# The *rib1* Mutant of Arabidopsis Has Alterations in Indole-3-Butyric Acid Transport, Hypocotyl Elongation, and Root Architecture[1]

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Polar transport of the auxin indole-3-butyric acid (IBA) has recently been shown to occur in Arabidopsis (*Arabidopis thaliana*) seedlings, yet the physiological importance of this process has yet to be fully resolved. Here we describe the first demonstration of altered IBA transport in an Arabidopsis mutant, and show that the *resistant to IBA* (*rib1*) mutation results in alterations in growth, development, and response to exogenous auxin consistent with an important physiological role for IBA transport. Both hypocotyl and root IBA basipetal transport are decreased in *rib1* and root acropetal IBA transport is increased. While indole-3-acetic acid (IAA) transport levels are not different in *rib1* compared to wild type, root acropetal IAA transport is insensitive to the IAA efflux inhibitor naphthylphthalamic acid in *rib1*, as is the dependent physiological process of lateral root formation. These observed changes in IBA transport are accompanied by altered *rib1* phenotypes. Previously, *rib1* roots were shown to be less sensitive to growth inhibition by IBA, but to have a wild-type response to IAA in root elongation. *rib1* is also less sensitive to IBA in stimulation of lateral root formation and in hypocotyl elongation under most, but not all, light and sucrose conditions. *rib1* has wild-type responses to IAA, except under one set of conditions, low light and 1.5% sucrose, in which both hypocotyl elongation and lateral root formation show altered IAA response. Taken together, our results support a model in which endogenous IBA influences wild-type seedling morphology. Modifications in IBA distribution in seedlings affect hypocotyl and root elongation, as well as lateral root formation.

The auxin indole-3-butyric acid (IBA) occurs naturally at levels that are physiologically relevant in many plant species, including Arabidopsis (*Arabidopsis thaliana*; for review, see Ludwig-Muller, 2000). The auxin activity of IBA is well recognized, as it has long been used as the auxin of choice for root formation on cuttings (Zimmerman and Wilcoxon, 1935; Hartmann et al., 1997). Like the auxin indole-3-acetic acid (IAA), IBA affects lateral root induction and elongation of roots, shoots, and hypocotyls (Yang and Davies, 1999; Poupart and Waddell, 2000; Zolman et al., 2000; Rashotte et al., 2003).

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 *Zea mays* and *Pisum sativa* 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, as 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). 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).

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Polar auxin transport is a specialized delivery system that moves IAA from its point of synthesis in young apical tissues to the rest of the plant in a highly regulated manner (Muday and DeLong, 2001). IBA and IAA are transported in a polar fashion in roots and hypocotyls of Arabidopsis at similar rates and amounts, while in the inflorescence stem IBA transport is much lower than IAA transport (Rashotte et al., 2003). The transport of IBA is regulated differently than that of IAA; IBA transport is not sensitive to inhibition by IAA efflux inhibitors, and the *agr1/eir1/pin2/wav6* and *aux1* mutants, which are defective in IAA transport, have wild-type levels of IBA transport (Rashotte et al., 2003). These results suggest that IBA is transported by a pathway distinct from the IAA transport pathway and support the hypothesis that IBA has activities beyond action as a precursor to IAA. Identification of mutants with altered IBA transport will be an important step in understanding the biochemical mechanisms and physiological significance of IBA transport.

Proper regulation of auxin transport is important for plant growth and development. Disruption of IAA polar transport using IAA efflux inhibitors, such as naphthylphthalamic acid (NPA) and triiodobenzoic acid (TIBA), results in a variety of phenotypes including defects in embryo and vascular tissue patterning, inhibition of lateral root, leaf primordia, and floral organ formation, reduced hypocotyl elongation in the light, and altered response in the root to gravistimulation (Okada et al., 1991; Muday and Haworth, 1994; Hadfi et al., 1998; Jensen et al., 1998; Mattsson et al., 1999; Sieburth, 1999; Reinhardt et al., 2000). Many mutations that disrupt auxin transport and affect the above-mentioned aspects of plant development have been isolated, and proteins involved in auxin transport have been identified through analysis of such mutants. Three different families of putative carrier proteins have been identified that are products of the *AUX*, *PIN*, or *MDR* gene families (Noh et al., 2001, 2003; for review, see Parry et al., 2001; Luschnig, 2002; Friml, 2003). Regulatory proteins have also been identified, such as those encoded by *RCN1* or *PID*, which mediate reversible protein phosphorylation (Deruère et al., 1999; Christensen et al., 2000; Benjamins et al., 2001; Rashotte et al., 2001; Friml et al., 2004), and flavonoids, which have been suggested to act as endogenous regulators of auxin efflux (Brown et al., 2001; Buer and Muday, 2004; Peer et al., 2004). The current model is that expression of different carriers and regulatory proteins in specific cells allows precise regulation of auxin transport in a tissue- and cell-specific manner (Friml, 2003). It has also been suggested that different transporters could have different substrate specificities or affinities (Luschnig, 2002).

In addition to endogenous factors, exogenous factors also affect auxin transport, such as light and gravity stimulation (Muday, 2001; Blancaflor and Masson, 2003). Redistribution of auxin following tropic stimulation has been shown by the activation of an auxin-inducible reporter gene on one side of stems (Li et al., 1991) and roots (Rashotte et al., 2001; Ottenschläger et al., 2003), coinciding with differential growth. Light quality, intensity, and direction can all affect auxin response and transport (Swarup et al., 2002). Dim red light was shown to cause an increase in polar auxin transport in cucumber (*Cucumis sativus*) hypocotyls (Shinkle et al., 1992). Steindler and coworkers (1999) have shown that shade-induced hypocotyl elongation was dependent on auxin transport, as it can be inhibited by NPA, and the auxin response mutant *axr1-12* is defective in this response. Arabidopsis hypocotyls grown in low light, but not in the dark, are sensitive to growth inhibition by NPA (Jensen et al., 1998). Finally, hypocotyl basipetal transport of IAA is inhibited by NPA only in low-light conditions and not in the dark (Rashotte et al., 2003). Together these results suggest light can affect auxin transport. The cloning of the *BIG* gene also supports a link between auxin transport and light response. Surprisingly, it was determined that two Arabidopsis mutants, *doc1* (dark overexpressor of chlorophyll *a/b*-binding protein), which was isolated because it expresses abnormally high levels of light-induced genes in the dark, and *tir3* (transport inhibitor response), which is defective in auxin transport and in NPA binding, are both mutations of the *BIG* gene (Gil et al., 2001; Kanyuka et al., 2003; Cluis et al., 2004).

