a 1-mm line at the root-shoot junction or in a 5-mm line along and below the root tip. A larger application area was necessary at the root tip so that the agar covered the root tip during the length of the experiment. Controls for these experiments were performed by the addition of an agar line without added compound.

Gravity Response and Waving

The gravity response was measured using 4-d-old light-grown plants. The plants were transferred to plates containing either control agar for local application of NPA or agar supplemented with NPA at the indicated concentrations for global application of NPA. After the application of NPA, the plants were grown in vertically oriented Petri dishes for 24 h and then rotated 90°. After an additional 24 h, the amount of growth during 48 h and the angle of curvature after 24 h were measured, and the average and SE are reported. The exposure to NPA was through the entire 48-h period.

The gravity response was measured in 5-mm root tips and in entire roots from which the shoot was excised. The

excised 5-mm root tips from a 4-d-old plant were transferred to an agar plate containing control agar or NPA-containing agar. The tips were allowed to grow for 24 h after NPA application and then the plates were rotated 90°. Root growth and angle of curvature were measured after an additional 24 h of contact with NPA, and the average and SE are reported. In the analysis of entire excised roots, the roots were transferred to control agar and allowed to grow for 4 d before gravity stimulation. A longer period before gravity stimulation was used so that lateral roots would develop. The root growth and number of lateral roots formed during this 4-d period and the gravitropic bending 24 h after reorientation with constant exposure to NPA are reported.

Root waving was measured using 4-d-old light grown plants or excised root tips. The plants were transferred to 1.5% (w/v) agar plates containing either control agar or agar supplemented with NPA at the indicated concentrations. The plates were placed at an angle of 60° from horizontal in continuous light and allowed to grow in this position for 7 d. During these assays, gravity directed roots toward the agar, but they could not penetrate the hard agar surface. The roots continually reversed the direction of growth forming S-shaped curves or waves. The number of waves and the total root length were measured after 7 d, and the average and SE are reported.

Auxin Transport Assays

Basipetal auxin transport was measured in 7-d-old vertically grown plants. Plants were transferred to control plates with root tips aligned. Agar at 1% (v/v) was mixed with 100 nM [^{3}H]IAA in the presence or absence of 10^{-4} M NPA. Control and NPA-containing agar both had a final DMSO concentration of 1% (v/v). The agar was hardened in 3-mL vials. Narrow stem transfer pipettes were inserted into the hardened agar mixture to form a cylinder. This cylinder or line of agar was applied such that it just touched the root tip. Plates remained vertically oriented for 5 h in the dark to minimize IAA breakdown. The root growth was minimal during this period, and the agar remained in contact with only the root tip. However, if the assay were continued for 18 h, the roots would grow out from under the agar line. To control for the simple diffusion of a weak acid, 4 μM [^{14}C]BA was also used in this assay in place of [^{3}H]IAA. The [^{14}C]BA diffusion was also measured in the presence and absence of NPA. IAA transport and BA diffusion were measured after 5 h by first removing the apical 1 mm in contact with the agar line, then cutting each root into either 2- or 5-mm segments back from the root tip. Individual root segments were placed into 2.5 mL of scintillation fluid, and radioactivity was measured for 2 min using a scintillation counter (model LS 6500, Beckman, Fullerton, CA). There was no measurable root growth during the length of this assay.

Acropetal auxin transport was measured in 7-d-old vertically grown plants according to the method of Reed et al. (1998), with several modifications. Plants were transferred to plates containing control agar such that their root-shoot junctions were roughly in a horizontal line. [^{3}H]IAA (100 nM) and cold IAA (10 μM) were thoroughly mixed in 1% (w/v) agar and allowed to harden in a 3-mL vial. Cold IAA was added only to the acropetal transport assay, as it was found to increase the amount of [^{3}H]IAA transport by about 2-fold. In contrast, in the basipetal transport assay, cold IAA decreased the movement of [^{3}H]IAA. The total amount of 10 μM cold IAA reaching the root tip in the acropetal IAA transport assay could be estimated at 0.5 μM by using the [^{3}H]IAA as a radiotracer. Lines or cylinders of agar were formed as described above and placed directly onto the root-shoot junction of the transferred plants.

NPA at a concentration of 10^{-4} M or DMSO at a concentration of 1% (w/v) was applied either by addition to the agar containing IAA or as a second agar line placed onto the roots of the plants just below the agar line containing radiolabel IAA. Both approaches give similar values. Weak acid diffusion controls using [^{14}C]BA (4 μM) in the presence and absence of NPA were also used in this assay in place of [^{3}H]IAA. Plates were vertically oriented in the dark to minimize IAA breakdown. There was no difference in transport when shoots were oriented above or below the root. The reported values are for inverted plants, with the shoot above the root. IAA transport and BA diffusion were measured in the apical 1 cm of the root after 18 h, as described above. Shorter transport periods were also examined, which led to lower amounts (closer to background levels) of radiolabeled IAA reaching the root tip.

