

Auxin-induced leaf blade expansion in *Arabidopsis* requires both wounding and detachment

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Elevation of leaf auxin (indole-3-acetic acid; IAA) levels in intact plants has been consistently found to inhibit leaf expansion whereas excised leaf strips grow faster when treated with IAA. Here we test two hypothetical explanations for this difference in growth sensitivity to IAA by expanding leaf tissues *in vivo* vs. *in vitro*. We asked if, in *Arabidopsis*, IAA-induced growth of excised leaf strips results from the wounding required to excise tissue and/or results from detachment from the plant and thus loss of some shoot or root derived growth controlling factors. We tested the effect of a range of exogenous IAA concentrations on the growth of intact attached, wounded attached, detached intact, detached wounded as well as excised leaf strips. After 24 h, the growth of intact attached, wounded attached and detached intact leaves was inhibited by IAA concentrations as little as 1 μ M in some experiments. Growth of detached wounded leaves and leaf strips was induced by IAA concentrations as low as 10 μ M. Stress, in the form of high light, increased the growth response to IAA by leaf strips and reduced growth inhibition response by intact detached leaves. Endogenous free IAA content of intact attached leaves and excised leaf strips was found not to change over the course of 24 h. Together these results indicate growth induction of *Arabidopsis* leaf blade tissue by IAA requires both substantial wounding as well as detachment from the plant and suggests *in vivo* that IAA induces parallel pathways leading to growth inhibition.

Introduction

The plant hormone auxin (i.e., indole-3-acetic acid; IAA) controls or influences most aspects of plant development and physiology.¹ Concerning leaf development alone, its morphogenic effects include control of the initiation of leaf primordia,² control of vascular differentiation,³ as well as control of leaf expansion during both the cell division leaf growth phase,⁴ and the cell enlargement leaf growth phase.⁵

The post-cell division cell-enlargement phase of leaf growth accounts for most final leaf size as cell division is generally complete when leaves have reached no more than 20% of their final surface area.^{6,7} Tissue auxin content (both in the active free form or in various inactive conjugated forms⁸) varies greatly in plant tissues with the highest levels in apical meristematic tissues and young leaves. As leaves expand auxin levels fall.^{9,10} It has been suggested that cell expansion in leaves is promoted by auxin only at lower concentrations.⁵ Indeed, in the intact plant sustaining high levels of leaf auxin by various means has consistently resulted in inhibition of leaf expansion

of expanding leaves. For example, *Petunia* transformed to constitutively overproduce IAA has smaller leaves.¹¹ The *sur* and *yucca* mutants of *Arabidopsis* also have elevated IAA levels and smaller leaves.¹² Application of the auxin transport inhibitor NPA (1-N-naphthylphthalamic acid) to expanding *Phaseolus* leaf petioles increased leaf auxin, slowed growth and decreased final leaf size.¹⁰ Though application of aqueous auxin solutions directly to expanding *Phaseolus* leaves does initially increase growth for up to several hours,¹³ growth rate subsequently slows so that after 24 h treated leaves are significantly smaller than controls.¹⁴

This auxin-induced growth inhibition of leaf expansion in intact plants contrasts with the response of isolated tissues. Leaf strips excised from expanding *Nicotiana* leaves¹⁵⁻¹⁸ and from expanding *Phaseolus* leaves¹⁴ grew faster in a range of auxin concentrations compared with non-auxin controls over 24 or 48 h periods. This growth begins within 60 min of treatment.¹⁶ Auxin-induced leaf strip growth is always epinastic (downward curving) as the adaxial (upper) blade surface responds more than the abaxial (lower) surface.

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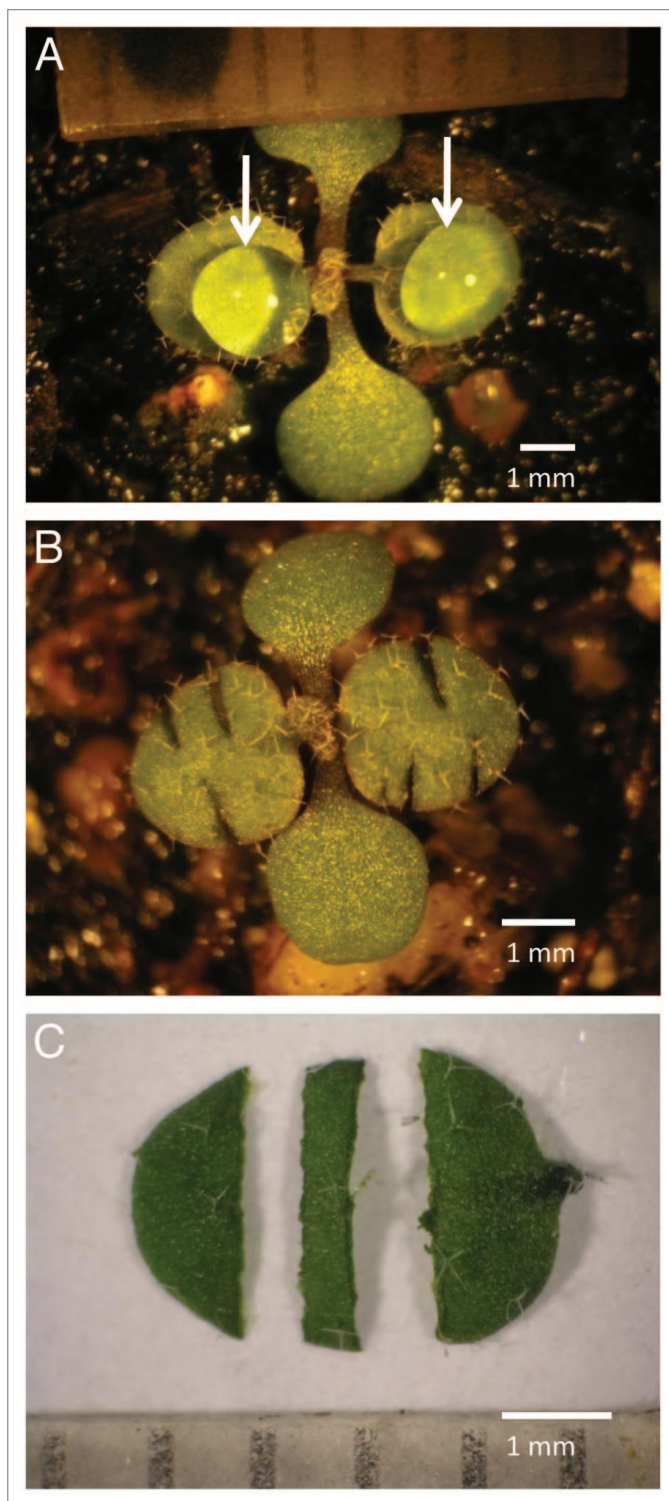