Links between auxin and photomorphogenesis in seedlings have also been supported by publications showing that mutants defective in auxin transport or response have defects in photomorphogenesis (Morelli and Ruberti, 2002). For example, dominant mutations in the *AXR2/IAA7*, *AXR3/IAA17*, and *SHY2/IAA3* auxin response genes all cause short hypocotyls, expanded cotyledons, and leaf production in the dark, phenotypes that are characteristic of photomorphogenesis and not seen in etiolated wild-type seedlings (Leyser et al., 1996; Tian and Reed, 1999; Nagpal et al., 2000; Tian et al., 2002). HY5 is a bZIP protein that is suggested to act as a positive regulator of light signaling. *hy5-1* mutants have defects in hypocotyl elongation in the light, but also in root gravitropism and lateral root formation (Oyama et al., 1997; Cluis et al., 2004). These auxin-related phenotypes in light response mutants further suggest a functional link between the light and auxin-signaling pathways.

In this study, we studied IAA and IBA transport in different tissues of the *resistant to IBA* (*rib1*) mutant. *rib1* was isolated in a screen for mutants with defects in root gravitropism and shown previously to have an altered response when the root is reoriented with respect to the gravity vector (Poupart and Waddell, 2000). Further characterization of the mutant revealed it was less sensitive to root elongation inhibition by the auxins IBA and 2,4-D, but had a wild-type response to IAA and the synthetic auxin NAA. *rib1* did not show resistance to other classes of plant hormones tested, but did show resistance to the IAA efflux inhibitors NPA, TIBA, and 9-hydroxy fluorene carboxylic acid. Phenotypically, *rib1* seedlings could be distinguished from wild type by their shorter primary root and

increased number of lateral roots. Adult *rib1* plants, however, were indistinguishable from the wild type in terms of shoot length and secondary inflorescence formation. Based on its phenotypes and the resistance of *rib1* to IAA efflux inhibitors, we had hypothesized that this mutant could be defective in IBA transport or response (Poupart and Waddell, 2000).

This study shows that *rib1* is defective in IBA transport but has wild-type levels of IAA transport. To the best of our knowledge, this is the first demonstration of such a phenotype. These defects in IBA transport can be correlated to defects in root elongation, lateral root formation, and hypocotyl elongation. Our results also suggest a role for IBA in hypocotyl response to light signals and that this response could be modulated by Suc. Finally, we characterize the responses of *rib1* and wild-type seedlings to exogenous IAA and IBA, and to IAA transport inhibitors NPA and TIBA. Together these results suggest RIB1 could be a regulator of IBA transport important for defining root architecture and in hypocotyl elongation response to light and Suc.

**Table I.** *Transport of IAA and IBA in wild-type and rib1 tissues*

| Transport Assay | Auxin Transported[a] | | *P* Value[b] |
|---|---|---|---|
| | Wild Type | *rib1* | |
| Hypocotyl basipetal[c,e] | | | |
| IAA | 3.6 ± 0.4 | 3.2 ± 0.2 | 0.40 |
| IBA | 3.1 ± 0.3 | 1.8 ± 0.2 | $1.51 \times 10^{-3}$ |
| Root basipetal[c] | | | |
| IAA[f] | 2.0 ± 0.1 | 1.8 ± 0.1 | 0.14 |
| IBA[g] | 9.9 ± 0.6 | 7.4 ± 0.5 | $1.66 \times 10^{-3}$ |
| Root acropetal[c,g] | | | |
| IAA | 9.3 ± 0.6 | 10.4 ± 0.6 | 0.22 |
| IBA | 17.1 ± 0.9 | 30.4 ± 1.4 | $1.55 \times 10^{-13}$ |
| Inflorescence basipetal[d,e] | | | |
| IAA | 1.1 ± 0.1 | 1.3 ± 0.1 | 0.22 |
| IBA | 0.04 ± 0.005 | 0.04 ± 0.006 | 0.67 |

[a]Average and standard error of the mean are presented. [b]Two-tailed Student's *t* test assuming equal variance. [c]Transport is reported in fmol. [d]Transport is reported in pmol. [e]*n* values are from three experiments with 15 to 30 plants. [f]*n* values are from 134 to 136 plants from 14 experiments. [g]*n* values are from six to eight experiments with 59 to 82 plants.

## RESULTS

### Transport of Auxin in *rib1*

In seedlings, both IAA and IBA are transported in three different flows: from the shoot apical meristem at the tip of the hypocotyl down toward the root (hypocotyl basipetal transport), from the root/shoot junction at the base of the root to the tip (root acropetal transport), and from the root tip back up toward the root base over a short distance (root basipetal transport; Muday and DeLong, 2001; Rashotte et al., 2003). We measured IAA and IBA transport in *rib1* and wild-type seedlings to directly assess the impact of the *rib1* mutation on auxin transport (Table I). Significant changes in IBA transport are seen in all three seedling transport streams. In *rib1* seedlings, hypocotyl basipetal transport of IBA is reduced to approximately 60% of the value of wild type, while IBA root basipetal transport is reduced to approximately 75% of wild type. Opposite to the decreases observed for basipetal transport, root acropetal IBA transport in *rib1* is increased relative to wild-type levels by approximately 180% of wild-type levels.

In contrast to the alterations in IBA transport, no significant alteration in IAA transport was observed in any of the seedling assays (Table I). Basipetal IAA transport in inflorescence stems is similar in Nossen-0 (No-0) and *rib1* adult plants, and at higher levels of transport than in the seedling assays. The reported amount of IAA transport is approximately 50- to 100-fold higher than IAA transport in the seedling assays. It is clear that auxin moves in greater amounts through the inflorescence than in the young seedling tissues, but it is not possible to directly compare the transport amounts. The inflorescence assays were performed with 3.3-fold greater concentration of radiolabeled auxin, over a longer transport period, and with auxin loaded from a liquid solution, rather than from agar. Additionally, there are large differences in the tissues, with hypocotyls and roots being much thinner and younger tissues than an adult inflorescence.

A more accurate comparison is the amount of IBA and IAA basipetal transport in the inflorescence tissue, as those assays are done in similar ways by assessing transport in inverted inflorescence segments. The amount of IBA transport is extremely low and at the levels of background. At most, the amount of IBA transport in the inflorescence is 4% of the levels of IAA transport performed in identical assays, consistent with a previous report (Rashotte et al., 2003). The *rib1* mutation has no effect on inflorescence IBA transport (Table I). These results are consistent with the wild-type appearance of adult *rib1* plants (Poupart and Waddell, 2000).

### Effect of NPA on IAA Transport in Wild Type and *rib1*

We have examined the effect of NPA on IBA transport and have never found evidence of NPA inhibition of root basipetal or acropetal IBA transport, or on basipetal transport of IBA in the hypocotyls. These experiments have been extensively repeated in wild type (Rashotte et al., 2003), and preliminary experiments in *rib1* also show no regulation of IBA transport by NPA in all three transport streams (data not shown). While our transport studies indicate that IBA transport is not regulated by NPA, the roots of *rib1* are altered in sensitivity to NPA.

