Transport of the Two Natural Auxins, Indole-3-Butyric Acid and Indole-3-Acetic Acid, in Arabidopsis[1]

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Polar transport of the natural auxin indole-3-acetic acid (IAA) is important in a number of plant developmental processes. However, few studies have investigated the polar transport of other endogenous auxins, such as indole-3-butyric acid (IBA), in Arabidopsis. This study details the similarities and differences between IBA and IAA transport in several tissues of Arabidopsis. In the inflorescence axis, no significant IBA movement was detected, whereas IAA is transported in a basipetal direction from the meristem tip. In young seedlings, both IBA and IAA were transported only in a basipetal direction in the hypocotyl. In roots, both auxins moved in two distinct polarities and in specific tissues. The kinetics of IBA and IAA transport appear similar, with transport rates of 8 to 10 mm per hour. In addition, IBA transport, like IAA transport, is saturable at high concentrations of auxin, suggesting that IBA transport is protein mediated. Interestingly, IAA efflux inhibitors and mutations in genes encoding putative IAA transport proteins reduce IAA transport but do not alter IBA movement, suggesting that different auxin transport protein complexes are likely to mediate IBA and IAA transport. Finally, the physiological effects of IBA and IAA on hypocotyl elongation under several light conditions were examined and analyzed in the context of the differences in IBA and IAA transport. Together, these results present a detailed picture of IBA transport and provide the basis for a better understanding of the transport of these two endogenous auxins.

Auxins are phytohormones involved in mediating a number of essential plant growth and developmental processes. The majority of the research conducted on endogenous auxin has focused on the primary free auxin in most plants, indole-3-acetic acid (IAA), yet there are other abundant auxins in plants. Indole-3-butyric acid (IBA) comprises approximately 25% to 30% of the total free auxin pool in Arabidopsis seedlings (Ludwig-Muller et al., 1993). Although there have been great advances in understanding the molecular mechanisms behind IAA action and transport (Muday and DeLong, 2001; Friml and Palme, 2002; Leyser, 2002), it is not yet clear whether IBA and IAA act and move by similar mechanisms.

In vivo studies on the function of IBA are rather limited (Ludwig-Muller, 2000; Bartel et al., 2001). IBA has been identified in a number of plant species from maize (*Zea mays*) and pea (*Pisum sativum*) to Arabidopsis, and concentrations of free IBA approach the levels of free IAA in a number of plants (Ludwig-Muller, 2000). IBA, like IAA, is also found in conjugated forms, yet at significantly lower levels than IAA (Ludwig-Muller et al., 1993). IBA and IAA can be interconverted (Bartel et al., 2001), which has led to the suggestion that IBA may act as a precursor to IAA. Arabidopsis mutants whose roots have reduced sensitivity to growth inhibition by IBA but normal sensitivity to IAA have been isolated recently (Bartel et al., 2001), and many of these have defects in beta-oxidation, which is the pathway by which IBA is thought to be converted to IAA (Zolman et al., 2001a, 2001b). These findings support a role for IBA as an IAA precursor.

Other lines of evidence suggest that IBA might also act directly as an auxin, rather than solely being an auxin precursor. First, IBA is the preferred auxin for the induction of root formation because it is much more potent than IAA or synthetic auxins (Ludwig-Muller, 2000). Several studies have demonstrated that internal IBA levels, not IAA levels, increase and stay elevated during IBA-induced root formation (Nordstrom et al., 1991; van der Krieken et al., 1992). Finally, the occurrence of several IBA resistant, IAA-sensitive mutants that do not have defects in beta-oxidation also suggest that IBA could act directly and not necessarily through conversion to IAA (Poupart and Waddell, 2000; Zolman et al., 2000).

To understand the endogenous role of IBA and the defects in these IBA-insensitive Arabidopsis mutants, it is necessary to examine how IBA is transported and the relationship between transport and action of IBA. This question has been examined using several approaches in plants other than Arabidopsis. Early

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studies relied on bioassays to detect auxin movement through tissues. Such studies report a polar movement of IBA in a basipetal direction in stems with similar or slower rates of movements to those of IAA (Went and White, 1938; Leopold and Lam, 1961). Interpretation of these studies is hampered by differences in sensitivity of bioassays to IBA and IAA (Thimann, 1952) and the effects of application of high auxin concentrations on auxin movement (Parker and Briggs, 1990). Further evidence for basipetal transport of IBA can be found in the work of Yang and Davies (1999). These authors showed that apically applied IBA can stimulate elongation of subtending nodes, suggesting IBA is transported basipetally in intact pea plants, but with slower kinetics than that of IAA (Yang and Davies, 1999). Additional studies have examined the distribution of radiolabeled IBA after application of a rooting solution to the base of explants. In most cases, however, these studies were not designed to distinguish between movement of auxin in the plant's vascular system and polar auxin transport (for example, see Epstein and Lavee, 1984; Wiesman et al., 1988; van der Krieken et al., 1992; Epstein and Ackerman, 1993). In one notable exception, IBA polar transport was examined in excised citrus leaf midribs and found to be twice as high in the basipetal direction as in the acropetal direction (Epstein and Sagee, 1992).

There is one report in the literature comparing IBA and IAA transport in the inflorescence axis of Arabidopsis ecotype Landsberg *erecta* (Ludwig-Muller et al., 1995). Using a nonstandard assay, transport of both IAA and IBA in the inflorescence axis was found to occur mostly in the acropetal direction but with some basipetal transport of both auxins (Ludwig-Muller et al., 1995). The authors of this study suggested this acropetal auxin movement could occur through the transpiration stream, possibly because of water loss at wound sites where leaves and siliques were removed from the axes (Ludwig-Muller et al., 1995). This is the only report in the literature in which there is more acropetal IAA movement than basipetal IAA movement in the Arabidopsis inflorescence axis in either Landsberg *erecta* or other ecotypes (Okada et al., 1991; Bennett et al., 1995), leaving it difficult to conclude whether the methods used in that report (Ludwig-Muller et al., 1995) were measuring cell-to-cell polar transport of either IAA or IBA in the Arabidopsis inflorescence axis. To the best of our knowledge, polar IBA transport in roots or hypocotyls of Arabidopsis and its regulation by auxin transport inhibitors, such as naphthyphthalamic acid (NPA), has not been examined. These are the tissues that have a clear IBA response and an altered sensitivity to IBA in mutant plants.

The major goals of this study were to gain a more detailed understanding of IBA transport in Arabidopsis and to use this information to clarify the role of this auxin in plant growth and development. Auxin transport was examined in several different tissues to determine where, and in which direction, IBA is transported. Furthermore, the rate and quantity of IBA transport was examined, as well as how this transport is affected by IAA efflux inhibitors and mutations in genes encoding putative IAA transport proteins. In addition, we have compared the effects of IAA and IBA on elongation of hypocotyls because differences in transport of the two auxins are detected in this tissue. Together, these results present a detailed picture of IBA transport with insights into its physiological role and provide the necessary background to interpret IBA mutant phenotypes.