Statistical analysis of the data from transport assays was performed using Microsoft Excel. Multiple experiments were analyzed simultaneously, using each root as an independent sample. The IAA transport data were analyzed by a one-tailed Student's *t* test for equal variance, since the assumption being tested was that NPA treatment or the mutant phenotype would reduce IAA movement. The BA diffusion data was analyzed by a two-tailed Student's *t* test, since no difference in BA movement was expected in response to NPA treatment or in the mutant.

Automated Video Digitizer Analysis of Root Gravitropism

Ecotype Columbia plants were germinated on control plates for 5 d and transferred to agar plates with or without 50 μM NPA. The plants were covered with liquid agar (1× Murashige and Skoog medium, 0.8% [w/v] agar, described above) with or without NPA cooled to 32°C, to prevent damage to the plants. Embedding the plants in agar increases the contrast for the image analysis program. The plants remained on the agar plates for less than 5 min until the agar had solidified before image analysis began. Root growth was similar when the plants were allowed to recover after exposure to the warm agar. Images were captured with a CCD camera connected to a computer by a frame-grabber circuit board. The Petri dishes were oriented vertically and held in place with a micromanipulator. The plants were illuminated from behind with an infrared LED. The CCD camera, computer, infrared LED, and software were purchased from the Plant Growth Imaging Facility at Ohio State University.

The images were analyzed using the Multi-ADAPT software (Ishikawa and Evans, 1997). This software divides the

root into segments of consistent length based on user-defined reference points at the tip and in the non-elongating region of the root. The angle of each segment and the length on both sides of each segment are then recorded at user-specified time intervals. For this analysis, segments of 160 μm were examined at 60-s intervals. To simplify the data, the angle of curvature relative to the vertical and the length are plotted for samples collected every 5 min. An angle of 90° was defined as horizontal growth, and an angle of 0° as roots that had completely reoriented relative to gravity.

RESULTS

Effect of Global NPA Application on Root Growth, Gravity Response, and Waving

Arabidopsis roots were germinated on control agar plates and transferred to agar plates containing a range of concentrations of NPA (10 nM–5 μM). A representative experiment examining the effect of NPA on the ability of the roots to elongate, respond to gravity, and form root waves is shown in Figure 1. All three of these processes were inhibited by NPA in a dose-dependent manner. The concentrations for 50% inhibition (IC_{50}) for these processes were calculated from three separate experiments and the averages are compared in Table I. The NPA concentrations for inhibition of the gravity response and root waving were very similar, but 10-fold higher concentrations of NPA were needed to inhibit growth by 50%.

To examine the immediate effect of NPA on root gravitropism, roots were transferred to agar plates containing 50 μM NPA and imbedded in agar containing the same NPA concentration. The growth and gravitropic curvature of the roots were examined using Multi-ADAPT software (Ishikawa and Evans, 1997). Figure 2 shows a comparison of the gravitropic angle and root elongation of NPA-treated roots with roots grown on and embedded in control agar. Roots on control agar showed an initial gravity response within 75 min and were almost completely reoriented by 300 min (Fig. 2A). Root curvature occurs due to the greater growth rate on the upper side of the root (Fig. 2B). In contrast, the NPA-treated roots exhibited no curvature (Fig. 2A), although the growth rates during gravitropic bending were close to the control (Fig. 2B). The growth rates on the upper sides of the NPA-treated root were 3.7 μm min^{-1} and the control rate was 4.1 μm min^{-1}. In this particular experiment, NPA-treated roots grew against the gravity vector, due to a slightly elevated growth rate on the lower side than the upper side, 4.5 μm min^{-1} compared with 3.8 μm min^{-1}, respectively. The growth rate of the plants was also determined before gravity stimulation, and the rates were similar to plants that were gravity stimulated. Also, roots that were allowed to recover from the treatment with warm control agar exhibited similar growth and gravitropic bending profiles.

Table I. *Effect of NPA on the gravity response, waving, and growth in root tips and intact plants*

Response	NPA Concentration Causing 50% Inhibition[a]	
	Intact plants	Excised root tips
Gravity response	0.5 μM ± 0.08	0.6 μM ± 0.08
Waving	0.4 μM ± 0.11	0.6 μM ± 0.24
Growth	4.8 μM ± 0.15	1.9 μM ± 0.14

[a] Reported values are the average and SE of four separate experiments using NPA concentrations between 10 nM and 5 μM.