To explore the regulation of auxin transport in *rib1*, IAA transport was examined in the presence of NPA, as shown in Table II. Overall levels of IAA transport are unaffected by the *rib1* mutation under the

**Table II.** *Effect of NPA on IAA transport in wild-type and rib1 tissues*

| Plants | NPA | IAA Transported[a] | | | |
|---|---|---|---|---|---|
| | | IBT[b] | HBT[c,d] | RBT[c,e] | RAT[c,f] |
| Wild type | − | 1.1 ± 0.1 | 3.4 ± 0.4 | 2.3 ± 0.2 | 11.4 ± 1.3 |
| Wild type | + | 0.03 ± 0.01 | 1.4 ± 0.3 | 1.4 ± 0.1 | 8.0 ± 0.4 |
| *P* value[g] | | $6.0 \times 10^{-13}$ | $2.0 \times 10^{-10}$ | $4.8 \times 10^{-5}$ | $7.1 \times 10^{-3}$ |
| *rib1* | − | 1.3 ± 0.1 | 3.3 ± 0.4 | 2.0 ± 0.2 | 13.4 ± 1.1 |
| *rib1* | + | 0.05 ± 0.02 | 1.3 ± 0.2 | 0.9 ± 0.1 | 15.0 ± 1.2 |
| *P* value[g] | | $7.4 \times 10^{-11}$ | $1.2 \times 10^{-13}$ | $1.4 \times 10^{-9}$ | 0.16 |

[a]Average and standard error of the mean are presented. [b]IBT: Inflorescence basipetal transport, reported in pmol of auxin transported with *n* values from 14 to 15 segments from three experiments. [c]Seedling transport is reported in fmol of auxin transported. [d]HBT: Hypocotyl basipetal transport; *n* values are from 29 to 30 plants from three experiments. [e]RBT: Root basipetal transport; *n* values are from 65 to 66 plants from seven experiments. [f]RAT: Root acropetal transport; *n* values are from 28 to 35 plants from three to four experiments. [g]One-tailed Student's *t* test assuming equal variance for the presence and absence of NPA.

conditions of our assays (Table I). Table II shows that all flows of IAA transport are significantly reduced by NPA in wild-type seedlings and adult plants, but with the magnitude of reduced transport much greater in the inflorescence stems. Likewise, IAA basipetal transport in the inflorescence stem, hypocotyl, and root are inhibited by NPA in *rib1*. Analysis of the effects of NPA on IAA transport in *rib1* reveals one difference in regulation of IAA transport relative to wild type. NPA does not inhibit root acropetal IAA transport in *rib1*. This finding is interesting in light of the fact that the only IBA transport stream elevated in *rib1* is this acropetal auxin transport in the roots.

### Hypocotyl Elongation in *rib1*

As *rib1* exhibits a significant decrease in hypocotyl basipetal transport of IBA, we examined whether hypocotyl elongation was also affected in the *rib1* mutant under several light intensities and two Suc concentrations, as shown in Figure 1. Under the conditions used for the transport assays, low white light (5 $\mu$mol $m^{-2}$ $s^{-1}$) and 1.5% Suc, *rib1* hypocotyls are significantly longer than wild-type hypocotyls (Fig. 1). In the dark on the same media, *rib1* hypocotyls are also significantly longer, but with a greater difference (20% as compared to 35%, respectively). However, there is no difference in hypocotyl length under high white-light conditions (95 $\mu$mol $m^{-2}$ $s^{-1}$) and 1.5% Suc. This last result was surprising as we had previously noted that *rib1* had a long hypocotyl under different growth conditions, i.e. high white light with a 16-h-day/8-h-night cycle and 1% Suc. This prompted us to further examine the effects of light on *rib1* hypocotyl length.

We examined hypocotyl elongation in the absence of Suc in the growth media and under several light levels and wavelengths (Fig. 1). In the absence of Suc, *rib1* hypocotyls are significantly longer than wild type in high white (approximately 30% longer) and red light (approximately 45% longer), but not in the dark or blue-light conditions. There is also a small, yet significant (*P* value = 0.04), 5% reduction in hypocotyl elongation under far-red light conditions in *rib1* relative to wild type. Our results suggest a modification in IBA transport results in defects in hypocotyl elongation reminiscent of those of phytochrome mutants. As Murashige and Skoog salt concentrations were the same for experiments presented in Figure 1, the only difference between these media conditions was the presence of Suc. This Suc difference results in an important change in hypocotyl elongation in *rib1* relative to wild type: *rib1* hypocotyls are longer in the dark or low light in the presence of 1.5% Suc and longer in high light in the absence of Suc. Surprisingly, in the presence of both high light (95 $\mu$mol $m^{-2}$ $s^{-1}$) and Suc, *rib1* hypocotyl lengths are similar to wild type (Fig. 1).

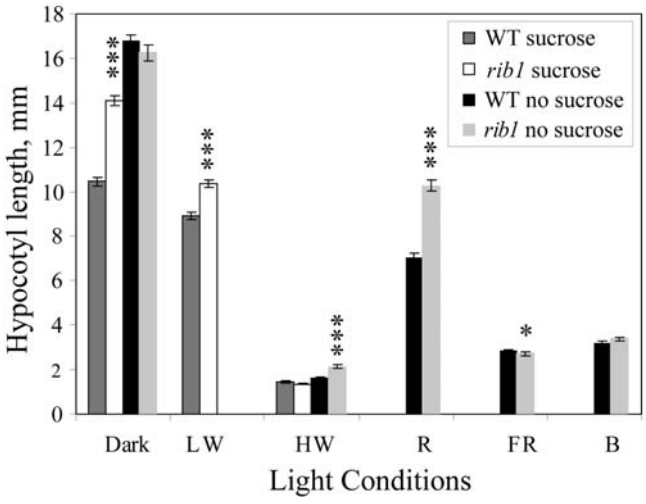


**Figure 1.** Hypocotyl length in wild-type and *rib1* seedlings grown in different light and Suc conditions. Wild-type and *rib1* seedlings were plated on media containing 1.5% Suc or Suc-free media. Plates were placed in dark, low white (LW), high white (HW), red (R), far red (FR), or blue (B) light, and hypocotyl length was measured after 5 d (with Suc) or 7 d (without Suc) of growth. Average length and SE of 138 to 209 individuals from seven to nine trials are presented for conditions with Suc. Average length and SE of 32 to 143 individuals are presented from one representative trial without Suc. Similar results were obtained in one to five additional trials. Asterisks indicate *P* values from two-tailed Student's *t* test comparing wild type to *rib1* under each condition. *, *P* value $< 0.05$; ***, *P* value $< 0.001$; actual values range from $3.9 \times 10^{-5}$ to $2.5 \times 10^{-32}$.

These results uncover a difference in the combined effects of light and Suc on hypocotyl elongation in *rib1*.