RESULTS

Survey of IBA Transport in Several Arabidopsis Organs

IAA polar transport occurs in several distinct pathways in Arabidopsis. In the hypocotyl and inflorescence axis, IAA moves in a single direction from the apex to the base (basipetal transport). In the root, there are two distinct polar transport pathways. The first flows from the base of the root to the root tip (acropetal transport) and the second flows from the root tip back toward the base (basipetal transport).

To detect auxin movements in seedlings, tritiated auxin was applied from a 1-mm-diameter agar cylinder placed on top of roots or hypocotyls grown on agar and followed by measuring the amount of radioactivity that was taken up and transported into a distant tissue. Auxin transport was determined for several tissues, in which either ^{3}H-IAA or ^{3}H-IBA was applied continuously for the duration of the assay. For hypocotyls, radioactive auxin was applied below the cotyledons to examine basipetal transport toward the hypocotyl base. For acropetal transport in the hypocotyl, radioactive auxin was applied at the root shoot junction, and its movement to the hypocotyl apex was quantified. Radioactive auxin was applied at the root tip to examine root basipetal transport (RBT) within the first 5 to 10 mm of the root. For root acropetal transports (RATs), tritiated auxin was applied at the root shoot junction, and transport of auxin to the root tip was measured.

Measurements of both ^{3}H-IAA and ^{3}H-IBA movements in Arabidopsis tissues are reported in Table I and Figure 1A. IBA, like IAA, is transported in the root in both polarities and in the basipetal direction in the hypocotyl. In both roots and hypocotyls, IBA is transported at greater levels than is IAA. The elevated movement of IBA could be due to either greater IBA uptake or elevated polar IBA transport, but we did not differentiate between these two possibilities.

In contrast, there was no detectable IBA transport above background levels in the inflorescence stem at 18, 24, or 36 h after application in either a basipetal or an acropetal manner (Table I; data not shown). To determine if low levels of IBA transport could be

Table I. *Transport of IBA and IAA in Arabidopsis seedlings*

Transport Assay	Control	NPA[a]
	pmoles transported[b]	
Root acropetal		
IAA	4.5 ± 0.4	3.3 ± 0.2[c]
IBA	11.4 ± 1.4	11.8 ± 1.4
Root basipetal		
IAA	2.7 ± 0.2	1.6 ± 0.1[c]
IBA	10.2 ± 0.7	10.6 ± 0.6
Inflorescence basipetal		
IAA	1.4 ± 0.1	0.04 ± 0.02[c]
IBA	0.03 ± 0.005	0.02 ± 0.002

[a] For root transport assays, NPA concentration was 100 μM and for inflorescence transport assays NPA concentration was 15 μM. [b] Average and SE of 14 to 86 seedlings from two to seven experiments. [c] $P < 0.05$ as determined by one-tailed Student's *t* test for control vs. NPA. For all IBA transport experiments, the control vs. NPA treatments had $P > 0.05$.

detected in the inflorescence axis, an additional pulse-chase assay was used because this assay can identify small local amounts of auxin movement. ^{3}H-IAA was transported in a single wave, and the transport was completely inhibited by addition of the transport inhibitor NPA, whereas no detectable ^{3}H-IBA transport was observed in the presence or absence of NPA (Fig. 2). These results suggest that IBA is not transported in the Arabidopsis inflorescence axis, although IBA transport is readily measurable in the hypocotyl and root.

Polarity of IBA Transport in Roots and Hypocotyls

To identify the polarity of auxin transport in the hypocotyl, a comparison of ^{3}H-IAA or ^{3}H-IBA movement after application at the hypocotyl base and apex was performed (Fig. 1A). Both IBA and IAA are transported basipetally from the tip to the base of the hypocotyl, whereas neither IBA nor IAA is transported acropetally at levels above background.

IAA is transported basipetally in the first 5 to 7 mm of the Arabidopsis root tip, whereas acropetal IAA transport occurs along the whole length of the root (Rashotte et al., 2000). To determine if IBA is transported similarly in roots, ^{3}H-IAA or ^{3}H-IBA was applied mid-root in an agar cylinder 10 mm back from the root tip, and the amount of transport was measured in both directions (Fig. 1B). Because this site of auxin application is behind the zone of basipetal auxin transport, IAA and IBA movement is predominantly in the acropetal direction.

An additional assay was performed that determined how far IBA traveled from the tip. Labeled auxin was applied to the root tip, and after 5 h, the radioactivity in several 2-mm segments from the root tip back toward the base was quantified (Fig. 3A). These results indicate that root basipetal auxin transport occurs over the same distance for both IBA and IAA and is confined to the apical 7 mm of the root tip.

Rates of IBA Transport

To compare the rates of IAA versus IBA movement, a pulse-chase method for measuring the rate of root acropetal auxin transport was developed. It is easier to measure the rate of acropetal transport than basi-

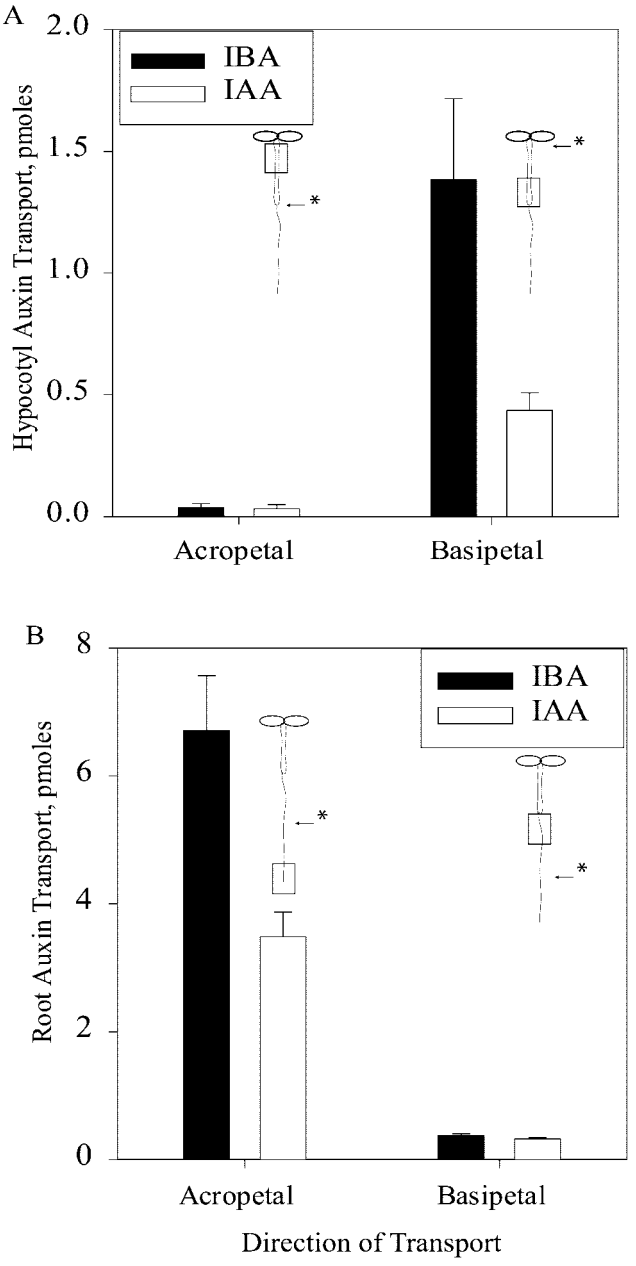


Figure 1. Polarity of IBA and IAA transport. A, Direction of IAA and IBA movement in hypocotyls was examined by applying ^{3}H-IBA or ^{3}H-IAA at the base or the tip of the hypocotyl and measuring radioactivity in distant 5-mm segments, as shown in inset (±SE; $n = 30$). B, Acropetal and basipetal movement of IBA and IAA in roots was examined by applying ^{3}H-IBA or ^{3}H-IAA at mid-root and measuring radioactivity in distant 3-mm segments, as shown in inset (±SE; $n = 10$). For the inset diagrams, the arrow and asterisk indicate the site of ^{3}H-IBA or ^{3}H-IAA application, and the boxes indicate the segments in which radioactivity was measured.