Figure 1. View of young *Arabidopsis thaliana* plant with intact attached leaves as used in this study (A), a similar plant shown with wounded attached leaves (B) and a detached leaf illustrating preparation of leaf strips (C). For all experiments, soil grown plants were selected with a partially expanded, similarly sized, pair of the first and second true leaves (each leaf measuring between 2.7 and 3.3 mm across). Shown in (A) are leaves as treated as “intact attached leaves,” with 5 μ l droplets (indicated by arrows) of control and of IAA containing solution administered to the first and second leaves of each plant. For “wounded attached leaves” (B), each of the first and second leaves were wounded with three incisions administered as shown from leaf margin to close to the midrib before the application of droplets (not shown). For “detached leaf” experiments, both leaves were excised in the distal portion of the petiole and floated, adaxial side down in most experiments, one of each leaf pair, on the control and on the IAA containing solution (not shown). Excised “leaf strips” were prepared 0.5 mm wide with a double-bladed cutter from the central portion of each leaf blade as shown (C) of both first and second leaves. The excised leaf strips, one from each leaf pair, were floated either on control or IAA containing solutions as with detached leaves.

or detachment from the plant and thus loss of some shoot or root derived growth controlling factors that otherwise condition expanding leaf cells to respond to auxin with slower growth. For our experiments we chose to experiment with young soil-grown *Arabidopsis* seedlings. This system offered the same advantage as young *Phaseolus* plants used in previous studies^{10,14} as the paired first and second true leaves in both plant species develop simultaneously allowing opposite untreated leaves to serve as a within plant control. Unlike *Phaseolus*, however, the first and second true leaves emerge nearly flat allowing for nondestructive initial sampling of leaf size. The results of the experiments described here suggest that both wounding and detachment are required for expanding leaf cells to show auxin-induced growth.

Results

To test whether leaf blade growth induction, as opposed to growth inhibition, by IAA treatment depends upon leaf wounding or upon leaf detachment from the plant we conducted a series of experiments in which the effect of applied IAA on the growth (increase in surface area) of leaf blade tissue was tested across a range of IAA concentrations with excised leaf strips, intact attached leaves, wounded attached leaves, detached (but otherwise intact) leaves and wounded detached leaves.

Figure 1 illustrates the conduct of the various growth studies in this investigation. For all experiments soil grown *Arabidopsis* seedlings were selected with approximately equal sized 1st and 2nd true leaf pair, each leaf blade measuring 2.7–3.3 mm across the lamina at mid-blade (**Fig. 1A**). By this point in their development the cells of these two leaves have ceased dividing with the continuing rapid leaf growth due entirely to cell expansion.⁷ In order to eliminate the effect of between plant variance in growth from our results, for all experiments, leaves were treated and compared pair-wise with one randomly chosen leaf of the 1st and 2nd true leaf pair on each plant serving as a control and the other as the experimental leaf. **Figure 1A** also shows leaves as treated as intact attached leaves. In those experiments the control was administered a 5 μ l droplet of control solution. The experimental

Reported here are the results of an investigation into why auxin both induces increased growth in excised leaf lamina tissues but also acts to slow growth in same tissues in the intact plant. Two hypothetical explanations for this difference were considered. First we asked if auxin-induced growth of excised tissues is dependent on the changed gene expression environment produced by the wounding required to excise tissue. We also asked if auxin-induced growth of excised tissues results from separation

Figure 2. Effect of light intensity and of IAA on the growth and epinastic curvature of leaf strips cut from expanding *Arabidopsis* leaves floated on either a control or IAA containing solutions in either high light ($275 \mu\text{mol s}^{-1} \text{m}^{-2}$), low light ($135 \mu\text{mol s}^{-1} \text{m}^{-2}$) or dark. Shown in (A) is growth (increase in area as % of initial area) of pooled control leaf strips. (B) shows the difference in relative growth between IAA treated and control leaf strips (i.e., the growth of each IAA treated strip minus that of the control strip from the same plant expressed as % of initial area) over 24 h in high light (●, ○) low light (■, □) and dark (▲, △). (C) shows the difference (IAA treated minus control) in epinastic curvature (measured as the angle created by the tangent of terminal portion of each strip viewed in profile) expressed in degrees per mm of the same pairs of strips shown in (B). Data are means \pm standard errors ($n = 24$). Significant differences (Student paired t-test) between IAA treated and controls leaf growth and curvature are indicated by filled symbols. Open symbols indicate no significant differences.

leaf blade received the same solution augmented with IAA. For wounded attached leaves (Fig. 1B), the leaves were wounded with three incisions administered as shown from leaf margin to the midrib before the application of droplets the control or IAA containing solutions (not shown). For detached leaf experiments, both leaves of the true leaf pair were excised in the distal portion of the petiole and floated, one of each leaf pair, on the same control solution and one on the IAA containing solution (not shown). Excised leaf strips were prepared 0.7 mm wide with a double-bladed cutter from the central portion of each leaf blade as shown (C) of both first and second leaves. The excised leaf strips, one from each leaf pair, were floated on control or IAA containing solutions as with detached leaves.

Figure 2 shows the effect of light conditions and of IAA on growth of excised leaf strips over 24 h. Control strips floated 24 h on a nutrient solution in continuous high light ($275 \mu\text{mol s}^{-1} \text{m}^{-2}$), low light ($135 \mu\text{mol s}^{-1} \text{m}^{-2}$) or in darkness grew significantly (Fig. 2A). Others have shown that growth of excised leaf tissues is enhanced by light.¹⁹ Here as well, growth was significantly greater in lower light and higher light conditions than in darkness. The high light treatment was evidently supra optimal for growth as these strips grew less than those in low light. Also, conspicuous anthocyanin accumulation, a hallmark of plant stress,²⁰ occurred in the abaxial epidermis of the high light strips. Strips floated on a nutrient solution also including IAA tended to grow more than control strips (Fig. 2B). The growth of strips in the apparently stressful high light was most sensitive to IAA as strips treated with between 10 and 300 μM IAA grew significantly more than control strips. Strips treated with IAA in low light only grew significantly faster than controls in the highest concentration tested (300 μM), while strips treated in darkness showed no significant response at either of the tested IAA concentrations 10 and 100 μM . These results show that, as in *Phaseolus* and *Nicotiana*, IAA induces increased growth of *Arabidopsis* leaf strips though with a clear sensitivity to light conditions.

With other species (i.e., *Nicotiana*¹⁵⁻¹⁷ and *Phaseolus*¹⁴) IAA-induced leaf strip growth is always epinastic. Figure 2C shows that IAA treatment also produces epinasty in *Arabidopsis* leaf strips. The epinastic response was more sensitive to IAA than surface area growth as all IAA treatments of 10 μM or higher concentration produced measurable epinasty regardless of light treatment.