### Effect of Exogenous Auxin on Hypocotyl Elongation

The effects of exogenous IAA and IBA on hypocotyl elongation were investigated in the dark and in two different light conditions, including the low white-light condition used for the hypocotyl basipetal transport assays (Fig. 2). Media with 1.5% Suc was used, as this is the media used in auxin transport assays. In the dark, both IAA (Fig. 2A) and IBA (Fig. 2B) inhibit hypocotyl elongation in *rib1* and wild type. Small differences in *rib1* response to IAA under dark conditions were detected (Fig. 2A). More notably, the dose-response curve of *rib1* to IBA is clearly shifted toward higher concentrations, indicating a reduced sensitivity of *rib1* to IBA under these conditions (Fig. 2B). Exogenous auxin also inhibits hypocotyl elongation under low-light conditions. Under these light conditions, the response of *rib1* to both IAA (Fig. 2C) and IBA (Fig. 2D) requires higher concentrations relative to the wild-type curve, indicating that hypocotyl elongation in *rib1* is also less sensitive to inhibition by both auxins in these conditions. A comparison of the concentrations of auxins that lead to a 50% inhibition ($IC_{50}$) of hypocotyl elongation confirms that *rib1* dramatically affects the response to IBA in the dark (5.1 versus 11.5 $\mu$M for No-0 and *rib1*, respectively), while the response to IAA is only marginally affected (2.0 and 2.8 $\mu$M for wild type and *rib1*, respectively). In low-light conditions, however, both IAA and IBA responses are affected by *rib1* with the $IC_{50}$ for *rib1* hypocotyls being 4.4-fold higher for IAA and 2.6-fold higher for IBA than wild type.

IBA significantly stimulates hypocotyl elongation under high light at concentrations ranging from 1 $\mu$M to 10 $\mu$M in the wild type (Fig. 2F), as previously reported (Rashotte et al., 2003). At a higher concentration (100 $\mu$M), IBA inhibits hypocotyl elongation in wild type. In high light, greater amounts of auxins are required to inhibit hypocotyl elongation, compared to low light and dark conditions (compare the dose-response curves in Fig. 2, E and F, to those of Fig. 2, A to D). In these conditions, the dose-response curve of *rib1* to IBA (Fig. 2F) is again shifted toward higher concentrations relative to the wild-type curve. This can

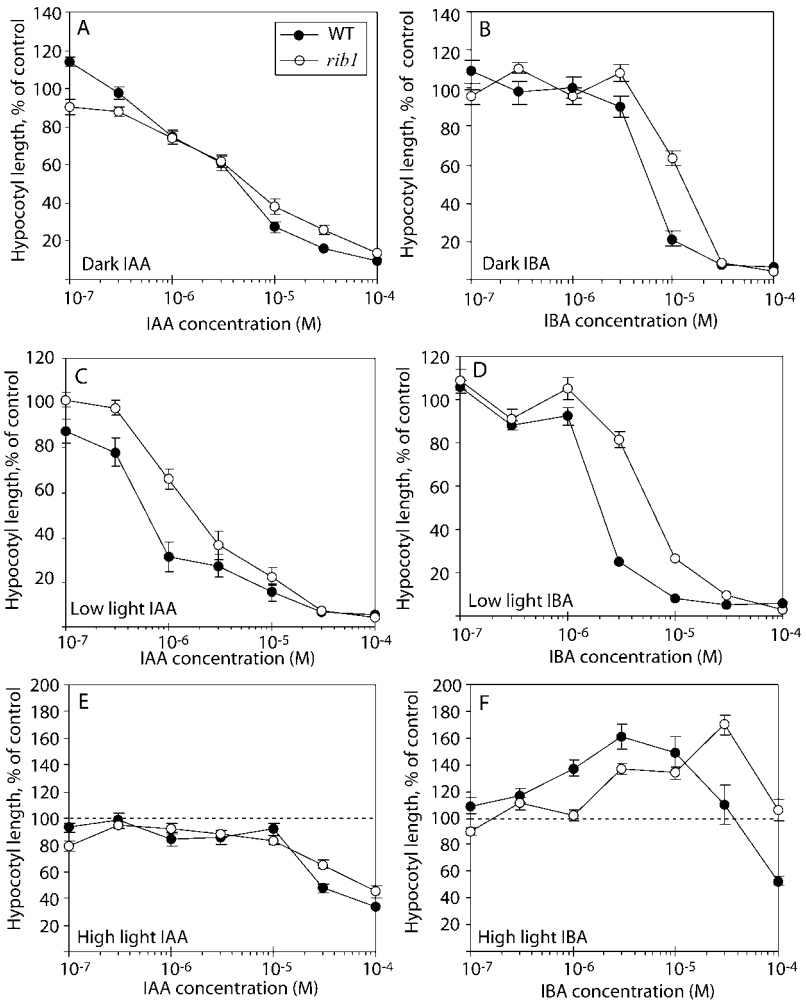


**Figure 2.** Dose response of hypocotyl elongation to auxins in different light conditions in the presence of 1.5% Suc. Wild-type and *rib1* seedlings were plated on media containing the indicated amounts of IAA (A, C, and E) or IBA (B, D, and F). Plates were placed in dark (A and B), low-light (C and D), or high-light (E and F) conditions, and hypocotyl length was measured after 5 d of growth. Average and SE is presented as the percent of elongation in the absence of auxin for each genotype. Error bars smaller than the symbols are not visible. The average of six to 26 seedlings from one representative trial is shown. Similar results were obtained in two or three additional trials.

be seen by the fact that a higher concentration of IBA is required to stimulate elongation (1 $\mu$M results in longer wild-type hypocotyls, while 3 $\mu$M is required to stimulate *rib1*), but also to inhibit elongation (wild type is significantly inhibited at 100 $\mu$M IBA, while the *rib1* curve has not yet fallen below the 100% line).

### Effect of Exogenous Auxin on Lateral Root Formation

Lateral root formation in response to exogenous auxin was investigated in wild-type seedlings grown in low light (5 $\mu$mol $m^{-2}$ $s^{-1}$) and the presence of 1.5% Suc. Figure 3 shows a dose-response curve of lateral root formation in response to IAA and IBA in wild-type and *rib1* seedlings. The graph indicates that IBA is more potent at inducing lateral roots. Lateral root induction occurs at lower concentrations of IBA than IAA and the slope of the IBA graph is steeper.

The lateral root number and sensitivity to induction by auxins were also compared in No-0 and *rib1*. Under these conditions, No-0 and *rib1* have similar numbers of lateral roots in the absence of added auxins, as shown in Figure 3. In contrast, if roots are grown at higher light levels (80–90 $\mu$mol $m^{-2}$ $s^{-1}$) and with lower Suc (1%) for four additional days, *rib1* has a greater number of lateral roots than wild type, indicating that Suc and light level differentially affect root development in No-0 and *rib1* (Poupart and Waddell, 2000). Figure 3 shows the effect of increasing concentrations of IAA and IBA on the number of elongated lateral roots in wild type and *rib1*. For both auxins, there are differences in the concentration dependence of the lateral root induction between wild type and *rib1*. The differences in number of lateral roots between *rib1* and wild type are greater in the presence of IBA, with 2.3-fold greater root development in wild type than in *rib1* at $10^{-5}$ M IBA and only a 1.6-fold greater effect on wild type than *rib1* at this concentration of IAA. The *rib1* mutation therefore confers reduced sensitivity to both auxins, but with a larger difference in IBA response. The induction response seems to have reached a plateau in wild type at concentrations of IBA greater than $10^{-5}$ M. In contrast, in *rib1*, increasing the levels of IBA above $10^{-5}$ M results in a higher number of lateral roots, suggesting the response is not yet maximal in the mutant at the concentration used.