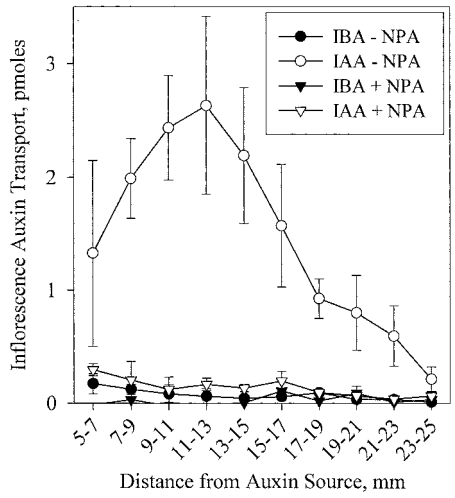


Figure 2. Inflorescence axis transport of IBA and IAA. IBA and IAA basipetal transport was examined in 25-mm inflorescence axis segments. The apical end of each segment was placed in ^{3}H-IBA or ^{3}H-IAA for 10 min, followed by a 90-min chase of the respective unlabeled auxin, either with or without NPA. The radioactivity in 2-mm segments was determined (±SE; $n = 3$).

petal transport because it occurs over a longer distance and with higher amounts of auxin movement. Using this assay, it was possible to identify the leading edge of auxin movement to estimate the rates of auxin movements (Fig. 3B). In 1 h, both auxins are transported the same distance to the segment 10 mm from the site of auxin application. Radioactivity levels in segments that are 12 mm or further from the site of labeled auxin application are at background levels. This experiment shows that IBA and IAA are transported at the same rate of 8 to 10 mm per hour.

Regulation of Auxin Transport by IAA Efflux Inhibitors

Polar IAA transport is reduced by inhibition of IAA efflux using inhibitors such as NPA and 2,3,5-triiodobenzoic acid (TIBA). These two inhibitors block efflux by binding to two different sites on the auxin efflux carrier complex, either a regulatory subunit or to the auxin-binding site, respectively (Rubery, 1990; Muday and DeLong, 2001). The mechanism of action of these compounds is not completely clear, but they may either directly block auxin movements or indirectly alter the cycling of auxin transport proteins to or from the plasma membrane (Muday and DeLong, 2001). The effect of NPA on IAA and IBA transport in roots and hypocotyls was determined (Tables I and II). There was no effect on the transport of IBA in any of these tissues, even with concentrations of NPA as high as 100 μM, which significantly reduce IAA transport. The ability of TIBA to block root acropetal IAA and IBA transport was also tested, and a concentration of 100 μM significantly reduced IAA movement (3.3 ± 0.2 pmol) as compared with untreated controls (4.5 ± 0.5 pmol), but did not significantly affect IBA movement (12.2 ± 1.4 pmol) as compared with controls (11.4 ± 1.4 pmol). These results indicate that IBA transport is not regulated by IAA efflux inhibitors and suggest that the inhibitor-sensitive auxin efflux carrier protein complexes that transport IAA differ from the protein complexes that transport IBA.

IBA Transport in Arabidopsis Mutants with Defects in Auxin Transport

To test the hypothesis that IBA is transported by different transport proteins than IAA, measurements of IBA and IAA transport were made in the *aux1* and *eir1* mutants, which have defects in genes predicted to encode IAA influx and efflux carriers, respectively (Parry et al., 2001b; Friml and Palme, 2002). *aux1* and *eir1* have been shown previously to have significant reductions in IAA accumulation and basipetal IAA transport in the root (Chen et al., 1998; Marchant et al., 1999; Rashotte et al., 2000, 2001). Levels of IBA and IAA transport in the roots of *aux1-7* and *eir1-1*

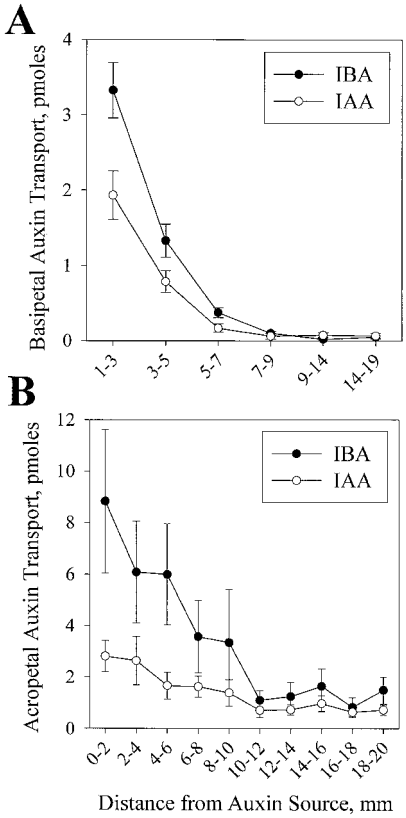


Figure 3. Distance and rate of IAA and IBA transport. A, Distance of IBA and IAA basipetal transport from the root tip was examined by applying ^{3}H-IBA or ^{3}H-IAA at the root tip and measuring radioactivity in either 2- or 5-mm segments spanning the indicated distance from the root tip. B, Rate of IBA and IAA acropetal transport was examined by applying ^{3}H-IBA or ^{3}H-IAA 20 mm from the root tip and measuring radioactivity in 2-mm segments at the indicated distance from the site of application (±SE; $n = 10$).

Table II. *Effect of light on hypocotyl basipetal transport of IBA and IAA*

Auxin Transported	Low Light		Darkness	
	Control	+NPA	Control	+NPA
	pmoles transported[a]			
IAA	1.4 ± 0.2	0.54 ± 0.09[b]	0.52 ± 0.04[b]	0.43 ± 0.06[b]
IBA	2.3 ± 0.3	2.2 ± 0.4	1.2 ± 0.1[b]	ND[c]

[a] Average and SE of 20 to 30 seedlings from two to three experiments. [b] Indicates values that were significantly different from low-light controls with $P < 0.001$ as determined by one-tailed Student's *t* test for control vs. 10^{-4} M NPA and by two-tailed Student's *t* test for low light vs. darkness. IAA transport in darkness was not significantly different in the presence and absence of NPA with a *P* value > 0.05. [c] N.D., Not determined

and in the wild-type Columbia background are shown in Table III. Basipetal IAA transport is significantly reduced in *aux1-7* and *eir1-1*, and acropetal IAA transport is reduced in *aux1-7*. We have found previously that acropetal IAA transport is unaffected by the *eir1-1* mutation (Rashotte et al., 2000). Basipetal IBA transport is similar to wild type in both *aux1-7* and *eir1-1*, and acropetal IBA transport is similar to wild type in *aux1-7*. These results suggest that IBA transport does not require the activity of either the EIR1 or AUX1 proteins, whereas IAA transport requires both of these proteins. Differences in values for basipetal transport in Table III, as compared with other values, reflect ecotype differences in transport and refinements in technique during the course of this work.