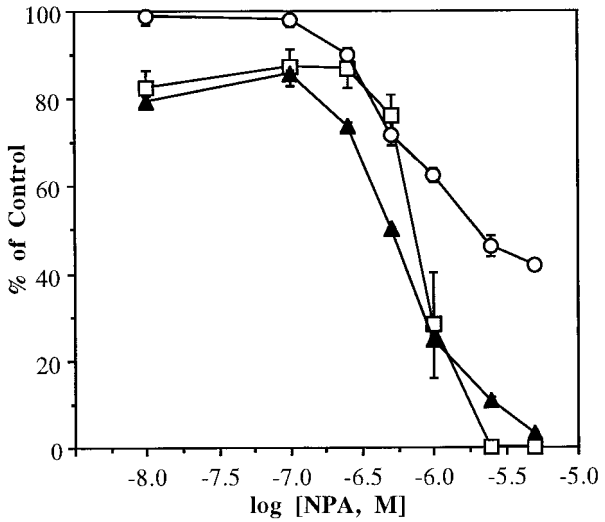

Figure 1. NPA inhibits the gravity response, root waving, and root elongation. Roots were in continuous contact with NPA for the duration of this experiment. The gravity response (□) was measured 24 h after gravity stimulation of roots. Waving (▲) and root growth (○) were measured after 7 d on 1.5% (w/v) agar plates. The average and SE for 10 plants are shown. Control values were: gravity response = 87.6°; length = 47 mm; and waving = 8.5 waves per root.

Measurement of Phytotropin-Sensitive Basipetal IAA Transport

An assay to measure basipetal IAA transport in Arabidopsis roots was developed. Agar lines containing 100 nM [^{3}H]IAA in the presence and absence of NPA were applied to the tip of roots. After 5 h, the apical 1 mm of the root that was in contact with the agar was removed. The remaining part of the root was divided into 2-mm segments, and the amount of radioactivity in individual root segments was determined by scintillation counting. A comparison of the IAA levels as a function of distance from the root tip in the presence and absence of NPA are shown in Figure 3. This figure shows that most of the IAA is transported in the apical end of the root and that very little IAA travels beyond the apical 5 mm of the root tip. As it is difficult to work with 2-mm segments of Arabidopsis roots, all other measurements of transport were done with 5-mm root segments, as shown in Table II.

The amount of [^{3}H]IAA transported in the basipetal transport experiments were routinely over 400 dpm. There was a statistically significant reduction in basipetal auxin transport in NPA-treated roots, as shown in Table II. In parallel samples with [^{14}C]BA as a weak acid diffusion control, NPA did not decrease [^{14}C]BA movement (Table

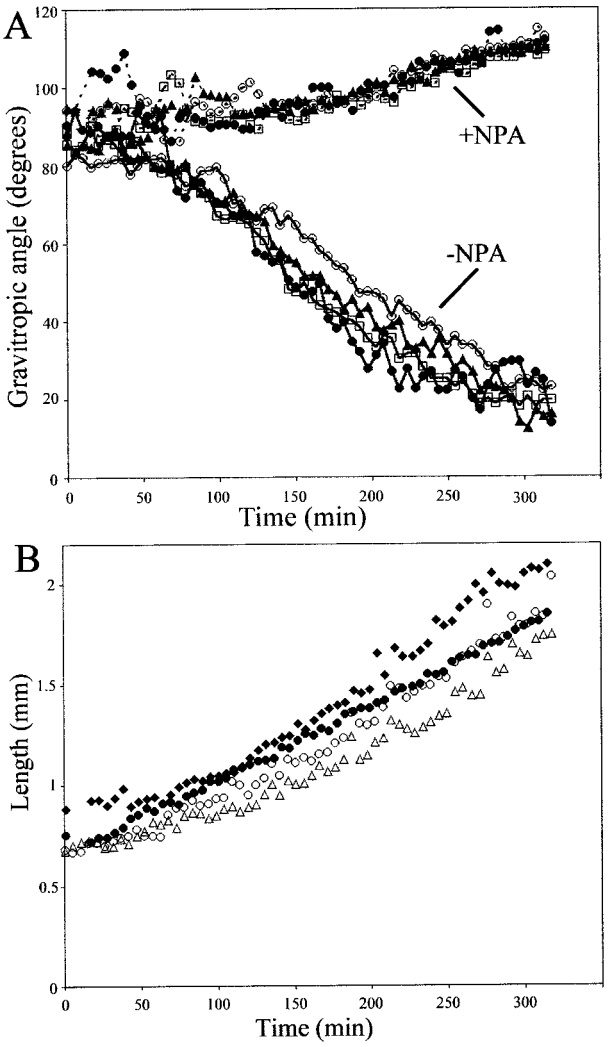

Figure 2. Kinetics of the gravity response and elongation of Arabidopsis roots in the presence and absence of NPA. A, The angles of four 160-μm segments are compared with control samples having solid lines and NPA-treated samples having dotted lines. ●, 0 to 160 μm; □, 80 to 240 μm; ▲, 160 to 320 μm; ○, 240 to 400 μm. B, The length along each side of the root is shown over time for plants grown in the presence or absence of NPA. △, Lower control; ○, upper control; ♦, lower NPA treated; ●, upper NPA treated.

II). This suggests that auxin is transported basipetally in Arabidopsis root tips and this transport is regulated by NPA. There was greater uptake and/or movement of IAA than of BA even though there was 40 times more [^{14}C]BA applied than [^{3}H]IAA. Higher levels of BA were necessary to obtain a sufficient amount of radioactivity in each sample to accurately quantify BA movement. Therefore, a 400-fold greater proportion of applied IAA than BA is taken up and transported.