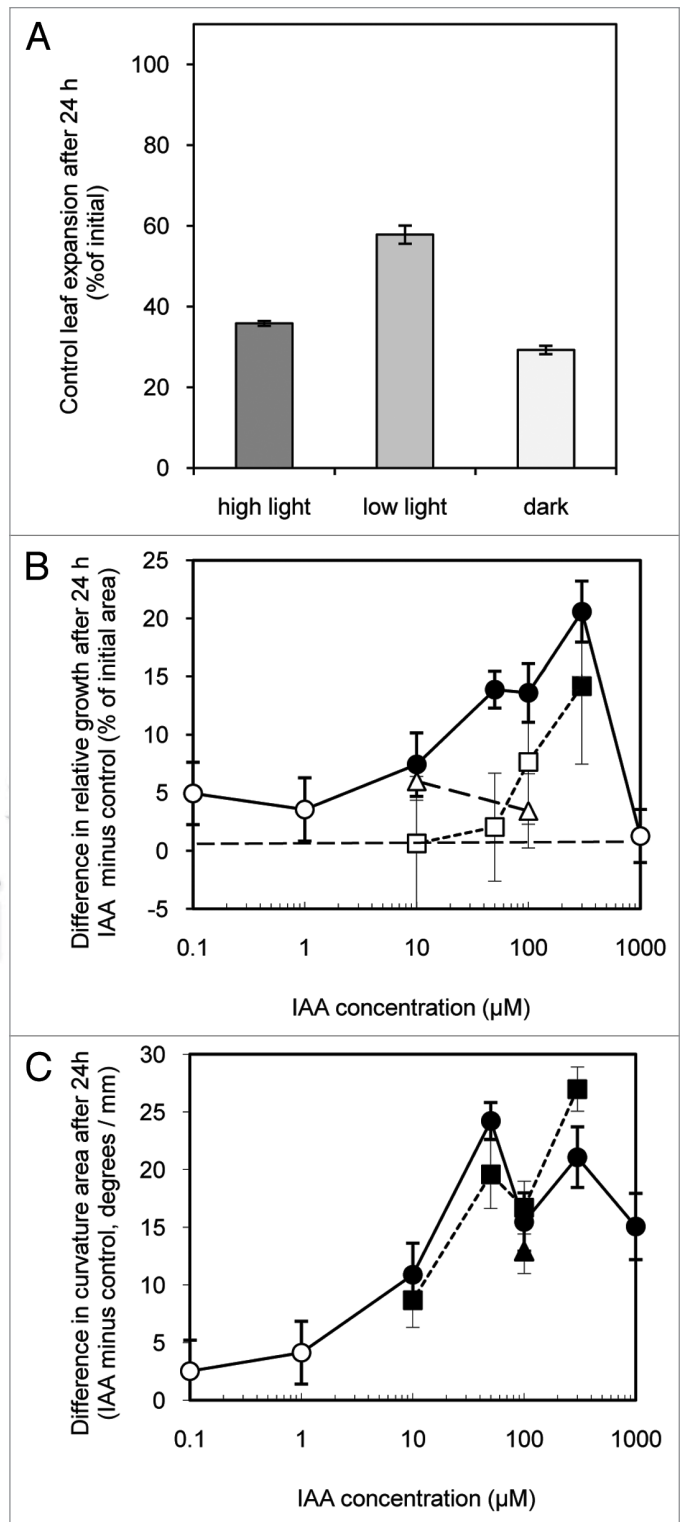


Figure 3 shows the effect of wounding and of IAA on growth and epinastic curvature of intact attached leaves (as shown in Fig. 1A) and of wounded attached leaves (Fig. 1B) under low light conditions ($135 \mu\text{mol s}^{-1} \text{m}^{-2}$) after 24 h. Intact attached leaves treated with control solution (on plants with an opposite IAA treated leaf) approximately doubled in size over 24 h (Fig. 3A), not significantly different for that of control leaves of plants in

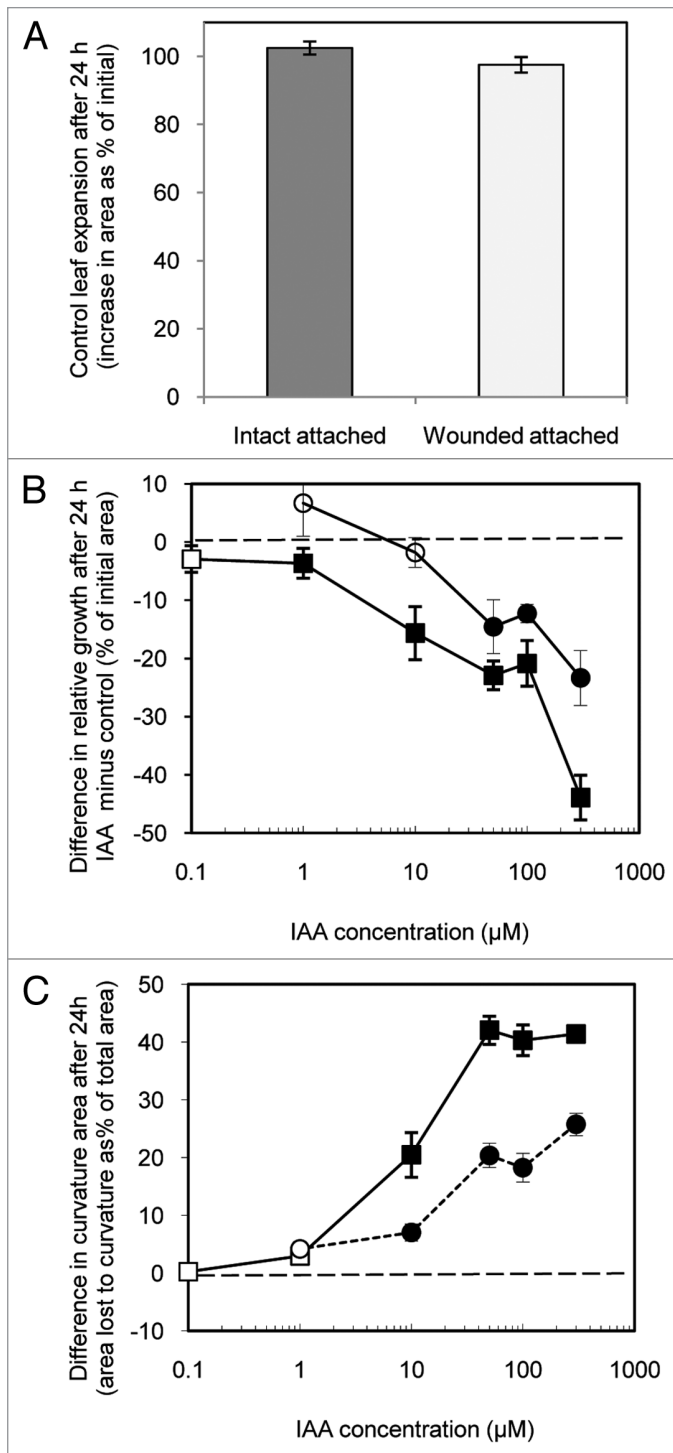


Figure 3. Effects of (A) wounding on the expansion of attached *Arabidopsis thaliana* leaves, (B) IAA on the expansion of both intact attached and of wounded attached leaves and (C) IAA on the curvature of both intact attached and of wounded attached leaves under low light. Shown in (A) is growth (increase in area as percent of initial area) of the pooled values from control leaves after 24 h. (B) shows the difference in relative growth between IAA treated and control leaves (i.e., the growth of each IAA treated leaf minus that of the control leaf of the same plant expressed as percent of initial area) over 24 h while intact attached (■, □) or while wounded attached (●, ○). For the same leaf pairs, (C) shows the difference (IAA treated minus control) in epinastic curvature after 24 h as the difference in area before and after flattening expressed as percent of the flattened area. Data are means \pm standard errors ($n = 25\text{--}39$). Significant differences (Student paired t-test) between IAA treated and controls leaf growth and curvature are indicated by filled symbols. Open symbols indicate no significant differences.