Examination of Transport Saturation

If IBA transport is protein mediated, then it should saturate at high auxin concentrations. Increasing concentrations of unlabeled IBA or IAA were added to an agar cylinder containing a constant level of radioactive auxin in an RBT assay (Fig. 4). Transport of IAA and IBA are both saturated at similar high concentrations of IAA and IBA, suggesting that IBA transport, like IAA transport, is carrier mediated.

Table III. *Transport of IBA and IAA in auxin transport mutants*

Transport	Columbia	*aux1-7*	*eir1-1*
	pmoles transported[a]		
Root basipetal			
IAA	1.1 ± 0.1	0.6 ± 0.03***	0.8 ± 0.05***
IBA	1.7 ± 0.08	1.7 ± 0.1	1.8 ± 0.1
Root acropetal			
IAA	3.7 ± 0.4	2.7 ± 0.2**	N.D.[b]
IBA	5.6 ± 0.6	5.6 ± 0.6	N.D.[b]

[a] Average and SE of 23 to 30 seedlings from three experiments. The *P* values were obtained by two-tailed Student's *t* test for Columbia vs. mutant plants. **, $P < 0.01$; ***, $P < 0.001$. No significant differences in IBA transport were detected for these mutants. [b] N.D., Not determined.

Relationship between Physiological Effects of IBA and Transport

Previous studies have examined the IBA and IAA sensitivity of Arabidopsis developmental processes, such as root elongation and lateral root formation (Poupart and Waddell, 2000; Zolman et al., 2000), but have not examined the response of hypocotyls to these two auxins. We examined the sensitivity of hypocotyl elongation to IBA and IAA using hypocotyls grown in dark, low, or high light. Figure 5 shows that IBA, but not IAA, is able to stimulate hypocotyl elongation significantly in high-light conditions, at concentrations ranging from 1 to 10 μM, with a maximum of about 50% stimulation at a concentration of 3 μM IBA. In all light conditions, hypocotyl elongation is more sensitive to inhibition by exogenous IAA than to IBA. This can be seen in dark and low-light conditions at concentrations ranging from 0.3 to 3 μM and in high-light conditions at concentrations ranging from 10 to 100 μM. These experiments also show that about 30-fold higher concentrations of auxin are required to inhibit hypocotyl elongation in high-light conditions relative to low-light or dark conditions.

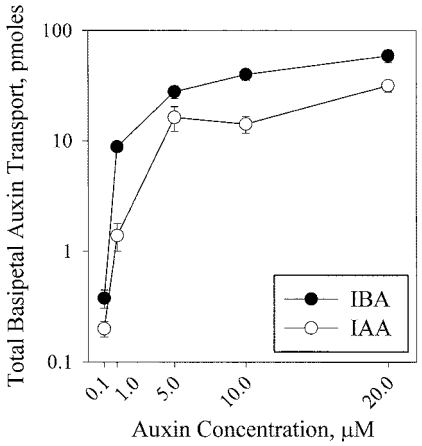
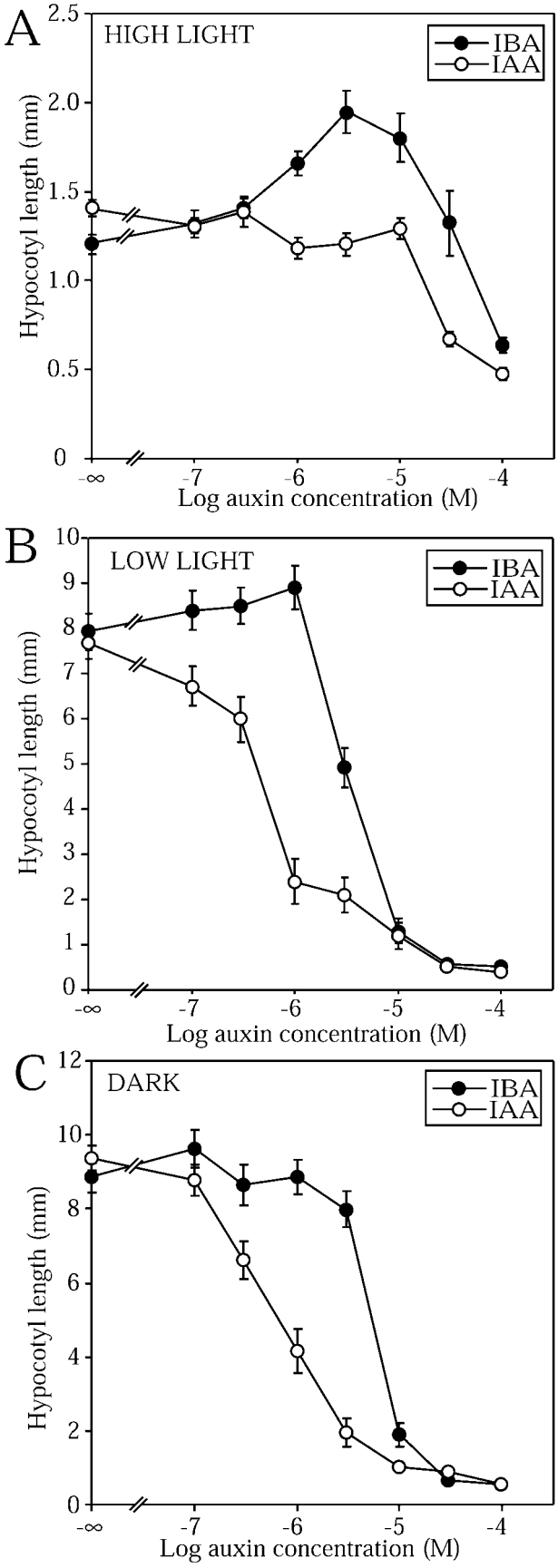


Figure 4. Saturation of auxin transport. The ability of IBA and IAA to saturate RBT was measured as a function of the movement of a constant amount of ^{3}H-IBA or ^{3}H-IAA with increasing unlabeled amounts of the respective auxin in distant 5-mm segments (±SE; $n = 10$).



Consistent with the differences between IBA and IAA on growth in hypocotyls under different light conditions are differences in hypocotyl IBA and IAA transport under similar conditions. The amount of IBA and IAA transport in hypocotyls under low light and in the dark are shown in Table II. In low light, there is more IBA transport than IAA transport, and only IAA transport is NPA sensitive. In the dark, both IAA and IBA transport are reduced about 2-fold (Table II), and the IAA transport is no longer sensitive to NPA.