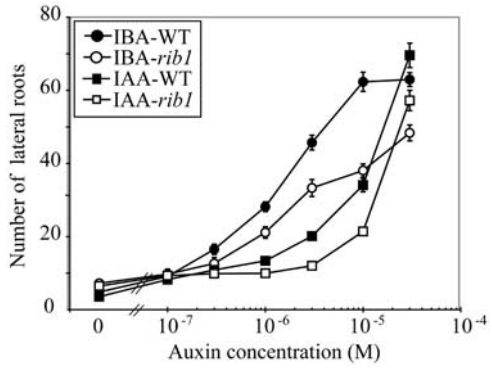


**Figure 3.** Comparison of IAA and IBA effects on lateral root formation in wild-type and *rib1* seedlings. Four-day-old seedlings were transferred to media containing the indicated amounts of auxin. After 6 d of growth in the presence of auxin the number of emerged lateral roots was counted from 17 or 18 seedlings in each trial. The averages and SEs from two combined trials are shown.

In order to examine the statistical significance of the differences between lateral root numbers in wild type and *rib1* in the presence of these two auxins at a range of concentrations, a three-way factorial ANOVA was performed. The differences between genotype ($F_{1,974}$ = 120.9, $P < 10^{-3}$), between auxin used ($F_{1,974}$ = 183.3, $P < 10^{-3}$), and concentration ($F_{6,974}$ = 599.7, $P < 10^{-3}$) were all significant. The two-way interactions of auxin type and genotype, auxin type and concentration, and genotype and concentration were also significant ($F_{1,974}$ = 10.0, $P < 0.002$; $F_{6,974}$ = 55.1, $P < 0.001$; $F_{6,974}$ = 24.1, $P < 0.001$, respectively). These statistical tests allow us to conclude that wild type and *rib1* have significantly different responses to each auxin and each dose of auxin. Yet, the precise comparison that should be made is whether *rib1* and wild type are significantly different at each auxin concentration used. This statistical comparison was performed using a Neuman-Keuls post-hoc comparison of means. This post-hoc analysis is justified based on the statistical differences in genotype by auxin type and genotype by auxin concentration revealed in the two-way interactions identified by ANOVA, as described above. For these comparisons, the differences between wild type and *rib1* become significant at IBA doses of 1 $\mu$M and greater and for IAA at doses of 3 $\mu$M or greater (degrees of freedom = 974; $P < 0.007$). Therefore, the *rib1* mutation reduces the sensitivity to both IBA and IAA, but with a significant difference in response detected at a lower concentration of IBA than IAA and with a greater magnitude of effect with IBA.

### Effect of Auxin Transport Inhibitors on *rib1* Seedlings

The *rib1* mutant has been shown to be less sensitive not only to IBA and 2,4-D but also to the IAA efflux inhibitors NPA, TIBA, and 9-hydroxy fluorene carboxylic acid by root elongation assays (Poupart and Waddell, 2000). Figure 4 quantifies the effects of the IAA efflux inhibitors NPA and TIBA on lateral root formation in wild-type and *rib1* seedlings. The concentration of NPA used (0.1 $\mu$M) inhibits lateral root formation in wild type to approximately 20% of the numbers in nontreated wild-type seedlings. In contrast, NPA reduces lateral root formation in *rib1* much less, to about 77% of nontreated levels. *rib1* is also less sensitive to the effects of TIBA; at 0.1 $\mu$M, TIBA reduces the number of lateral roots formed in wild type to approximately 45% and in *rib1* to 71% of nontreated controls. Interestingly, in wild type NPA is significantly more effective in inhibiting lateral root formation than TIBA (20% versus 45%; Student's *t* test, $P = 6 \times 10^{-3}$), while the difference between the effect of 0.1 $\mu$M NPA or TIBA is not significant in *rib1* (77%

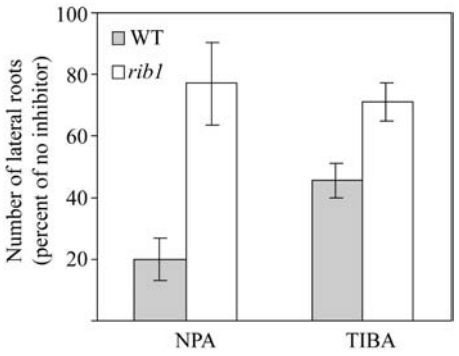


**Figure 4.** Effect of auxin transport inhibitors on lateral root formation in wild-type and *rib1* seedlings. Wild-type (gray bars) and *rib1* (white bars) seedlings were grown for 4 d on control media and then transferred to media containing 0.1 $\mu$M NPA or TIBA. After 6 d of growth in the presence of auxin, the number of emerged lateral roots was counted. Data are expressed as a percentage of the number of lateral roots formed in the absence of inhibitor. Average and SE of 36 individuals from two assays (NPA) or 54 individuals from three assays (TIBA) are presented.

versus 71%; $P = 0.66$). Taken together with the previous report (Poupart and Waddell, 2000), these results show that *rib1* is less sensitive to IAA efflux inhibitors for both primary root elongation and lateral root formation responses. Additionally, the reduced ability of NPA to inhibit lateral root development in *rib1* parallels the insensitivity of acropetal IAA transport to NPA, further linking these two processes.

## DISCUSSION

Although IBA has recently been shown to be transported in a polar fashion in Arabidopsis, the role or importance of this transport remains to be defined precisely (Rashotte et al., 2003). Disruptions in IAA transport caused by inhibitor treatments or mutations have helped dissect the role of the different flows of this auxin in plants. IAA efflux inhibitors do not affect IBA transport (Rashotte et al., 2003); therefore, other means must be used to study the transport of this endogenous auxin. Changes in IBA transport in the *rib1* mutant can be linked to specific modifications in root and hypocotyl morphology, and these phenotypes are reminiscent of those of seedlings in which IAA transport has been disrupted. This study shows that all flows of IBA transport are affected in *rib1* seedlings, while IAA transport levels are unchanged relative to wild type. Therefore, the analysis of the phenotypes and auxin-response profiles of *rib1* can help provide valuable information to dissect the role of IBA transport in Arabidopsis and other plants.

Basipetal IAA transport has been implicated in the control of Arabidopsis root growth and gravitropism (Rashotte et al., 2000; Blancaflor and Masson, 2003). Blocking root basipetal IAA transport by localized application of auxin transport inhibitors, or by mutations in genes encoding IAA transport proteins such as AGR1/EIR1/PIN2/WAV6 or AUX1, results in defects in gravity-induced reorientation and root elongation (Maher and Martindale, 1980; Bell and Maher, 1990; Chen et al., 1998; Rashotte et al., 2000). *rib1* has a slowed response to gravity and a shorter primary root (Poupart and Waddell, 2000), indicating a defect in elongation growth. *rib1* has wild-type levels of root basipetal IAA transport but has significantly reduced root basipetal IBA transport, suggesting a role for IBA transport in controlling root elongation growth and gravitropic curvature.