24 h (Fig. 3A). As well, the growth of control solution treated leaves, whether wounded or intact, did not appear to be effected by IAA treatment of the opposite leaf blade with IAA as comparison between groups differing in the IAA concentration administered to the opposite leaf blade found no consistent trends (data not shown).

IAA treatment of leaves slowed their growth relative to the control leaves on the same plants (Fig. 3B) after 24 h. This was especially true of the intact leaves which were growth inhibited by as little as $1 \mu\text{M}$ IAA. Wounded attached leaves were also growth-inhibited but only by concentrations of $50 \mu\text{M}$ and higher suggesting that wound stress decreases the growth inhibition sensitivity of leaves. Together these data indicate that, while wounding does appear to lessen the inhibiting effect of IAA on leaf blade growth, wounding alone is not sufficient to allow the IAA-induced growth seen with excised strips.

Applied at concentrations of $10 \mu\text{M}$ or more, whether to intact or wounded leaves, the growth inhibiting effect of IAA was associated with often dramatic epinastic leaf curvature after 24 h. This same effect was observed earlier with *Phaseolus*.¹⁰ Here (Fig. 3C) this epinastic effect (measured as the area of the flattened leaf minus the area presented by the leaf unflattened) was particularly dramatic with intact leaves treated with high concentrations of IAA.

Treatment of intact attached *Phaseolus* leaves with IAA and other auxins results in growth inhibition and epinastic curvature within 24 h^{10,14} this is preceded, however, by an initial transient hyponasty. This response, which develops within 2 h and lasts about 6 h, has been well characterized^{10,21-25} and is due to an auxin-induced relative increase in abaxial surface growth¹³ associated with an initial surge in auxin-induced leaf growth.¹⁴ Figure 4 shows that no similar response to the initial hyponastic growth surge seen in *Phaseolus* occurs in intact attached *Arabidopsis* leaves treated with $50 \mu\text{M}$ IAA. While significant IAA-induced epinasty is evident within 4 h and relatively slower growth is evident in IAA treated leaves within 8 h, no initial hyponasty is evident in these data or in casual observation of treated plants. The results suggest that the transient hyponasty seen with auxin treated *Phaseolus* leaves is not a general characteristic of all plant leaves.

Figure 5 shows the effect of light conditions and of IAA on growth and epinastic curvature of detached, but otherwise

which both 1st and 2nd true leaves were treated with control solution ($100.55 \pm 2.76\%$, $n = 120$). The leaves treated with control solution did, however, grow dramatically more than excised strips subjected to similar light conditions (Fig. 2A). Slower growth by strips might simply result from slower water uptake due to greater hydraulic resistance resulting from severed vasculature. Alternatively, altered metabolism within wounded strips might reduce the osmoregulation driving water uptake.

Wounding attached leaves appeared to have no consistent effect on subsequent control solution treated leaf expansion after

intact, leaves after 24 h. Detached leaves floated on control solution differed in growth depending on light treatment as those floated in low light ($135 \mu\text{mol s}^{-1} \text{m}^{-2}$) grew significantly more than those floated in high light ($275 \mu\text{mol s}^{-1} \text{m}^{-2}$). Growth of detached leaves was similar to that of excised strips grown under the same light conditions (Fig. 2A) and less than that of intact attached or wounded attached leaves (Fig. 3A). IAA treatment of detached leaves slowed their growth relative to the control leaves on the same plants (Fig. 5B) after 24 h. Leaves grown in low light were especially sensitive, growing significantly slower in as little as $10 \mu\text{M}$ IAA. Detached leaves grown in high light were inhibited by $50 \mu\text{M}$ or more IAA. As with attached leaves (wounded or intact) and with excised strips IAA treatment induced epinastic curvature with the response greatest by low light grown detached leaves (Fig. 5C). The results of these experiments with intact detached leaves suggested that detachment alone cannot be responsible for the growth-induction response seen in excised strips.

As the growth of detached leaves is generally inhibited by IAA while the growth of excised strips is increased or induced by IAA (above) it would seem that substantial wounding (i.e., more than just leaf detachment) is a requirement for the IAA growth induction response by Arabidopsis leaf blade tissue. A question then is whether wounded attached leaves as we prepared them (Fig. 1B) are sufficiently wounded to have a growth induction response when treated with IAA. Is the lack of IAA-induced growth found with those leaves due to continued attachment to the plant or due to insufficient wounding? Figure 6 shows the effect of IAA on the growth and epinastic curvature of wounded detached leaves (i.e., wounded as for wounded attached leaves then detached in the distal portion of the petiole) and floated on solutions for 24 h in low light ($135 \mu\text{mol s}^{-1} \text{m}^{-2}$). Wounded detached leaves on control solutions grew $70.54 \pm 1.26\%$ over 24 h, less than intact attached or wounded attached leaves (Fig. 3A) but, perhaps surprisingly however, more than either control leaf strips (Fig. 2A) or intact detached leaves controls (Fig. 5A). It may be that the wound incisions, by disrupting the cuticle barrier, reduced the hydraulic resistance to water uptake thereby enhancing growth.

IAA treated wounded detached leaves (unlike detached intact leaves and like excised leaf strips) grew more relative to the control leaves on the same plants (Fig. 6A) over 24 h. Since the growth of wounded attached leaves is inhibited by IAA treatment while the growth of wounded attached leaves is induced by IAA, it is apparent that leaf blade detachment (separated from wounding) is a requirement for a growth induction response to IAA by Arabidopsis leaves. As with all other leaf blade preparations (i.e., strips, intact attached, wounded attached, detached intact), wounded detached leaves were made epinastic by IAA treatment over 24 h (Fig. 6B).

Wounding has been shown to induce a drop in leaf free IAA in tobacco leaves.²⁶ One possible explanation for a wounding requirement for leaf blade growth induction by IAA could be that wounding lowers endogenous free IAA levels within leaves to below that optimal for growth. Thus exogenous IAA would restore the levels within the leaf to optimal levels and restore growth. We tested this possibility by analyzing leaf blade free