Effect of Local NPA Application on the Gravity Response

To determine if IAA moving from the tip controls growth and the gravity response, lines of agar containing NPA were applied directly to the root tip. Plants were grown for

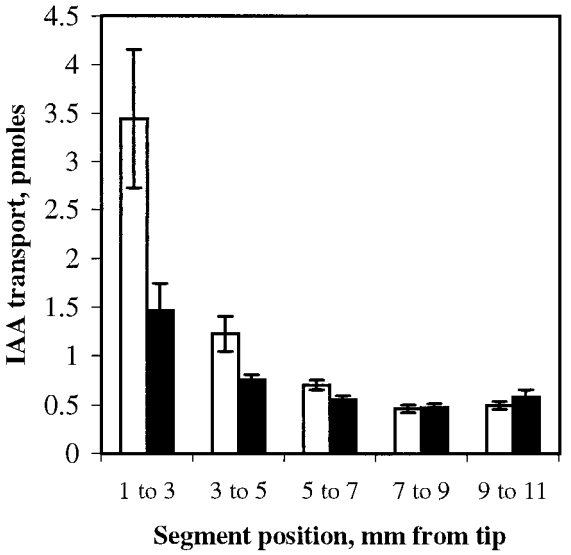

Figure 3. Basipetal [^{3}H]IAA movement is greater at the tip of Arabidopsis roots. An agar line containing 100 nM [^{3}H]IAA with (black bars) or without (white bars) 100 μM NPA was applied to the root tip, and after 5 h of transport the apical 1 mm was excised and discarded. The root tip was sectioned into 2-mm segments, and the amount of [^{3}H]IAA was determined in each region. The data are the average and SE of 14 plants.

24 h vertically, followed by a 90° reorientation and an additional 24 h of growth in continuous contact with NPA. The length and angle of curvature of each root were measured and the average and SE are shown in Figure 4. NPA applied at the root tip inhibits both the gravity response and root elongation, with lower levels of NPA needed to inhibit the gravity response.

The effect of the site of NPA application on the gravity response was also examined. Plants were grown on control agar plates and lines of agar containing similar concentrations of NPA were applied at either the root-shoot junction or the root tip. Global application of NPA was performed by growth on agar plates containing the indicated NPA concentrations. The roots were in contact with the NPA for 48 h, with the first 24 h in the vertical position and the second 24 h after a 90° reorientation. The gravity response was measured after these three treatments and is shown in Figure 5. The gravity response was inhibited similarly by

Table II. *Basipetal IAA and BA movement in ecotype Landsberg erecta roots*

Assay	[^{3}H]IAA Transport		*P* Value[a]
	−NPA	+NPA	
	picomoles		
IAA transport[b]	7.03 ± 0.91	4.73 ± 0.47	0.011
BA diffusion[c]	0.65 ± 0.05	0.53 ± 0.08	0.224

[a] The difference between samples with and without NPA was determined by Student's *t* test of equal variance. [b] The average and SE of 18 plants from four separate experiments are reported. [c] The average and SE of 11 plants from three separate experiments are reported.

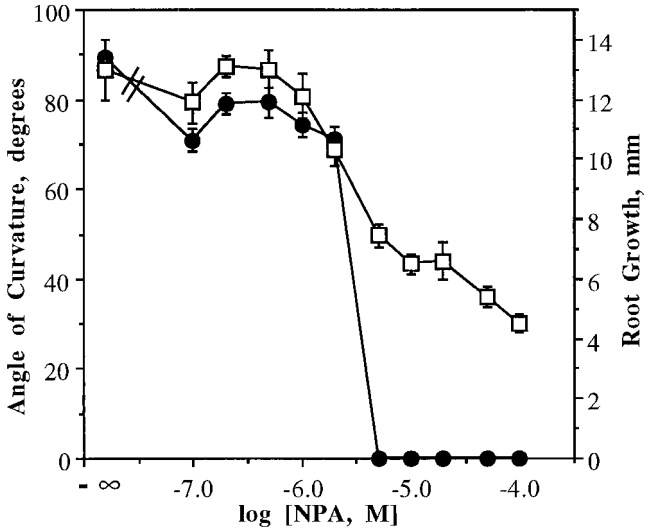

Figure 4. Localized application of NPA at the root tip inhibits the gravity response. Plants were continuously treated with agar with or without NPA and grown for 24 h vertically and another 24 h after reorientation by 90°. New growth and the angle of curvature were measured and the average and SE for 10 plants are shown. ●, Gravity response; □, growth.

low concentrations of NPA when applied globally or at the root tip, whereas even at the highest NPA concentration applied to the root-shoot junction, there was only partial reduction in the gravity response.