DISCUSSION

The major goal of this work was to determine if the natural auxin, IBA, is transported in Arabidopsis with similar polarity, rate, and regulatory properties as is IAA. The effects of IAA and IBA on hypocotyl elongation were also examined to explore the relationship between polar transport and action of these two natural auxins. Polar transport of IBA was found to occur in hypocotyls and roots of Arabidopsis seedlings. IBA transport in hypocotyls occurred in a basipetal direction, with no detectable acropetal movement. In the roots, IBA transport occurred acropetally from the root shoot junction to the root tip at a rate of 8 to 10 mm h^{-1} and basipetally for a short distance back from the root tip. These results indicate that IBA transport mirrors the directional transport of IAA found in Arabidopsis seedlings (Rashotte et al., 2000, 2001), which was first demonstrated over 30 years ago in *Phaseolus coccineus* (Davies and Mitchell, 1972).

In stark contrast to the results with hypocotyls and roots, no IBA transport was detected in the inflorescence axis of Arabidopsis. Numerous experiments were performed to try to detect IBA transport in this tissue because there is one previous report of IBA movement in the inflorescence axis of the Landsberg *erecta* ecotype (Ludwig-Muller et al., 1995). In that paper, IAA and IBA were reported to have a predominantly acropetal movement in the inflorescence axis but were also found to move in a basipetal direction with both auxins transported at similar rates (Ludwig-Muller et al., 1995). These results conflict with other published papers on IAA movement in the inflorescence. Other studies have shown that IAA moves solely in the basipetal direction in the inflorescence and have confirmed that this movement is polar transport by using efflux inhibitors (Okada et al., 1991; Bennett et al., 1995). The acropetal transport observed by Ludwig-Muller et al. (1995) was suggested to be movement in the transpiration stream, and it is unclear whether the basipetal auxin

Figure 5. Effect of auxins on hypocotyl elongation. Dose response curves for hypocotyl elongation in response to 5 d on IAA or IBA under high white light (90 μmol m^{-2} s^{-1}; A), low light (5 μmol m^{-2} s^{-1}; B), or dark (C) conditions are reported (±SE; n = 6–26).

movements reported in that work were truly polar transport.

To look for IBA transport in our system, we performed several assays for time periods spanning from 5 to 36 h, with a range of IBA concentrations, and with tissue segments from different positions in the inflorescence axis from the Nossen ecotype and from plants of different ages. IBA transport was not detected in any of these experiments (J. Poupart and C.S. Waddell, data not shown). The pulse-chase experiment reported here examined IBA and IAA movement in the inflorescence axis by dividing this tissue into a number of small segments. Even when IBA transport was examined within 5 mm from the site of IBA application, no ^{3}H-IBA was detected above background levels. It remains a formal possibility that ^{3}H-IBA moves differently in the inflorescence of Landsberg *erecta*, which we did not specifically test in our experiments, although no ecotypic differences in inflorescence auxin transport have been reported previously.

To determine if the same auxin carrier protein complexes might mediate both IAA and IBA transport, auxin transport was examined in plants with mutations in genes believed to encode IAA transport proteins and in the presence of IAA efflux inhibitors. IBA and IAA transport were measured in roots of *eir1-1* and *aux1-7*, plants with mutations in genes predicted to encode IAA efflux and influx proteins, respectively (Parry et al., 2001b; Friml and Palme, 2002). There was no reduction or alteration in the transport of IBA in either the *eir1-1* or *aux1-7* mutant background, whereas basipetal IAA transport was significantly reduced in both mutants, and acropetal IAA transport was reduced in *aux1-7*. These results suggest that IBA is not transported by proteins encoded by the allelic *AGR1/EIR1/PIN2/WAV6* gene or the *AUX1* gene. Because the influx and efflux proteins are members of large gene families (Parry et al., 2001b; Friml and Palme, 2002), it is possible that other members of these gene families mediate IBA transport. Alternatively, IBA transport might be mediated by other proteins such as the AtMDR and AtPGP proteins, which have been implicated recently in IAA transport (Noh et al., 2001).

In experiments using the IAA transport inhibitors NPA and TIBA, concentrations as high as 100 μM had no effect on IBA transport in any tissues examined, whereas there was a significant reduction in IAA transport in the same tissues. This result suggests that IAA efflux carrier protein complexes sensitive to these inhibitors are unlikely to transport IBA. In the root and hypocotyl, in contrast to the inflorescence axis, polar IAA transport is not completely inhibited by these IAA efflux inhibitors. The residual level of IAA transport in these tissues may be mediated by auxin efflux carrier complexes that are insensitive to inhibitors, and these complexes may also mediate transport of IBA. Two additional lines of experimentation support this hypothesis. In roots of the *eir1-1* mutant, basipetal IAA transport is reduced, and the remaining transport of IAA is insensitive to NPA (Rashotte et al., 2000), consistent with the presence of a remaining NPA-insensitive carrier. This putative NPA-insensitive efflux carrier complex does not appear to act in the inflorescence axis; in this tissue, NPA treatment results in the almost complete inhibition of IAA transport. The absence of this putative NPA-insensitive efflux carrier complex in the inflorescence correlates with the absence of IBA transport.

One formal possibility to explain the lack of effect of IAA efflux inhibitors and mutations in putative IAA transport proteins is that IBA transport is not protein mediated. However, the strict polarity and tissue specificity of IBA movement argue against this possibility. Ludwig-Muller (1995) reported that IBA uptake is saturable for young Arabidopsis seedlings grown in culture. We also asked whether IBA movement is saturable in our transport assays. ^{3}H-IBA and ^{3}H-IAA movement were examined in the presence of increasing amounts of unlabeled IBA or IAA, respectively. The transport of IBA and IAA saturated at high concentrations, in a manner consistent with IBA and IAA transport being protein mediated. This saturation result, combined with the absence of IBA movement in the inflorescence axis and the strict directionality of IBA movement in other tissues, suggest that IBA transport is protein mediated but by proteins with different tissue specificity and regulatory properties than IAA carriers.

The levels of radioactive IBA transported in roots and hypocotyls are in general 2 to 4 times greater than those of IAA. The higher amounts of IBA transport could be due to either a greater uptake of IBA or more transport of IBA after it has been taken up. It is difficult to experimentally resolve these possibilities because we measured the radioactive auxin at a distance and did not measure the radioactivity at the site of application in these tissues. In contrast, in experiments with the inflorescence axis, IBA and IAA uptake into the first segment was compared. Even though IBA is not transported in this tissue, higher levels of tritiated IBA were found in the segment in direct contact with the solution containing radioactive auxin (data not shown). This suggests higher IBA uptake, although it could also reflect the absence of movement of IBA out of this tissue. The pulse-chase experiment in Arabidopsis roots, shown in Figure 3B, also suggests that IBA uptake is higher than IAA uptake. However, the higher amounts of IBA may result from an increased capacity for IBA transport. Without a compound that can effectively inhibit the transport of both IAA and IBA, we are unable to distinguish between higher uptake and higher transport capacity. Therefore, we are unable to resolve whether there is a greater total flux of IBA movement or whether the initial uptake of IBA is greater than

for IAA and the subsequent higher levels of transport represent a larger initial pool for transport.