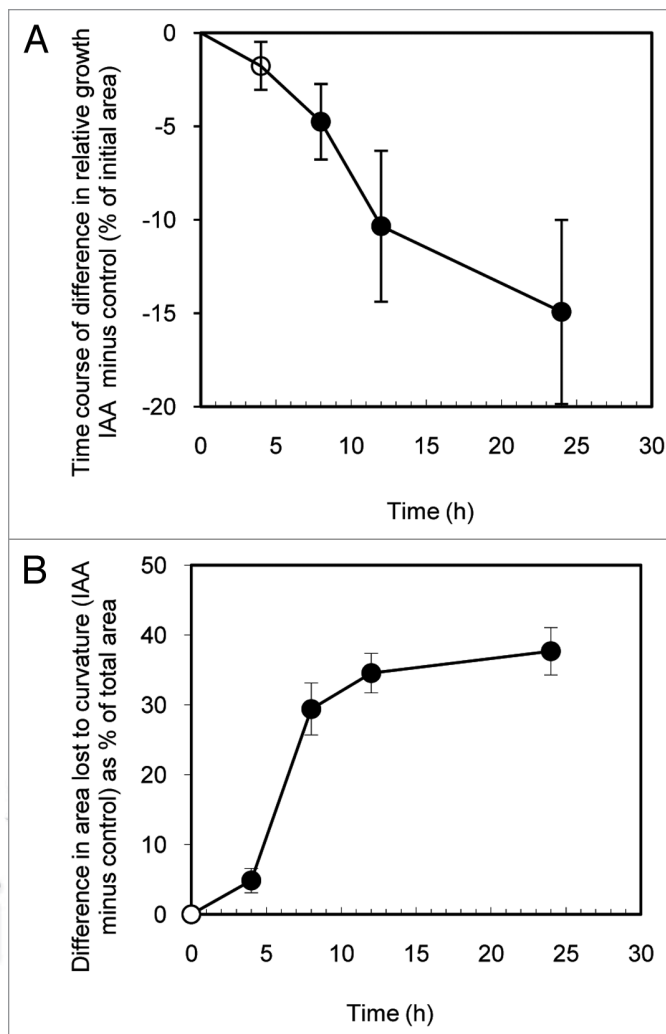


Figure 4. Time course for IAA-induced growth inhibition (A) and development of epinasty (B). Shown in (A) is the difference in relative growth between IAA treated ($50 \mu\text{M}$) and control intact attached leaves (i.e., the growth of each IAA treated leaf minus that of the control leaf of the same plant expressed as percent of initial area). For the same leaf pairs, (B) shows the difference (IAA treated minus control) in epinastic curvature as the difference in area before and after flattening expressed as percent of the flattened area. Data are means \pm standard errors ($n = 24$). Significant differences (Student paired t-test) between IAA treated and controls leaf growth and curvature are indicated by filled symbols. Open symbols indicate no significant differences.

IAA content in variously prepared leaves and leaf strips. Table 1 shows that the free IAA concentration in intact attached leaves treated with control solution for 0, 6, 12 or 24 h does not change significantly. The mean of sample values at the different times ranging from 20.3 to 33.2 ng free IAA/g fresh wt are consistent with other estimates of young leaf IAA levels.^{9,10} Table 1 also shows that the free IAA content of freshly excised strips is approximately the same as that of entire leaves. Floating excised strips on control solution and subjecting them to high light [thus making them susceptible in IAA-induced growth (see above)] found the IAA content of excised strips not to change significantly after 6, 12 and 24 h. As well, the free IAA content of wounded attached

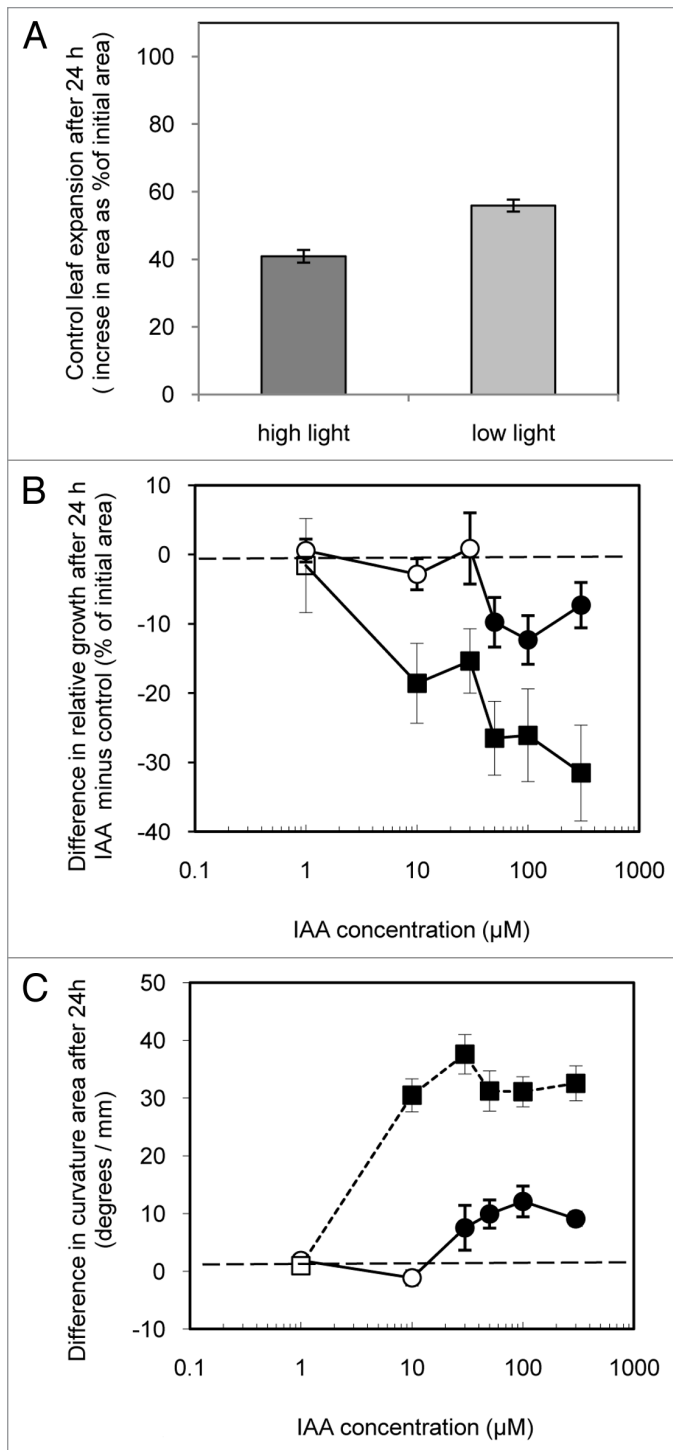


Figure 5. Effects of light intensity and IAA on the growth and curvature of detached intact *Arabidopsis thaliana* leaves. Shown in (A) is growth (increase in area as % of initial area) of the pooled values from control leaves in high light ($275 \mu\text{mol s}^{-1} \text{m}^{-2}$) and in low light ($135 \mu\text{mol s}^{-1} \text{m}^{-2}$). (B) shows the difference in relative growth between IAA treated and control leaves i.e., the growth of each IAA treated leaf minus that of the control leaf of the same plant expressed as percent of initial area after 24 h while adaxial surface down in high light (●, ○) or in low light (■, □). For the same leaf pairs, (C) shows the difference (IAA treated minus control) in epinastic curvature after 24 h as the difference in area before and after flattening expressed as percent of the flattened area. Symbols in (C) as in (B). Data are means \pm standard errors ($n = 24$). Significant differences (Student paired t-test) between IAA treated and controls leaf growth and curvature are indicated by filled symbols. Open symbols indicate no significant differences.

low relative to the exogenous concentrations found effective for induction of growth and epinasty or inhibition of growth (i.e., $10 \mu\text{M}$ in most experiments). Assuming *Arabidopsis* leaf blade tissue in our experiments to 95% water and that the hormone was uniformly distributed, the highest mean IAA content measured (e.g., 52.9 ng/g for excised strips in low light) would have the endogenous free IAA within the tissue at $0.32 \mu\text{M}$. While effective permeation of the leaf by exogenous IAA in our experiments is a potential question, it would appear that the effects on leaf growth and curvature we report require raising the leaf free IAA by perhaps an order of magnitude or more.