Root acropetal transport of IAA has been shown to be important for lateral root formation, as inhibition of this flow of transport results in a reduction of lateral root formation (Reed et al., 1998) and movements of free IAA into the root are timed to drive lateral root elongation (Bhalerao et al., 2002). Four characteristics of *rib1* are consistent with altered acropetal IBA transport also being linked to the control of lateral roots. First, when seedlings are grown under high light, there are also elevated levels of IBA transport in *rib1* relative to No-0 that occur specifically in the root acropetal transport flow; acropetal IAA transport is similar in the two genotypes. Second, *rib1* mutant seedlings have approximately 60% more lateral roots than wild type at 14 d when grown in high light (Poupart and Waddell, 2000), consistent with the higher root acropetal IBA transport under similar light levels. Third, the regulation of root acropetal IAA transport is modified in *rib1* and is no longer sensitive to NPA. This is reflected in the reduced sensitivity of seedlings to inhibition of lateral root formation by NPA. Fourth, lateral roots are induced by application of exogenous auxin, either IBA or IAA, and *rib1* exhibits a higher level of insensitivity to IBA than to IAA. These results support the hypothesis that root acropetal transport of IBA modulates lateral root formation, like the acropetal transport of IAA. The root phenotypes of *rib1* (increased number of lateral roots in light-grown seedlings, shorter primary root, and slowed gravity response) are therefore suggested to be the result of IBA transport disruptions, indicating that IBA, in addition to IAA, has an important role in defining root architecture.

One point that is still not clear about these results is the relationship between the action of auxin transport inhibitors and the *rib1* mutation in modulation of root growth and transport. NPA and TIBA don't directly affect IBA transport, yet *rib1* is less sensitive to the effect of NPA on acropetal IAA transport. *rib1* is also less sensitive to the inhibitory effect of both TIBA and NPA on lateral root formation. *rib1* also shows resistance to NPA inhibition of hypocotyl and primary root elongation (Poupart and Waddell, 2000). This scenario partially parallels the *rcn1* mutant, which has a defect in a gene encoding a regulatory subunit of protein phosphatase 2A and which shows a similar phenotype: though root acropetal IAA transport in this mutant is normal, it shows defects in regulation of this transport by NPA (Rashotte et al., 2001). By

analogy, *RIB1* could encode a protein that regulates IBA transport, directly affecting IBA transport and affecting the response of some flows of IAA to NPA. Alternatively, RIB1 could have a more direct role in the regulation of root acropetal IAA transport. In the presence of RIB1, acropetal IAA transport occurs through an NPA-sensitive pathway, while in the absence of RIB1 acropetal IAA transport occurs through a second pathway, which is NPA insensitive. This second pathway would be the transport stream normally utilized by IBA. A third possibility is that the *rib1* mutation affects IBA specificity in a protein that normally functions in both IAA and IBA transport. The mutated form of the protein may no longer recognize or respond to IBA, but retains most of its wild-type IAA activity with some limited changes in the regulation of IAA transport and subtly altered responses to IAA (see below).

An additional link between the altered IBA transport in *rib1* and lateral root formation is identified in assays that examine lateral root induction in response to exogenous auxin. IBA is a more potent inducer of lateral roots than IAA in wild-type seedlings grown under the low-light conditions used for our physiological assays. These results contrast with the results of Zolman et al. (2000) who found that IAA induced more lateral roots than IBA when both auxins were used at similar concentrations. Many factors vary between our studies and these factors have been shown to affect lateral root formation in Arabidopsis: age of seedlings, Suc and nitrogen salt composition of media, length of hormone treatment, ecotype, and light conditions (Forde, 2002; Malamy, 2005). For example, in an earlier study comparing lateral root formation of *rib1* and No-0, in which plants were grown in high light with lower Suc levels, *rib1* had elevated lateral root formation (Poupart and Waddell, 2000), while under the lower light and higher Suc conditions used in this study, *rib1* and No-0 have similar levels of lateral roots. As noted earlier, *rib1* is less sensitive than wild type to lateral root induction by IBA and IAA, but the magnitude of the insensitivity to IBA is greater. Nonetheless, these experiments revealed one physiological condition (low light and 1.5% Suc) in which *rib1* roots (and as discussed below, *rib1* hypocotyls) display some modest resistance to IAA and suggest either cross talk or direct interaction between the two auxins.

Hypocotyl basipetal transport of IBA is reduced in the *rib1* mutant, while IAA transport is unaffected in *rib1* hypocotyls. We also found hypocotyl elongation to be affected by the *rib1* mutation in a range of conditions. In the absence of Suc, *rib1* hypocotyls are longer than wild type in white and red light, not significantly different in the dark or blue light, and shorter in far-red light. In the presence of Suc, *rib1* hypocotyls are longer than wild type in dark- and low-light conditions, but not different in high-light conditions. Our data therefore suggest IBA transport has a role in hypocotyl elongation both in the light (without Suc) and in the dark (with Suc). Our results also suggest interaction between light and Suc signaling on hypocotyl elongation of *rib1*. Exactly where and how RIB could be integrated in this complex regulatory web is unclear at present, but it would affect IBA transport in response to light. Light has been shown previously to affect auxin transport in cucumbers (Shinkle et al., 1992), but also to specifically affect IBA transport in Arabidopsis (Rashotte et al., 2003).

Previous research has suggested auxin transport is not required for hypocotyl elongation in the dark in Arabidopsis (Jensen et al., 1998). This conclusion was based on the fact that an IAA efflux inhibitor reduced hypocotyl elongation in light-grown seedlings but not in dark-grown seedlings. These results can be reconciled with ours if we consider the specificities of IBA transport. IBA transport in Arabidopsis occurs in the same directions as IAA transport, but IBA movements are not inhibited by NPA. Therefore, lack of inhibition by NPA of hypocotyl elongation in the dark does not rule out a role for IBA transport in etiolated hypocotyl growth. Additionally, IAA transport itself is not completely abolished by NPA in hypocotyls, unlike what is observed in inflorescence stem, where NPA causes almost complete inhibition of IAA transport (Rashotte et al., 2003). Furthermore, IAA transport in the hypocotyl becomes completely insensitive to NPA in the dark (this study; Rashotte et al., 2003). Taken together, these results suggest that some IAA transport also occurs through an NPA-insensitive pathway, which could use the same pathway as IBA transport. Interestingly, Jensen et al. (1998) noted that NPA had a lesser effect on hypocotyl elongation in red light than in white-, far-red, or blue-light conditions. Based on Jensen's results and the results presented here, it is tantalizing to suggest that NPA-insensitive transport is more important for the phytochrome B-mediated hypocotyl elongation inhibition response.