We considered the possibility that applied IBA was converted to IAA before transport. The radiolabeled versions of IAA and IBA used in our experiments are labeled on the indole ring; therefore, interconversion of these auxins will not affect the associated radioactivity. Several lines of evidence argue against this possibility. First, the complete absence of IBA transport in the inflorescence axis is inconsistent with conversion of IBA into IAA, at least in this tissue, because some transport should be detectable if IBA is converted to IAA. In both roots and hypocotyls, IAA transport is inhibited by the efflux inhibitors, NPA and TIBA. The complete absence of inhibition of polar IBA transport by these inhibitors suggests that significant quantities of ^{3}H-IBA are not converted to ^{3}H-IAA during these assays either. Finally, we failed to detect conversion of IBA to IAA in seedlings. Experiments were performed in which Arabidopsis seedlings were incubated with ^{3}H-IBA for time periods from 5 min to 24 h, and the labeled metabolites were extracted and then separated by thin-layer chromatography (TLC; for details, see "Materials and Methods"). No free IAA was detected in any of the assays. We estimate that free IAA must be present in levels equal to 5% to 10% of IBA levels to be detected in these assays.

The phenotypes of a number of mutants need to be examined in the context of these results. Analysis of the auxin resistant *axr* and *aux* mutants of Arabidopsis, which are resistant to root growth inhibition by IAA, has helped dissect the mode of action and transport of this auxin in plants (Leyser, 1997; Muday, 2001; Kepinski and Leyser, 2002). By analogy, mutants specifically resistant to IBA, but retaining wild-type sensitivity to IAA also have been identified and analyzed to help dissect the role of IBA in plant growth and development (Poupart and Waddell, 2000; Zolman et al., 2000). A subset of these mutants are resistant to the synthetic auxin 2,4-dichlorophenoxyacetic acid and to the auxin transport inhibitors NPA, TIBA, and 9-hydroxyfluorene-9-carboxylic acid. It is difficult to reconcile the inhibitor resistance phenotype of these mutants with the fact that IBA transport is not sensitive to such inhibitors. Many mutants that are insensitive to IAA exhibit reduced sensitivity to auxin transport inhibitors (Muday et al., 1995). The IBA-insensitive mutants are normally sensitive to IAA; therefore, in response to NPA treatment, local IAA accumulation at the root tip may cause an increase in IAA conversion to IBA, to which the roots are insensitive. The local accumulation of IAA after such IAA efflux inhibitor treatments has been reported (Casimiro et al., 2001), although the accumulation of IBA in response to elevated IAA levels under these conditions has not been investigated.

Previous reports suggest that IBA is not a substrate for the EIR1 protein because differential root growth in the *eir1* mutant can be stimulated by IAA but not by IBA (Poupart and Waddell, 2000; Zolman et al., 2000). This result is consistent with the transport experiments reported here. A similar conclusion for the role of AUX1 in mediating IBA transport is not as clear. In this study, we find no role for AUX1 in mediating IBA transport, yet two previous lines of experimentation have suggested IAA and IBA uptake may occur by similar mechanisms. The first is the ability of excess IAA to prevent labeled IBA uptake (Ludwig-Muller et al., 1995). These results can be reconciled by the hypothesis that IAA, but not IBA, is transported into the cell through the AUX1 carrier, whereas both IAA and IBA are transported into the cell through an alternative influx carrier, which is the only mode for IBA entry into the cell. If this was the case, then excess IAA would compete with labeled IBA uptake to the cell as reported (Ludwig-Muller et al., 1995). The second line of experimental evidence supporting a role for AUX1 mediation of IBA influx is the report that the *aux1-7* mutant is reduced in root growth inhibition by IBA (Zolman et al., 2000). However, the reduced growth inhibition is modest; *aux1-7* root growth is reduced 35% in the presence of IBA relative to untreated plants as compared with approximately 55% growth inhibition for wild-type plants. Furthermore, the *aux1-7* mutant responds in a wild-type manner to IBA induction of lateral roots. The reduced sensitivity of *aux1-7* to growth inhibition by IBA, therefore, may be the indirect result of excess conversion of IBA to IAA that then affects root growth (Bartel et al., 2001). Overall, these data suggest that if *AUX1* plays a role in IBA transport, it is an indirect one.

The tissue specificity of IBA transport reported here supports the possibility that this endogenous auxin plays a role in growth and development of some Arabidopsis tissues. Several previous studies have shown that IBA, like IAA, inhibits root elongation and induces lateral root formation (Poupart and Waddell, 2000; Zolman et al., 2001b). IBA affects stem elongation in pea seedlings (Yang and Davies, 1999), but its effect on Arabidopsis hypocotyl elongation has not been examined previously. Therefore, we examined the growth sensitivity of Arabidopsis hypocotyls to IAA and IBA. Hypocotyls were sensitive to growth stimulation by low concentrations of IBA in high-light conditions, but insensitive to growth stimulation if grown in low light or dark. In contrast, hypocotyls were insensitive to growth stimulation by IAA at any concentration tested or under any light condition. Interestingly, in pea epicotyls, both auxins can stimulate growth at low concentrations, and the growth-promoting effect moves in a basipetal polarity, consistent with the data reported here for IBA and IAA polar transport in hypocotyls (Yang and Davies, 1999). Both dark- and light-grown Arabidop-

sis hypocotyls were sensitive to growth inhibition by both auxins but were more sensitive to IAA in this assay.

The amounts of auxin transport in the hypocotyl change between low-light and dark conditions. In the dark, IAA transport and IBA transport are both reduced relative to low-light-grown hypocotyls, and IAA transport is no longer NPA insensitive, suggesting that similar mechanisms may control both IBA and IAA transport in the dark. Previous reports on hypocotyl growth are consistent with auxin transport in the dark being mediated by an IAA efflux carrier inhibitor-insensitive mechanism (Jensen et al., 1998). The interactions between light and auxin signaling are only now becoming apparent (Tian and Reed, 2001; Swarup et al., 2002). There is a complex interaction between light and auxin levels, transport, and hypocotyl elongation. Low concentrations of exogenous IBA stimulate hypocotyl elongation only in light-grown seedlings. Transgenic or mutant Arabidopsis plants with altered IAA levels show altered hypocotyl growth only in light-grown seedlings (Boerjan et al., 1995; Romano et al., 1995). These results suggest that light-grown hypocotyls are more sensitive to growth stimulation by auxin. There are much lower levels of IAA in dark-grown hypocotyls and roots as compared with light-grown plants (Bhalerao et al., 2002), indicating that light also controls the level of auxin synthesis. Finally, light-grown but not dark-grown hypocotyls show growth inhibition by NPA (Jensen et al., 1998) and NPA regulation of IAA transport (this report). Therefore, light influences auxin synthesis, transport, and response.