Discussion

The results reported here show that leaf strips excised from expanding *Arabidopsis* leaves (as with strips excised from leaves of *Phaseolus*¹⁴ and from *Nicotiana*¹⁵) were induced to grow faster by IAA. Also, as with *Phaseolus*,¹⁰ the growth of intact attached *Arabidopsis* leaves was inhibited by IAA (Fig. 3), although without (Fig. 4) the initial hyponastic growth surge found with *Phaseolus* leaves.^{13,14} Wounding attached *Arabidopsis* leaves did not reverse the effect on growth by IAA (Fig. 2), although the sensitivity of growth to inhibition by IAA is decreased by wounding. Detached intact leaves also respond to IAA treatment with inhibition of growth (Fig. 5) but wounded detached leaves are induced to grow faster by IAA (Fig. 6). Together these results indicate that growth induction of *Arabidopsis* leaf blade tissue by IAA requires both substantial wounding as well as detachment from the plant.

Both wounding and exogenous applications of IAA are known to induce the production of the plant hormone ethylene,²⁷ suggesting the possibility that the growth effects seen here are induced by ethylene especially as elevated plant ethylene is associated with petiole epinasty in some plants.²⁷ Earlier work, however, suggests ethylene induction is not responsible for either IAA-induced growth of excised leaf strips or for IAA-induced growth inhibition attached leaves. In *Phaseolus*, a poor correlation was found between ethylene biosynthesis and IAA-induced leaf strip growth and ethylene was unable to produce epinasty also in leaf strips.¹⁵ Growth inhibition by IAA of intact attached *Phaseolus* leaves was blocked by inhibition of ethylene. As well, the growth of attached leaves of ethylene insensitive *Arabidopsis*

leaves, detached leaves subjected to either high light or low light, wounded detached leaves subjected to low light was not significantly changed by these treatments. Only leaf strips subjected to low light for 24 h were found to have significantly different IAA content where the level was found to be elevated relative to all other treatments. Together the results suggests that wounding does not lower endogenous free IAA content in *Arabidopsis* leaves or that the results of our growth studies can be otherwise explained by changing endogenous IAA levels. Also, it should be noted that the endogenous IAA levels measured here are

ein-4 mutants was inhibited by IAA no differently than was the growth of the attached leaves of wild type plants.

Light is known to induce leaf cell expansion²⁸ with increased growth mediated by both increased photosynthesis as well as blue and red light receptors.¹⁹ In *Phaseolus* leaf discs light stimulated expansion is saturated by continuous white light at 100 $\mu\text{mol s}^{-1} \text{m}^{-2}$. In the current study, leaf strip expansion was similarly optimal under “low light” conditions (135 $\mu\text{mol s}^{-1} \text{m}^{-2}$) with “high light” (275 $\mu\text{mol s}^{-1} \text{m}^{-2}$) clearly supra optimal (Fig. 2A).

Our results suggest a model for the effect of elevated IAA on leaf blade expansion in *Arabidopsis*. We propose that elevation of IAA level within the leaf tissue leads to either decreased or increased cell expansion by two separate signal transduction pathways (A and B). Since leaves that are either unwounded or attached to the plant respond physiologically with decreased growth we suggest that two sub-pathways (A1 and A2) lead from IAA receptor binding to the a decrease in growth. The function of one of these sub-pathways (A1) is dependent on leaf attachment and presumably requires some continuously supplied root derived chemical factors (discussed below). The second sub-pathway (A2) is dependent on wounding and presumably requires the presence of wound-induced factors (discussed below). Since increased growth by leaf blade tissues requires both wounding as well as detachment a second signal transduction pathway leading to increased growth requires both presence of wound-induced factors and the absence of root derived factors. In our experiments, all IAA treatments that increased or decreased overall tissue expansion also produced epinastic curvature with the magnitude of the effect on growth, whether positive or negative, tightly correlated with the extent of curvature (Figs. 2C, 3C, 5C and 6B). When growth was slowed by IAA treatment (i.e., when tissue is either unwounded and/or attached to the plant), growth at the abaxial (lower) surface was slowed more than at the adaxial (upper) surface. When increased growth was induced by IAA treatment (i.e., when the tissue was both wounded and detached), growth at the adaxial (lower) surface was increased more than growth at the abaxial surface. These results suggests that pathways A and B not to be equally robust in the cells toward the adaxial and abaxial surfaces. In the adaxial epidermis (and perhaps immediately underlying cells) pathway B is apparently more sensitive to IAA than is the same pathway is in the cells of the abaxial epidermis (and underlying cells) while pathway A is more sensitive in the abaxial epidermis than in the adaxial epidermis.

The identity of an IAA receptor or receptors responsible for initiating pathways A and B (Fig. 7) are not apparent from our experiments. The principle IAA receptor proteins have been identified as TIR1^{29,30} and related AFB1–3 proteins³¹ which function to accelerate ubiquitin mediated removal of specific transcription repressor proteins that, in turn regulate the expression of genes associated with auxin-response DNA domains.¹ There is, however, considerable evidence for the existence additional receptors³² including auxin-binding protein 1 (ABP1). Though over-expression of ABP-1 has been shown to increase cell expansion in tobacco leaf strips¹⁸ and conditional repression of ABP-1 in