The IC_{50} was calculated from the data shown in Figure 5. The IC_{50} for growth and the gravity response when NPA was applied at the root tip were 20 and 3 μM, respectively. It is not possible to calculate the IC_{50} values for growth and

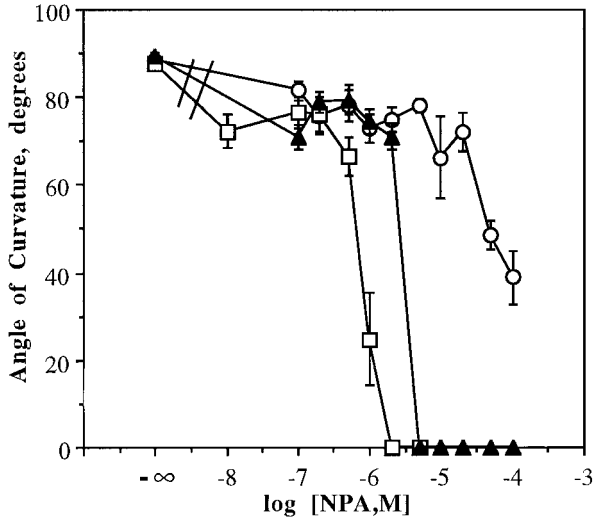

Figure 5. The gravity response depends on the location of applied NPA. NPA was continuously applied to roots for the entire experiment at the root tip or root-shoot junction, or by addition to the agar plate on which the plant was grown. During the first 24 h, the growth was vertical, then the plants were reoriented by 90°, and the angle of curvature was determined after an additional 24 h of growth. The average and SD of 10 roots are reported. □, Global application; ○, root-shoot junction application; ▲, root tip application.

Table III. *Inhibition of root growth and the gravity response in roots treated to inhibit auxin transport from the shoot*

The reported values are the averages and SE of 20 plants.

Treatment[a]	Angle of Gravitropic Curvature	Root Growth	No. of Lateral Roots
	degrees	*mm*	
Untreated	80.3 ± 2.3	13.1 ± 0.3	23.2 ± 1.7
NPA application	74.0 ± 4.0	11.1 ± 0.4	5.6 ± 0.8
Shoot excised	65.0 ± 4.4	10.2 ± 0.5	0.0 ± 0.0

the gravity response with NPA applied at the root-shoot junction, as 50% inhibition was only reached at the highest concentration. In three separate experiments in which 100 μM NPA was applied to the root-shoot junction, root growth was inhibited an average of 27%. NPA application to the root-shoot junction affected the gravity response by 56% or less. A greater than 30-fold higher level of NPA was needed to inhibit the gravity response when applied at the root-shoot junction compared with NPA applied at the root tip. Although the shape of the dose response curve for root tip application is similar to that for global application, more NPA is required to inhibit the gravity response when applied only at the root tip. The IC_{50} values for gravity inhibition for root tip versus global application are 3 and 0.7 μM, respectively, suggesting that root tip application is approximately 4-fold less effective at inhibiting the gravity response.

Since NPA application at the root-shoot junction did not abolish gravitropism, it was necessary to demonstrate that this treatment was sufficient to block acropetal auxin transport and processes that depend upon this polarity of auxin movement. As lateral root development has been shown to depend upon acropetal auxin transport (Reed et al., 1998), roots were treated with NPA at the root-shoot junction or the shoot was excised, and then gravitropism and lateral root development were compared. The treatments were for 4 d, and then the plants were reoriented by 90° and the roots allowed to grow for an additional day. The root growth and lateral root number during the 5-d experiment were determined and the angle of curvature after 24 h was measured. These two treatments profoundly reduced the number of lateral roots, but only slightly depressed the gravity response of primary roots, as shown in Table III.

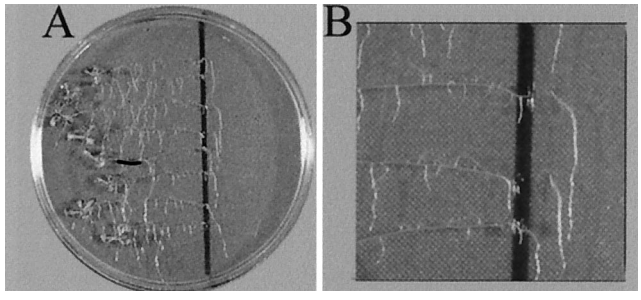

Figure 6. Excised root tips respond to gravity. The apical 5 mm of roots were excised and the plates reoriented 90°. The roots were grown for 4 additional d and then photographs were taken. A, Entire plate. B, Close-up of excised root tips.