The physiological significance of the absence of IBA transport in the inflorescence axis should also be considered. There are no reported measurements of IBA levels in the inflorescence, although this tissue is an abundant source of IAA (Brown et al., 2001) and is a site of conversion between IAA and IBA (Ludwig-Muller and Epstein, 1994). In addition, the mutants that have been isolated with altered IBA sensitivity largely have no apparent inflorescence phenotypes (Bartel et al., 2001). Two exceptions to this statement are the *pxa1* and *aim1* mutants, which have defects in fatty acid mobilization (Richmond and Bleecker, 1999; Zolman et al., 2001b). The inflorescences of *pxa1*, like all parts of this plant, are reduced in size, perhaps as a side effect of the fatty acid utilization, not IBA insensitivity (Zolman et al., 2001b). The inflorescence defects are much more striking in the *aim1* mutant, which has a defect in beta-oxidation of both lipids and auxins. This mutant is resistant to root growth inhibition by IBA (Zolman et al., 2000), so the inflorescence defects could be a result of altered IBA metabolism or altered lipid metabolism, although this has not been experimentally tested (Richmond and Bleecker, 1999). Given our finding that IBA is not transported in the inflorescence axis, any direct role that IBA has in the phenotype of these mutants must occur through local synthesis.

In summary, our study has revealed the basic outline of IBA transport within Arabidopsis, in terms of polarity, tissue specificity, distance, and rate. Our results suggest that different IAA efflux carrier protein complexes may mediate IAA and IBA transport. The best characterized IAA transport proteins, AUX1 and EIR1, do not have a role in IBA transport. Several of our results suggest the presence of an uncharacterized auxin efflux carrier complex, which is insensitive to NPA and transports both IAA and IBA. This study provides the groundwork necessary for understanding the differences and similarities between polar transport of IAA and IBA; this, in turn, will be critical for understanding the role of IBA in plant growth and development and in characterization of the recently isolated mutants with altered sensitivity to IBA.

MATERIALS AND METHODS

Chemicals

Chemicals were purchased from the following suppliers: NPA from Chemical Services (West Chester, PA), absolute ethanol from McCormick Distilling Co., Inc. (Weston, MO), and 3-[5(n)-^{3}H]-IAA (27 and 25 Ci mmol^{-1}) from Amersham (Arlington Heights, IL). 3-[^{3}H(G)]-IBA (25 Ci mmol^{-1}) was prepared in a custom synthesis under conditions designed to label the indole ring by American Radiolabeled Chemicals (St. Louis). All other chemicals were obtained from Sigma (St. Louis).

Seed Germination and Plant Growth

Wild-type Arabidopsis seeds (ecotype Columbia) and *aux1-7* and *eir1-1* seeds were obtained from the Arabidopsis Biological Resource Center (Ohio State, Columbus). All experiments were performed with ecotype Nossen-0, except where indicated. Seeds were soaked in distilled water for 30 min and surface sterilized with 95% (v/v) ethanol for 5 min and 20% (v/v) bleach with 0.01% (v/v) Triton X-100 for 5 min. After five washes in sterile distilled water, seeds were germinated and grown on 9-cm petri plates containing sterile control medium containing 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, and 0.5 μg mL^{-1} nicotinic acid. Seeds were grown in vertically oriented petri dishes in continuous 90 μmol m^{-2} s^{-1} fluorescent light at room temperature (22°C) for root auxin transport experiments. Seedlings used in hypocotyl assays were grown in horizontally oriented petri dishes at room temperature (22°C) but exposed to only 5 μmol m^{-2} s^{-1} of constant fluorescent light to increase hypocotyl length. Plants for continuous pulse inflorescence axis assays were grown on a 1:1:1 (w/w) mixture of perlite:vermiculite:Sunshine mix number 1 (Sun Gro Horticulture Inc., Bellevue WA). Plants were grown at 24°C under continuous white fluorescent light and fertilized twice during their growth period with 0.25× Hoagland solution. Light intensity was approximately 90 μmol m^{-2} s^{-1}. Plants grown for pulse-chase inflorescence axis assays were grown in metro mix 220 soil at room temperature (22°C) at 90 μmol m^{-2} s^{-1} fluorescent light on a 16-h-light: 8-h-dark cycle for 25 d.

Inflorescence Auxin Transport Assays

Inflorescence axis transport measurements were conducted on 25-d-old plants using a continuous pulse of radioactive auxin as described previously (Okada et al., 1991; Brown et al., 2001). In this assay, 100 nM ^{3}H-IAA or ^{3}H-IBA were applied to a 2.0-cm inflorescence axis segment in the presence or absence of 15 μM NPA, and transport into the basal 5 mm of that segment was measured after 18 h. Equivalent amounts of solvent only (dimethyl sulfoxide [DMSO]) were added to assays without NPA. Each segment was

placed into 2.5 mL of scintillation fluid overnight, and the amount of radioactivity within each sample was determined using a Beckman LS6500 scintillation counter (Beckman Instruments, Fullerton, CA) for 2 min.

Inflorescence axis transport was also measured using a pulse-chase experiment (modified from Parry et al., 2001a). This procedure is similar to the continuous pulse experiment above except that 400 nM ^{3}H-IAA or ^{3}H-IBA was applied to a 2.5-cm inflorescence axis segment for 10 min, then briefly rinsed and placed in a solution of nonradioactive auxin of similar concentration for 90 min. Higher concentrations of radioactive auxins were used in the pulse chase experiments because plants are in contact with radioactivity for very short periods, and higher levels of radioactivity are necessary to get sufficient counts in the segments. Transport was measured 100 min after the experiment started in the basal-most 10 2-mm segments as above.

Hypocotyl Transport Assay

Hypocotyl transport measurements were obtained for 5-d-old seedlings grown under low light or in the dark. Seedlings were transferred to control plates and oriented vertically along the surface of the agar. In experiments to examine hypocotyl basipetal transport, seedlings were aligned by their shoot apical meristems, and cotyledons were excised immediately preceding the experiment, leaving approximately 10 mm of hypocotyl. In experiments to examine acropetal transport, the root shoot junctions were aligned, and no tissues were removed. There was very little growth in a 5-h experiment. In these assays, mixtures containing 1% (w/v) agar, 100 nM ^{3}H-IAA, or ^{3}H-IBA with either 100 μM NPA or DMSO at the same concentration (1% [v/v]) were prepared in 3-mL scintillation vials. A narrow stem transfer pipette was carefully inserted into the hardened agar mixture such that a long 1-mm diameter cylinder of agar was removed. This cylinder containing radioactive auxin mixture was applied such that the agar was in contact with the cut surface of the hypocotyl for hypocotyl basipetal transport and on top of the seedling, just above the root shoot junction for hypocotyl acropetal transport. Plates remained vertically oriented in the dark to avoid auxin degradation (Stasinopoulos and Hangarter, 1989). Radioactive auxin transport was measured after 5 h by scintillation counting of a 5-mm segment of hypocotyl from the opposite end of the hypocotyl. The distance the auxin was transported was approximately 10 mm for dark- and low-light-grown seedlings from the cylinder of applied radioactive auxin. For experiments with dark-grown hypocotyls, all manipulations were performed with the aid of a green safelight.