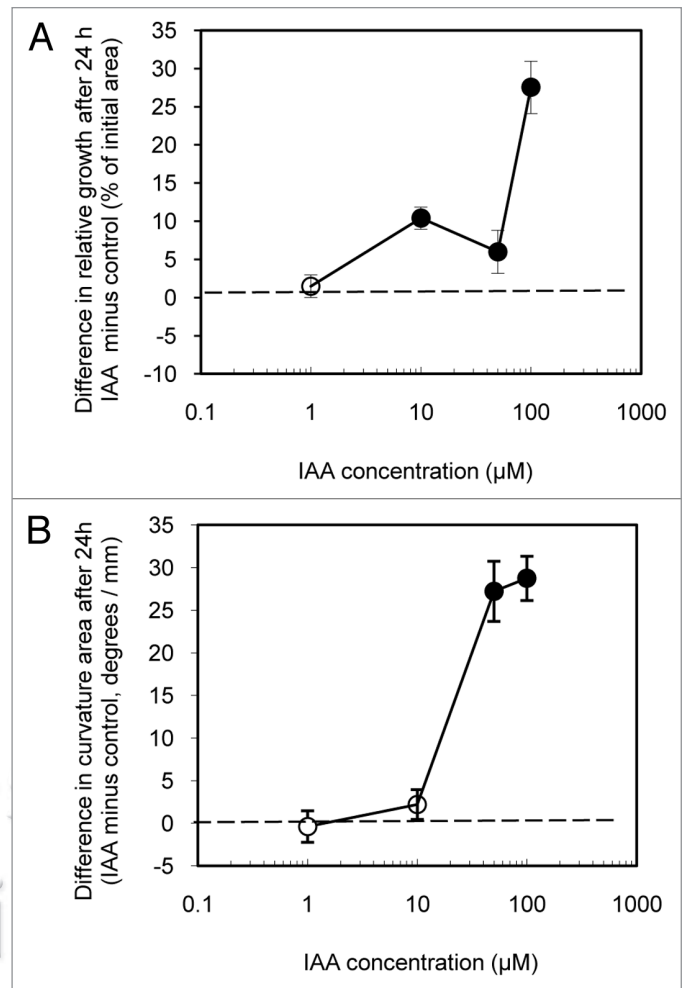


Figure 6. Effects of wounding and IAA on the growth and curvature of wounded detached *Arabidopsis thaliana* leaves. (A) shows the difference in relative growth between IAA treated and control leaves (i.e., the growth of each IAA treated leaf minus that of the control leaf of the same plant expressed as percent of initial area) after 24 h while adaxial surface down in high light while adaxial surface down in high light. For the same leaf pairs, (B) shows the difference (IAA treated minus control) in epinastic curvature after 24 h (measured as the area of the flattened leaf minus the area presented by the leaf unflattened) and expressed as percent of the flattened area. Data are means \pm standard errors ($n = 24$). Significant differences (Student paired t-test) between IAA treated and controls leaf growth and curvature are indicated by filled symbols. Open symbols, indicate no significant differences.

Arabidopsis retards leaf expansion,⁵ it is not clear that this receptor is specifically associated with signal transduction leading to IAA-induced growth or that involvement of other receptors are precluded from involvement.

Leaf wounding represents a threat to plants often associated with pathogen attack that must be effectively responded to. Not surprisingly, the transcriptional response to leaf wounding in *Arabidopsis* involves a substantial portion of the plant’s genome.³³ Interestingly, leaf wounding results in widespread downregulation of the expression of some IAA response genes.³³ In tobacco, however, genes responsible for increasing the hypersensitive

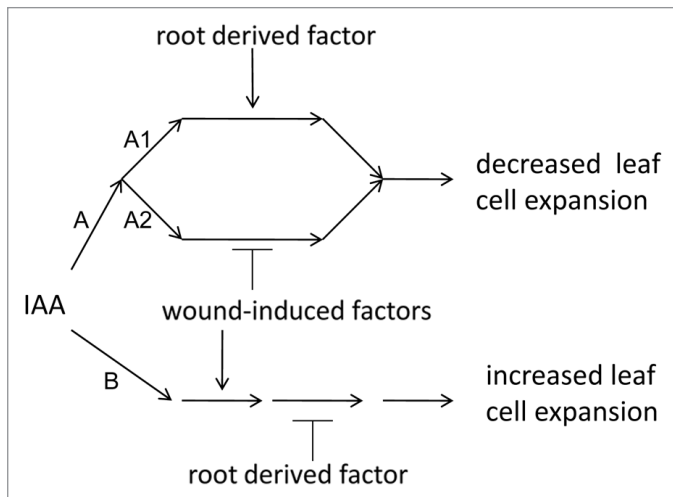


Figure 7. Simple general model for the effect of elevated IAA on growth of expanding leaf blades of *Arabidopsis*. The results presented here suggest that IAA applied to leaf blade tissues initiates two signal transduction pathways. Binding of IAA to an undetermined receptor (or receptors) might initiate separate signal transduction events leading in one case to decreased leaf cell expansion (A) or to increased leaf blade cell expansion (B). Since decreased leaf growth results from IAA treatment when leaves either remained attached to the plant or when the leaf blade tissue is unwounded, pathway (A) would appear to branch through two parallel sub-pathways (A1 and A2). A1 is induced in some fashion by leaf attachment possible due to provision to the leaf of a root derived factor. A2 is inhibited in some fashion by leaf wounding possibly involving wound-induced factors. Pathway (B) leading to increased leaf blade expansion appears to be upregulated by wounding possibly through wound-induced factors and downregulated by leaf attachment possibly through removal of a root derived factor.

response to infection are upregulated following wounding by binding of a specific transcription factor to the associated auxin-response domains of these genes.³⁴ How wounded-induced changes to the leaf gene expression environment contributes to a reversal in the growth response to IAA by expanding leaf cells is not clear.

In our results stress in the form of high light, sufficient to induce conspicuous anthocyanin expression, appeared to increase growth induction by IAA in the excised strips (Fig. 2B) and to decreased growth inhibition by IAA in detached intact leaves (Fig. 5B). It could be argued that the growth induction response to IAA by leaf strips in low light was simply less sensitive to IAA than high light treated leaf strips (Fig. 2B) because in low light control leaf strips grew much more than control strips in high light. Perhaps growth induction by IAA would be less evident in more rapidly growing tissues. Similarly, the reduced sensitivity of high light treated detached intact leaves to IAA growth inhibition compared with that of detached intact leaves in low light might be simply because control intact leaves in high light grew significantly less than control detached intact leaves in low light. This explanation is undermined by the effect of IAA on the growth of wounded detached leaves. Wounded detached leaves in low light without IAA grew more over 24 h ($70.54 \pm 1.26\%$) than either control excised strips (Fig. 2A) or detached leaves (Fig. 5A)

Table 1. Effects of wounding, light treatment and detachment on leaf free IAA content of *Arabidopsis thaliana* leaves

Leaf treatment	Time			
	0 h	6 h	12 h	24 h
intact attached	26.5 ± 7.1	24.7 ± 3.0	28.4 ± 4.9	33.2 ± 1.5
wounded attached				21.0 ± 4.7
detached (high light)				24.3 ± 2.1
detached (low light)				35.3 ± 3.3
wounded detached				19.4 ± 1.9
strips (high light)	27.4 ± 6.1	20.2 ± 1.4	23.3 ± 4.7	36.2 ± 3.4
strips (low light)				52.9 ± 13.4

First and second true leaves of young seedlings were variously treated. Using a high-throughput method, free IAA was extracted with organic solvents and purified using two solid-phase extraction steps from flash frozen leaves/leaf strips that had been variously treated (see Fig. 1) for the indicated times. Following methylation, tissue IAA was quantified by ion monitoring gas chromatography-mass spectrometry. Data are expressed as nanograms of free IAA per gram fresh weight, mean ± SE (n = 4–19). Strips treated 24 h in low light were found to contain significantly higher IAA content than tissue from all other treatments. No other significant differences (Duncan's multiple range test) between treatments were detected.

yet could still evidence substantial growth enhancement treated with IAA (Fig. 6A). A more attractive explanation for our data is that light stress results in similar changes to the leaf blade gene expression environment as does wound stress. There is extensive crosstalk within the signal transduction and altered gene expression among different stress responses.³⁵ Thus the gene expression environment induced in *Arabidopsis* leaves by wounding that results in reversing the physiological growth response to IAA from inhibiting growth to inducing growth would, at least in part, also be induced by high light stress. Perhaps other stresses (cold, salt, etc.) could also enhance the IAA growth induction response by *Arabidopsis* leaves.