Exogenous auxins have different effects on hypocotyl elongation depending on light intensity. Under low white-light or dark conditions, application of either IAA or IBA results in inhibition of hypocotyl elongation in wild type. *rib1* is less sensitive to inhibition of hypocotyl elongation by exogenous application of both IAA and IBA in low light, but only to IBA in the dark, showing that mutating *RIB1* differentially affects hypocotyl response to IAA and IBA in different light conditions. *rib1* phenotypes can be correlated to changes in response to IBA in this mutant: seedlings have longer hypocotyls in conditions under which application of low concentrations of IBA were shown to inhibit elongation (dark and low white light), but show hypocotyl lengths similar to wild type under high white-light conditions, where application of the same concentration of IBA had no significant effect on elongation. Stimulation of hypocotyl elongation in high light is seen in wild type with IBA concentrations ranging from 1 to 10 $\mu$M, a response not seen under dark or low-light conditions. Stimulation of hypocotyl elongation in *rib1* requires approximately 3-fold higher concentrations of IBA. The fact that exogenous IBA can stimulate hypocotyl elongation under conditions where IAA is inhibitory to

hypocotyl elongation suggests that IBA has a direct role in elongation of this organ in Arabidopsis.

Interestingly, our analysis of hypocotyl elongation and lateral root formation revealed a condition in which *rib1* differs from wild type in response to IAA. Under low-light conditions and in the presence of Suc, *rib1* is slightly less sensitive to the inhibitory effects that exogenous IAA has on hypocotyl elongation and the stimulatory effects on lateral root formation. The subtle changes in the mutant and the lack of any alterations to IAA transport or regulation in these tissues suggest that the effect is an indirect one and likely reflects the interaction and cross talk between the two endogenous auxins under these specific growth conditions. This interaction may be at the level of IBA to IAA conversion or at the convergence of IAA and IBA signaling or transport pathways.

This report contains evidence demonstrating that IBA transport is specifically altered in the *rib1* mutant while IAA transport levels are unchanged. *rib1* is the first mutant in which such a change in IBA transport has been identified. The changes in IBA transport parallel changes in IBA sensitivity in control of hypocotyl elongation and lateral root development. Interestingly, both acropetal IAA transport and lateral root formation in *rib1* mutant roots are altered in their response to the IAA transport inhibitor NPA, suggesting a change in the regulation of IAA transport, and cross talk between IAA and IBA in this specific transport pathway. We also have identified one specific growth condition, low light and 1.5% Suc, in which *rib1* exhibits altered IAA sensitivity. In all other conditions, we find this mutant to have wild-type IAA response. Additionally, IBA sensitivity differs under different light and Suc concentrations in both wild-type and *rib1* hypocotyls and roots, consistent with a complex interaction between the transport and action of IBA and Suc and light signaling.

## MATERIALS AND METHODS

Isolation and preliminary characterization of *rib1* has been described previously (Poupart and Waddell, 2000). *rib1* arose in a *Ds*-mutagenized population but does not carry a *Ds* element. The *rib1* mutation is in the Nossen ecotype of Arabidopsis (*Arabidopsis thaliana*); Nossen is used as the wild-type control in all assays. All experiments reported here were done with *rib1* homozygotes derived from the original isolate. *rib1* lines backcrossed to No-0 two times retained the suite of phenotypes described by Poupart and Waddell (2000), including resistance to IBA and 2,4-D in root elongation assays, a wild-type response to IAA in root elongation assays, an elongated hypocotyl, and shorter primary root under conditions of cycling light and increased root slanting. In addition, IAA and IBA transport in the inflorescence has been examined in this line and occurs at similar levels to those reported in Table I for *rib1* (data not shown). Although the backcrossed lines show no evidence of segregation of the *rib1* phenotypes, given the propensity of *Ds* to transpose to linked sites, the possibility exists that the *rib1* phenotype is the result of more than a single gene mutation. Resolution of this issue awaits cloning of the *rib1* mutant gene and complementation of the mutant phenotype with the wild-type *RIB1* gene.

### Chemicals

3-[5(n)-$^3$H]-IAA (27 and 25 Ci mmol$^{-1}$) was purchased from Amersham and 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. NPA was purchased from Chem Service. All other chemicals were purchased from Sigma, unless stated otherwise.

### Growth Conditions for Auxin Transport Assays

Seeds were soaked in distilled water for 30 min and surface sterilized with 95% ethanol for 5 min and 20% bleach with 0.01% 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% agar (Sigma type M, plant tissue culture), 1× Murashige and Skoog salts, pH 6.0; 1.5% Suc; 1 $\mu$g mL$^{-1}$ thiamine; 1 $\mu$g mL$^{-1}$ pyridoxine HCl; and 0.5 $\mu$g mL$^{-1}$ nicotinic acid. Seeds were grown in vertically oriented petri dishes in continuous 90 $\mu$mol 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 $\mu$mol m$^{-2}$ s$^{-1}$ of constant fluorescent light to increase hypocotyl length. Light values indicate the amount of light on the outside of petri dishes.

Plants for inflorescence assays were grown on a 1:1:1 mixture of perlite, vermiculite, and Sunshine mix number 1 (Sun Gro Horticulture). 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 $\mu$mol m$^{-2}$ s$^{-1}$.

### Hypocotyl Basipetal Transport Assay

Hypocotyl transport measurements were made on 5-d-old seedlings grown under low light to elongate the hypocotyl. Seedlings were transferred to control plates and oriented vertically such that the shoot apical meristems were aligned. In this assay, mixtures containing 1% agar, 100 nM $^3$H-IAA, or $^3$H-IBA with either 100 $\mu$M NPA or dimethyl sulfoxide at the same concentration (1%) were prepared in 3-mL scintillation vials. A narrow stem transfer pipette was carefully inserted into the hardened agar mixture to produce a 1-mm-diameter cylinder of agar. This cylinder containing radioactive auxin mixture was applied such that the agar just touched the tip of the hypocotyl from which the shoot apical meristem and cotyledons were cut. Plates remained vertically oriented in the dark, to avoid auxin degradation by light (Stasinopoulos and Hangarter, 1989). Radioactive auxin transport was measured after 5 h by scintillation counting of a 5-mm segment of the base of the hypocotyl. In Table I, the average and standard error of the mean of 30 individuals from three assays are reported in fmol of auxin transported.

### Root Transport Assays

Basic root auxin transport measurements were made on 6- or 7-d-old vertically grown seedlings as by Rashotte et al. (2001). 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. Standard placement of radioactive agar lines was at the root tips for root basipetal transport and just on the root side of the root shoot junction for root acropetal transport. In each of these assays, mixtures containing 1% agar, 100 nM $^3$H-IAA or $^3$H-IBA with either 100 $\mu$M NPA, TIBA, or 1% dimethyl sulfoxide were prepared in 3-mL scintillation vials and prepared and applied as above. Auxin transport was measured after 5 h for root basipetal transport by first removing the 1 mm of tissue in contact with the agar line, then cutting a 5-mm segment from the site of application along the desired length. In root acropetal transport, measurements were made after 18 h from an application site at the root shoot junction using a 5-mm segment at the root tip. Segments were measured as above. In Table I, the average and standard error of the mean of 59 to 80 individuals from five to seven assays are reported in fmol of auxin transported.