Root Transport Assays

Basic root auxin transport measurements were made on 6- or 7-d-old vertically grown seedlings as in Rashotte et al. (2001), which is a modification of the original protocol developed in Rashotte et al. (2000). In all root transport assays, seedlings were transferred to control plates and oriented vertically such that the site where radioactive auxin would be applied was aligned. In each of these assays, mixtures containing 1% (w/v) agar, 100 nM ^{3}H-IAA, or ^{3}H-IBA with either 100 μM NPA, TIBA, or 1% (v/v) DMSO were prepared in 3-mL scintillation vials and prepared and applied as above. Standard placement of radioactive agar cylinders was so there was just contact with the root tips for RBT and on top of the seedlings, just on the root side below the root shoot junction for RAT. Auxin transport was measured after 5 h for RBT by first removing the 1 mm of tissue in contact with the agar cylinder, then cutting 2- or 5-mm segments (as indicated) from the site of application along the desired length. In RAT, measurements were made either after 18 h from an application site at the root shoot junction using a 5-mm segment at the root tip, which was approximately 15 to 20 mm from the site of auxin application. The amount of radioactivity in each segment was determined as described above.

For the experiments to determine polarity of auxin transport in the root (Fig. 2B), RBT and RAT were measured for each root. Radioactive agar cylinders, as described above, were placed 10 mm back from the root tip in this experiment, and transport occurred during a 5-h assay. RBT was determined by measurement of radioactivity in a 3-mm segment at the root base, which was approximately 7 mm from the site of application. RAT was quantified by determination of the radioactivity in a 3-mm segment at the root tip, which measured auxin movement 7 mm from the site of application.

For RAT pulse-chase experiments (Fig. 3B), seedlings were placed on agar plates with the root shoot junction aligned, and a cylinder containing 400 nM ^{3}H-IBA or ^{3}H-IAA was applied 20 mm from the root tip. After 10 min, the radioactive agar cylinder was removed from the seedlings, and all seedlings were moved to a new agar plate where a nonradioactive agar cylinder of similar auxin concentration was applied in the same position on the seedling for a 50-min chase. Ten 2-mm segments were excised starting from the root tip and analyzed as above.

RBT assays to determine saturation kinetics were conducted as basic continuous pulse assays with a constant level of ^{3}H-IBA or ^{3}H-IAA and increasing amounts of unlabeled IBA or IAA from 0.1 to 20.0 μM, as indicted in Figure 4, in each agar cylinder. The amount of DMSO used as an auxin solvent was maintained at 0.1% (v/v) of the final concentration of each agar cylinder. A single 5-mm segment back from the root tip, excluding the 1 mm of root tip in contact with the agar cylinder, was collected and counted after 5 h as above.

Analysis of the Sensitivity of Hypocotyl Elongation to IBA and IAA

Seeds were surface sterilized using the vapor phase sterilization protocol (Clough and Bent, 1998). Seeds were placed in open microfuge tubes in a desiccating jar. One hundred milliliters of a 10% (v/v) sodium hypochlorite solution (commercial bleach) were placed in a 250-mL beaker in the jar with the seed in a fume hood. Three milliliters of concentrated hydrochloric acid was added to the bleach, and the desiccating jar was quickly closed. Seeds were left to sterilize for three to 6 h, after which the jar was opened carefully in a fume hood, the tubes were removed from the jar, and sterile water was added to each tube of seeds. Seeds were stratified 4 to 7 d in the dark at 4°C before being germinated. For growth analyses only, IAA and IBA were dissolved in 1 mL of 1 N NaOH and diluted with 49 milliliters of deionized water to a final stock concentration of 1 mg mL^{-1} and filter sterilized. These stocks had pH values of 11.5 and 11.3 for IBA and IAA, respectively. Appropriate amounts of the sterile stocks were added to media after autoclaving to obtain the different concentrations required. Because the growth media (GM) used in these studies is buffered (see below), addition of the stocks did not result in any pH change in the media.

The effects of auxins present in horizontally oriented GM plates on hypocotyl elongation were investigated. GM medium containing 0.8% (w/v) Difco agar was used instead of solidified nutrient solution. GM medium consists of 1× Murashige and Skoog basal salts, 1% (w/v) Suc, 0.5 g L^{-1} MES, 1 mg of thiamine, 0.5 mg L^{-1} pyridoxin, 0.5 mg L^{-1} nicotinic acid, and 100 mg L^{-1} myo-inositol, with pH adjusted to 5.7 with 1 N KOH (Valvekens et al., 1988). After stratification, seeds plated directly on auxin-containing plates, or control media were placed either in dark, high constant white light conditions (90 μmol m^{-2} s^{-1}) or low-light conditions (5 μmol m^{-2} s^{-1}). Hypocotyl length was determined on 5-d-old seedlings by tracing magnified seedlings (approximately 5-fold) using an overhead projector. A transparent ruler placed beside the hypocotyls was also traced for use as a scale bar. The tracings were then digitally scanned, and measured using the public domain National Institutes of Health Image program (http://rsb.info.nih.gov/nih-image/). Similar results were obtained in three separate trials for each light condition. Data from a single representative trial are presented.

Analysis of IBA Metabolism

Analysis of ^{3}H-IBA metabolism was performed using a protocol adapted from Delarue et al. (1999). Twenty-five 8-d-old seedlings were incubated in a 35-mm petri dish containing 2 mL of liquid GM media and 1 μCi of ^{3}H-IBA for periods of time ranging from 5 min to 24 h. Seedlings were removed from the incubation medium, rinsed twice with 2 mL of sterile distilled water, and blotted dry. The seedlings were transferred to a new microfuge tube containing 200 μL of methanol, crushed using a small plastic pestle (Kimble, Vineland, NJ), and left to extract overnight in methanol at 4°C. Extracts were centrifuged to clear debris, dried partially in a centrifugal evaporator (Speedvac, Savant Instruments, Holbrook, NY), and loaded onto silica gel TLC plates with aluminum backing (Merck, Darmstadt, Germany). Unlabeled IAA and IBA stocks were loaded in lanes at both sides of the plate and radioactive IAA and IBA were loaded in a control lane on one side of the plates. The mobile phase consisted of chloroform:methanol:water (84:

14:1 [v/v]; Piskornik and Bandurski, 1972) and resulted in well-separated IAA and IBA peaks (R_F values of 0.73 and 0.78, respectively). After migration, control lanes containing nonradioactive auxins were cut off the plate, sprayed with Ehmann's reagent (Ehmann, 1977), and heated to reveal the position of the IAA and IBA spots. The remaining plate was cut into individual lanes, and each lane was cut in 5-mm sections that were placed directly in scintillation vials containing 5 mL of scintillation cocktail. Pieces of TLC plate were allowed to extract overnight in the dark before radioactivity was measured using a scintillation counter. Free IBA was detected in the appropriate migration position. Radioactivity levels were never above background at the position of IAA migration, indicating that levels of conversion were below the detection limits of this assay.

Statistics

Statistical analyses of data were performed using Excel (Microsoft, Redmond, WA). Multiple experiments were analyzed simultaneously by comparing averages, using each root as an independent sample. The data were analyzed by a one-tailed Student's *t* test for equal variance for transport inhibitor treatments and by a two-tailed Student's *t* test for equal variance when comparing the wild type with mutant or inhibitor treated seedlings or to compare IAA and IBA treatments in physiological assays. The *P* values are reported.

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