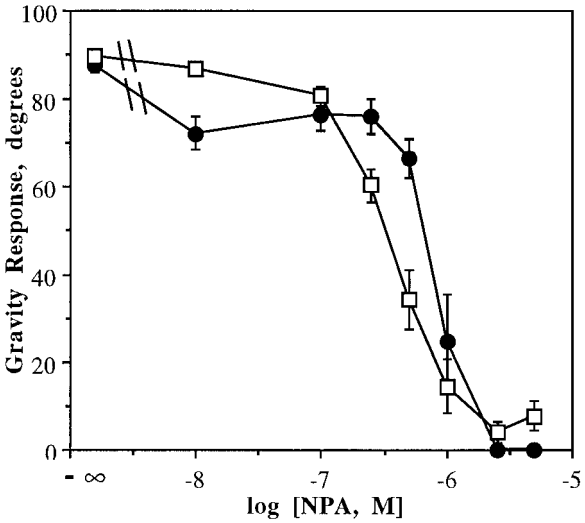

Figure 7. NPA inhibits the gravity response in root tips (□) and in intact plants (●). Four-day-old intact seedlings and excised root tips were placed on agar plates containing the indicated concentrations of NPA for the entire experiment. Plants were grown for 24 h in continuous light and then reoriented 90° relative to the gravity vector. Growth and angle of curvature were measured 24 h after reorientation, and the average and SE of 10 plants are shown.

Gravity Response in Excised Root Tips

If basipetal auxin transport from the tip controls the gravity response and auxin transport from the shoot is not required, then excised root tips should be fully gravitropic. To test this hypothesis, the apical 5 mm of Arabidopsis root tips were excised and gravity stimulated. When root tips were gravity stimulated immediately after excision from the plant, they responded to gravity, as shown in Figure 6. To further deplete the roots of shoot-derived auxin, the root tips were excised and allowed to grow for up to 4 d, and then gravity stimulated. These roots were still fully gravitropic, further suggesting that shoot-derived auxin is not necessary for the gravity response (data not shown). The ability of NPA to inhibit the gravity response in excised root tips was measured and compared with intact roots, as shown in Figure 7. The dose response curves for these two samples are very similar, as demonstrated by the similar IC_{50} values in Table I.

Measurement of IAA Transport in Roots of the *eir1* Mutant

To further understand the effect of basipetal auxin transport on the gravity response, we used an Arabidopsis mutant proposed to be altered in basipetal auxin transport (Luschnig et al., 1998). The *eir1* mutant (allelic to *agr1*, *pin2*, and *wav6*) is agravitropic, and the recently cloned *EIR1* gene has been proposed to encode an auxin efflux carrier (Luschnig et al., 1998). Both basipetal and acropetal auxin transport were measured in *eir1* and its wild-type background, ecotype Columbia, and the results are compared in Table IV. There was a statistically significant reduction in basipetal IAA transport in the *eir1* mutant compared with ecotype Columbia, but there was no change in acropetal IAA transport in this mutant. In wild-type Columbia plants, NPA significantly reduced both acropetal and basipetal movements of IAA. In *eir1*, there was no significant effect of NPA on basipetal IAA movement, suggesting that the levels of basipetal IAA movement in this mutant are not different from the background.

Acropetal transport of IAA and its regulation by NPA were similar in *eir1* and ecotype Columbia, which is consistent with the *eir1* mutation only altering basipetal IAA movement. The amount of basipetal IAA and BA movement into a 5-mm root tip segment is shown, although analysis of smaller segments yielded a similar trend in IAA movement. Examination of the movement of BA indicates that diffusion of this weak acid is not reduced in the mutant and that this BA diffusion is not affected by the addition of NPA in either wild-type or *eir1* plants. These transport results provide the first direct evidence that a mutation in the *EIR1* gene leads to a reduction in basipetal IAA transport. These results support the hypothesis that basipetal auxin transport controls root gravitropism in Arabidopsis.

DISCUSSION

Exciting genetic evidence has recently strengthened the link between auxin transport and the gravity response in Arabidopsis (Estelle, 1998; Jones, 1998; Chen et al., 1999), yet genetic analyses need to be combined with studies on the physiology of auxin transport to clarify this connection. The experiments in this study have focused on two goals. First, it was important to directly measure basipetal auxin

Table IV. *IAA transport and BA diffusion in ecotype Columbia and mutant eir1 roots*

The picomoles of IAA or BA transported were from at least two separate experiments and are the average and SD from between eight and 12 plants, accept for acropetal BA in *eir1*, which was obtained for 24 plants.

Transport Assay	Columbia			*eir1*			*P* Values[a]	
	−NPA	+NPA	*P* value[b]	−NPA	+NPA	*P* value[b]	−NPA	+NPA
Basipetal IAA	6.18 ± 0.46	4.02 ± 0.57	0.0042	4.40 ± 0.55	3.57 ± 0.53	0.15	0.012	0.29
Basipetal BA	0.43 ± 0.04	0.57 ± 0.06	0.051	0.61 ± 0.08	0.55 ± 0.09	0.63	0.049	0.85
Acropetal IAA	7.96 ± 0.74	4.68 ± 0.41	0.00037	7.39 ± 0.58	4.64 ± 0.50	0.00079	0.28	0.47
Acropetal BA	0.81 ± 0.06	0.81 ± 0.06	0.98	0.98 ± 0.10	0.79 ± 0.09	0.15	0.29	0.84

[a] *P* values (Columbia versus *eir1*) were obtained by Student's *t* test, as discussed in "Materials and Methods." [b] *P* values (−NPA versus +NPA) were obtained by Student's *t* test.

movement in Arabidopsis roots, and, second, it was necessary to show that this polarity of auxin movement controls root gravity response.