### Inflorescence Auxin Transport Assays

Inflorescence transport measurements were conducted on approximately 25-d-old plants as described previously (Okada et al., 1991; Brown et al., 2001). Care was taken to ensure wild-type and *rib1* inflorescence stems of matched length were used. In this assay 333 nM $^3$H-IAA or $^3$H-IBA were applied to a 20-mm inflorescence segment, and transport into the basal 5 mm of that inflorescence segment was measured after 18 h. Each segment was placed into

2.5 mL of scintillation fluid, and the amount of radioactivity within each sample was determined using a Beckman LS6500 scintillation counter for 2 min. As *rib1* segments tend to be more slender than wild-type segments, values were corrected for this weight difference. The corrected values were obtained as follows for wild type: counts for wild type were divided by the average weight of wild-type segments (=12.2 mg), to obtain counts/weight, and this value was multiplied by the average weight of wild-type and *rib1* segments (=9.99 mg), to obtain counts per average weight segments. For *rib1*, the values were calculated in a similar fashion: [(counts for *rib1*)/(average weight of *rib1* segments 7.8 mg)] × (average weight of wild-type and *rib1* segments = 9.99 mg). In Table I, the average and standard error of the mean of 14 to 15 individuals from two or three assays are reported in pmol of auxin transported.

## Calculation of Amount of Transported Auxin in All Assays

Transported tritiated auxin in each segment was determined by scintillation counting. The dpm transported in each sample was converted to fmol or pmol of auxin using the specific activity of the auxin. For seedling assays, both hypocotyl and root assays, the amount of auxin is reported in fmol. The amount of transport is comparable to previously published results (Rashotte et al., 2000, 2001, 2003), but in those previous papers the units of auxin transported in roots and hypocotyls were mislabeled as pmol and should have been reported as fmol. For inflorescence assays, higher levels of tritiated auxin were used (333 nM versus 100 nM) and higher counts were obtained. Inflorescence transport values are reported in pmol and were correctly reported as pmol in a previous report (Rashotte et al., 2003).

## Effects of Light on Hypocotyl Elongation

Hypocotyl length was determined by growing seedlings on horizontally oriented plates containing either Suc-free growth media (GM) solidified with 0.7% Difco agar or GM with 1.5% Suc and 0.8% Noble agar. GM consists of 1× Murashige and Skoog basal salts, 1% Suc, 0.5 g/L MES, 1 mg thiamine, 0.5 mg $L^{-1}$ pyridoxin, 0.5 mg $L^{-1}$ nicotinic acid, 100 mg $L^{-1}$ myo-inositol, with pH adjusted to 5.7 with 1 N KOH (Valvekens et al., 1988). Seedlings were germinated and grown in continuous dark, high white light (approximately 90 $\mu$mol $m^{-2}$ $s^{-1}$), low white light (approximately 5 $\mu$mol $m^{-2}$ $s^{-1}$), red light, or blue light. Light values indicate the amount of light on the outside of petri dishes. White light was provided by cool-white (Sylvania) fluorescent tubes; red light was provided by F40 gold (Sylvania) fluorescent tubes filtered by Rohm and Haas red plexiglas number 2423 (3.18-mm thick, Cadillac Plastic); and blue light was provided by F40 blue (Sylvania) fluorescent tubes filtered by Rohm and Haas blue plexiglas number 2424 (3.18-mm thick, Plastiques Marcon). The far-red light experiments were done in the laboratory of Professor X.-W. Deng at Yale University using F48T12/232/VHO red (Sylvania) fluorescent tubes filtered by far-red plexiglas FrF700 (3.18-mm thick, Westlake Plastics). Low white-light conditions were achieved by placing plates away from the light source, and plates received mostly indirect light in this case. Hypocotyl length was determined on 7-d-old seedlings grown in the absence of Suc or on 5-d-old seedlings grown on 1.5% Suc. Hypocotyls were measured either directly with a ruler or by tracing magnified seedlings using an overhead projector. The tracings were then digitally scanned, and measured using the public domain National Institutes of Health (NIH) Image program (developed at the U.S. NIH and available on the Internet at http://rsb.info.nih.gov/nih-image/). Data presented for hypocotyl elongation in the absence of Suc is from one representative assay; similar results were obtained in one to five other trials. Data presented for hypocotyl elongation with Suc is the average of seven or nine trials, as indicated.

## Effects of IAA and IBA on Hypocotyl Elongation

Seeds were surface sterilized by vapor phase sterilization (Clough and Bent, 1998), and then stratified 4 to 7 d in the dark at 4°C before being germinated. IAA and IBA were dissolved in 1 N NaOH and diluted in water to a final stock concentration of 1 mg $mL^{-1}$ and filter sterilized. Appropriate amounts of the sterile stocks were added to media after autoclaving to obtain the different concentrations required.

Hypocotyl elongation assays were performed on horizontally oriented GM plates containing 0.8% (w/v) Difco agar. After stratification, seeds plated directly on auxin-containing plates or control media were placed either in dark, high constant white-light conditions (90 $\mu$mol $m^{-2}$ $s^{-1}$) or low-light conditions (5 $\mu$mol $m^{-2}$ $s^{-1}$). Wild-type and *rib1* seedlings were plated to each of the two halves of the same plate, to ensure that they were being exposed to exactly the same conditions. 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 NIH Image program. Similar results were obtained in three separate trials for each light condition. Data from a single representative trial are presented.

## Lateral Root Formation

Lateral root formation assays were performed as described by Rashotte et al. (2001) with some modifications. Seedlings were germinated on vertically oriented plates containing GM with 1.5% Suc and 0.8% Noble agar under low white-light conditions (5 $\mu$mol $m^{-2}$ $s^{-1}$). After 4 d, seedlings were transferred to the same media containing the indicated amounts of auxins or transport inhibitor. The number of lateral roots on the whole primary root was counted under a dissecting microscope after six more days of growth; all lateral roots that had emerged from the primary root were counted. Each value is the average for 17 or 18 individual seedlings. Similar results were obtained in three separate trials. Figure 3 shows combined data from two trials for IAA and IBA. Figure 4 shows combined data from two (NPA) or three (TIBA) trials.

For all hypocotyl elongation and lateral root formation assays, wild-type and mutant seedlings were placed on two halves of the same plate to ensure exposure to identical conditions.

## Effect of NPA on Seedling Growth

Sterile and stratified wild-type and *rib1* seeds were plated on a horizontal line across two halves of a petri dish containing GM with 1% Suc and 0.1 $\mu$M NPA, and grown under high, white light for 14 d. A picture was taken on a Leica stereomicroscope at a magnification of 3.15×.

## Statistics

The data were analyzed by two-tailed Student's *t* tests for equal variance when comparing wild type and *rib1* using Microsoft Excel. A three-way factorial ANOVA was performed using Statistica v5.5 (Statsoft). A Neuman-Keuls post-hoc comparison of means was performed to determine for each auxin, whether there were significant differences between wild type and *rib1* at each concentration of IAA and IBA that was used.

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