Although many investigators now accept the conclusion that there are two polarities of auxin transport in roots, this conclusion has been debated in the literature (Davies and Mitchell, 1972). Several investigators have measured basipetal auxin transport (from the root tip toward the base) in *Phaseolus coccineus*, *Vicia faba*, and maize (*Zea mays*) (Davies and Mitchell, 1972; Mitchell and Davies, 1975; Tsurumi and Ohwaki, 1978; Young and Evans, 1996), other investigators were not able to measure this polarity of auxin movement in maize (Scott and Wilkins, 1968). Our first goal was to confirm the presence of basipetal auxin transport in intact Arabidopsis roots and to determine if this transport is regulated by the potent and specific auxin transport inhibitor NPA (Muday et al., 1993).

To examine basipetal IAA movement in Arabidopsis, a new method had to be developed to apply the radiolabeled IAA. The traditional approach of placing root or shoot segments between two agar blocks was not feasible due to the size of Arabidopsis roots. Instead, agar containing [^{3}H]IAA was hardened, and cylinders of agar were formed and laid such that they just contacted the root tip. [^{3}H]IAA transport was examined as a function of the distance from the site of application. After 5 h there was little [^{3}H]IAA detectable beyond the first 6 mm of root. Even after 18 h of transport, little IAA was detected beyond the first 11 mm (data not shown). Additionally, the movement of a weak acid control, [^{14}C]BA, was also examined and was shown to be insensitive to NPA application. These results confirm the presence of a phytotropin-regulated efflux system that controls basipetal IAA transport in the root tip of Arabidopsis.

The second goal of this work was to determine whether the basipetal movement of auxin controls the gravity response. Growth of Arabidopsis roots on agar containing NPA inhibited the root gravity response, elongation, and root waving. The gravity response and waving were more sensitive to inhibition by NPA than elongation, with IC_{50} values that were at least 10-fold lower. Therefore, concentrations of NPA exist at which the gravity response and waving were almost completely inhibited, yet elongation was greater than 50% of the initial values. The ability of roots to form waves when placed on a hard agar surface oriented at an angle of less than 90° relative to the gravity vector has been suggested to be a gravity-driven response (Simmons et al., 1994). The ability of NPA to inhibit root waving at concentrations similar to those required to block gravitropism supports this model.

As NPA has been shown to alter the structure of the root, presumably by altering the normal distribution of IAA (Ruegger et al., 1997), it was important to confirm that the gravity response was inhibited prior to changes in the architecture of the root tip. Indeed, when the gravity response was examined using Multi-ADAPT software (Ishikawa and Evans, 1997), it was clear that the effect of NPA on inhibition of the gravity response is immediate, even under conditions where growth occurs normally. Similarly, basipetal IAA transport was inhibited after less than 5 h of exposure to NPA.

When NPA was applied to the entire root, it was not possible to determine which polarity of auxin transport is required for gravitropic bending and waving. To dissect this further, three approaches were used to separately block the two distinct polarities of auxin movement. First, auxin transport inhibitors were applied in a local fashion. Application of NPA to the root tip abolished the gravity response and reduced root growth. In contrast, inhibition of acropetal auxin movement by application of NPA to the root-shoot junction only affected the gravity response at very high concentrations. The NPA concentrations for inhibition of the gravity response were more than 30-fold higher when NPA was applied at the root-shoot junction than when it was applied at the root tip.

These results are consistent with either of two explanations. NPA applied at the root-shoot junction may reduce the gravity response by diffusing to the root tip when NPA is applied at high concentrations. Alternatively, if the original source of the basipetally transported auxin is the shoot and that shoot-derived auxin is redistributed at the root tip, high concentrations of NPA might deplete IAA from the shoot, reducing the gravity response. Experiments were performed to examine the diffusion of [^{3}H]NPA applied to the root-shoot junction. These experiments indicate that less than 0.01% of the applied NPA diffuses away from the site of application, and there was no detectable [^{3}H]NPA at the root tip, where the gravity response occurs (data not shown). These results suggest that diffusion does not account for the reduction in root gravitropism by application of high concentrations of NPA to the root shoot junction. Therefore, from this experiment alone, it is not possible to determine if only basipetal IAA transport is needed for the gravity response.

An alternative explanation for the inhibition of the gravity response by NPA application at the root tip is that the NPA is in direct contact with the gravity-responsive tissues and is not just blocking basipetal auxin movement to them. This possibility cannot be eliminated by our experiments using Arabidopsis roots, as the cells that undergo differential growth in response to gravity are so close to the tip. Therefore, similar localized NPA treatments were performed with maize roots, with NPA applied to the apical 1 mm, which is outside the maize distal elongation zone, the site of gravitropic growth (Ishikawa and Evans, 1993). Maize plants treated with control agar contacting only 1 mm at the root tip completely reoriented within 6 h after gravitropic stimulation, whereas plants treated with 10 μM NPA showed no gravity response, although they continued to grow (data not shown). Consequently, local NPA application to the tip of roots completely inhibited the gravity response in the same time frame, without direct contact with the tissues that show differential growth. Therefore, it may also be the case that inhibition of gravity response by application of NPA to the Arabidopsis root tip is caused by inhibition of auxin movement to the gravitropic regions, rather than a direct effect on the tissues that show differential growth.

To determine if root gravitropism can occur in the absence of auxin transported acropetally from the shoot, root tips were excised from the plant. When Arabidopsis root tips were excised and gravity stimulated, the excised root tips responded to gravity. The root tips were nearly identical to intact roots in their sensitivity to growth and gravity inhibition by NPA, which indicates that the redistribution of IAA is necessary for the gravitropic response in these root tips. This result also implies that the effect of NPA on the gravity response may only be at the root tip. Additionally, the ability of excised root tips to respond to gravity supports the hypothesis that the only polarity of auxin transport important for the root gravity response is basipetal auxin transport. These data also suggest that auxin from the shoot is not required for the gravity response, since roots grown for 4 d after excision to deplete endogenous IAA from the shoot still responded to gravity (data not shown). This result suggests that IAA synthesis would need to occur in roots, specifically at the root tip. It has been reported that isolated and sterile Arabidopsis roots can convert a heavy-isotope-labeled Trp into similarly labeled IAA (Muller et al., 1998b). Additionally, the same authors showed that GUS expression driven by a promoter from the gene encoding nitrilase, an IAA biosynthetic enzyme, is localized to the root tip. These results are consistent with auxin synthesis being located in the Arabidopsis root tip.

The third approach to link basipetal transport to the gravity response was to examine a mutant Arabidopsis plant that does not respond to gravity and has been suggested to contain a mutation in a gene encoding an auxin transport protein. The *agr1/eir1/pin2/wav6* mutation, which leads to agravitropic root growth, has recently been cloned and the *EIR1* gene has been suggested to encode an auxin efflux carrier (Chen et al., 1998; Luschnig et al., 1998; Muller et al., 1998a; Utsuno et al., 1998). Chen et al. (1999) measured auxin uptake into wild-type and *agr1* mutant root tip segments and found that more IAA was retained in the *agr1* mutant root tips. Their result is consistent with the absence or reduced activity of an auxin efflux carrier protein, although that assay could not differentiate the polarity of IAA transport that was altered in the mutant.

To determine whether the agravitropic phenotype of the *eir1* mutant was due to an alteration in basipetal auxin transport, [^{3}H]IAA transport was measured in *eir1* and compared with the amount of [^{3}H]IAA transport in wild-type plants of the Columbia ecotype. There was a statistically significant reduction in basipetal IAA transport in this agravitropic mutant, but no change in acropetal IAA transport nor its regulation by NPA. This difference was not due to tissue-level changes that could alter diffusion, as the levels of diffusion of [^{14}C]BA were not decreased in the mutant. The reduction in basipetal transport in this mutant provides physiological relevance to the observation that the expression pattern of the *PIN2/AGR1* mRNA (Chen et al., 1998; Muller et al., 1998a) and PIN2 protein (Muller et al., 1998a) are localized to the root tip. Additionally, immunoflourescence microscopy with an antibody raised against the PIN2 protein localizes to the apical membrane in root tip cells (Muller et al., 1998a), which is the appropriate distribution for this protein to control the basipetal movement of IAA.

These results also provide a more general test of the Cholodny-Went hypothesis, which suggests that auxin redistribution during the gravity response is required for the root gravity response. The simplest form of this hypothesis has been questioned (Trewavas, 1992; Ishikawa and Evans, 1993), since application of exogenous IAA does not alter the gravity response in roots of maize and tomato, respectively (Ishikawa and Evans, 1993; Muday and Haworth, 1994). However, the results of the present study and those from many other studies have shown that treatment with auxin transport inhibitors abolishes the gravity response, thereby supporting the Cholodny-Went hypothesis. Although the specificity of action of inhibitors should always be questioned, the ability of a wide range of structural classes of auxin transport inhibitors to simultaneously block the gravity response and auxin transport (Rubery, 1990) suggests that the these inhibitors abolish the gravity response by inhibition of auxin movement. Additionally, we demonstrate that a loss of the gravity response occurs by mutation of a single gene that is required for basipetal auxin transport. These results support the idea that redistribution of endogenous IAA is required for root gravitropism, although exogenous IAA application may have unexpected results, which reflect the complexity of this process.

In conclusion, these results show that IAA is basipetally transported in the root tips of Arabidopsis through a phytotropin-sensitive efflux carrier. Reduction in basipetal auxin transport by inhibitors or a genetic lesion in an IAA transport protein leads to a loss of the gravitropic response. In contrast, acropetal IAA transport in roots does not appear to be required for the gravity response. These results suggest that basipetal auxin transport controls the gravity response and that shoot-derived auxin is not needed. These results also suggest that auxin synthesized in the root tip (Muller et al., 1998b) may be sufficient to control the gravity response.

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