Perhaps the most interesting aspect of our results is the requirement that leaves be detached from the plant to experience IAA-induced growth. Implied is that an entity, continuously supplied by the rest of the plant, somehow interacts with IAA or auxin signal transduction in leaves of the intact plant to inhibit growth and preventing IAA-induced growth. Our results give few clues to the nature of such a signal. Conceivably the signal could be electrical³⁶ but it seems unlikely that such signals would be continuously supplied. A chemical signal seems more likely. Such an entity could enter the leaf in either via phloem transport or the xylem stream. Long distance transfer of both mRNA and microRNA in the phloem is known to regulate gene expression.^{37,38} It seems likely, however, that the 1st and 2nd true leaves of *Arabidopsis* at the developmental stage we employed them in our experiments have already passed through the sink to source transition.³⁹ If they had, a role for these molecules in controlling the IAA response of expanding leaves would seem unlikely. The xylem stream could provide a more likely source of any number of root-derived potential modulators of IAA responses including abscisic acid, cytokinins, strigolactones, as well as an as yet unidentified signal molecule(s) evidenced by the bypass

mutant.⁴⁰ Of these, cytokinins seem unpromising in this respect as cytokinin deficient *Arabidopsis* transformants have impaired leaf expansion due to both fewer as well as smaller cells.⁴¹

Wounding can affect the level of endogenous free IAA as well as the biosynthetic pathway utilized. For example, slicing potato tubers results in a doubling in tissue IAA level between 12 and 24 h.⁴² In tobacco, however, crushing injury to leaf blade tissue results in a two- to 3-fold drop in the IAA content of adjacent leaf tissue within 6 h. This lower IAA level is sustained through 24 h post wounding.²⁶ This wound induced drop in leaf blade IAA levels is believed to induce both the plant defense gene encoding proteinase inhibitor II and nicotine production that occurs following wounding.^{43,44} In germinating bean, wounding by excision of the cotyledons resulted in a change in biosynthetic pathway from tryptophan dependent to tryptophan independent pathways and early excision doubled the rate of labeled anthranilate incorporation into IAA.⁴⁵ Our analysis of *Arabidopsis* leaf blade IAA content (Table 1) does not support a role for changing endogenous IAA levels in explaining the results seen here as no significant changes in IAA levels were found over the course of 24 h in either intact attached leaves (which respond to exogenous IAA with decreased growth) or in excised leaf strips subjected to high light (which are induced to grow by IAA).

Materials and Methods

Plant material. Columbia (Col-0) wild type *Arabidopsis thaliana* L. seeds (Lehle Seeds) were sown on moist potting medium [AIS (Enriched) *Arabidopsis* Growth Medium, Lehle Seeds] in plug flats enclosed within plastic trays and clear plastic humidity domes (all from Morton's Horticultural Products). Following stratification at 4° for 4 d, enclosed plug flats were placed in a growth chamber (Econair Bigfoot) at 19° under continuous light (approximately 135 $\mu\text{mol s}^{-1} \text{m}^{-2}$). After 4 d, during which the seeds germinated and the seedlings established themselves, the humidity covers were removed and the plants were grown an additional 7–9 d.

Growth studies. For all experiments individual seedlings were selected with approximately equal sized 1st and 2nd true leaf pair, each leaf measuring 2.7–3.3 mm across the lamina at mid-blade (Fig. 1A). For the “intact attached” experiments, plants were first digitally imaged and then one leaf of the 1st and 2nd true leaf pair was administered a 5 μl droplet of control solution containing full strength Murishige and Skoog nutrients (Caisson Laboratories) augmented with 10 mM KCl and pH 6 with 0.1 mM Mes/BTP. The osmolarity of control solution was determined by vapor pressure osmometer (Vapro 5520; Wescor) to be 102.4 ± 0.6 mmol/kg. The other (treatment) leaf received the same solution but also containing IAA (prepared from IAA stock solutions brought to pH 6 with BTP) at a range of concentrations. Humidity domes replaced over the plants kept the solutions from evaporating. After 24 h both the control and experimental leaf of each plant was detached and imaged first unflattened and a second time unrolled and gently flattened by the weight of a glass microscope slide.

For wounded attached experiments plants were similarly treated as above except that before application of control and IAA

droplets, three incisions were made to each leaf reaching from the leaf margin to close to the midvein (Fig. 1B).

For detached leaf experiments, both leaf blades of the true leaf pair were excised in the distal portion of the petiole and, after initial imaging, were floated individually, side down, in 3 ml of solution in 12 well tissue culture plates mounted on a rotary shaker (75 rpm) in either continuous high light (275 $\mu\text{mol s}^{-1} \text{m}^{-2}$) or low light (135 $\mu\text{mol s}^{-1} \text{m}^{-2}$) at 19°. One of each leaf pair was floated on the control solution and one on the IAA containing solution for 24 h after which they were patted dry and re-imaged unflattened and then flattened.

Excised leaf strips were prepared 0.5 mm wide with a double-bladed cutter from the central portion of each leaf blade leaf margin to leaf margin of both first and second leaves (Fig. 1C). The excised leaf strips, were first imaged gently flattened and subsequently were floated, one from each leaf pair, on control or IAA containing solutions as with detached leaves. After 24 h strips were reimaged both in profile (i.e., edge on) and then flattened.

For wounded detached experiments, leaves were wounded as for wounded attached (above), then imaged and floated, as described above, one of every leaf pair on control and one on IAA containing solutions for 24 h followed by reimaged as above.

Image J (public domain image analysis software; rsbweb.nih.gov/ij/) was used to quantify leaf and strip area before and after the 24 h treatment period. For analysis, the growth (increase in surface area as percent of initial area) of each control leaf was subtracted from that of the IAA treated leaf of the same plant. Curvature of strips was quantified as in reference 15 and 17, as the angle created by the intersection of the tangents described by the terminal portions of each strip when viewed in profile. Leaf curvature (for intact attached; wounded attached, detached and wounded detached experiments) was quantified as loss of area to curvature which was measured as the difference in leaf area before and after gentle leaf flattening following 24 h solution treatment. Analysis leaf curvature involved subtracting the curvature of the control leaf from that of the IAA treated leaf for each plant.

IAA quantification. Leaf blade tissue IAA was purified and quantified largely using a high-throughput method described previously in reference 46. Briefly, samples of 0.023–0.060 g consisting of variously pre-treated, flash-frozen and pooled leaves or leaf strips were homogenized along with an internal standard ($[^{13}\text{C}_6]$ IAA) in extraction buffer (65% isopropanol, 35% 0.2 M imidazole, pH 7.0). Following 1 h incubation on ice and centrifugation, extracts were loaded into 96-well microplates on a sample rack of an automated liquid handler programmed for sample purification. Sample purification consisted of elution first from a conditioned amino anion exchange column and then elution from a polymethacrylate column with the purified IAA of each sample finally in methanol.

The IAA of each sample was then methylated by combining with ethereal diazomethane containing 10% methanol from the sample, dried under N_2 and dissolved in 30 μL ethyl acetate. Actual IAA quantification was by GC-MS-selected ion monitoring as previously described by Ribnicky et al. using a model 6890N GC/5973Network MS (Agilent Technologies) equipped with a HP-5MS fused silica capillary column (Agilent Technologies). Injector temperature was at 280°C and an initial oven temperature of 70°C ramping 20°C min^{-1} to 280°C. The monitored ions

were mass-to-charge ratio 130 and 136 (quinolinium ions from sample IAA and from the $^{13}\text{C}_6$ -labeled internal standard, respectively) and mass-to-charge ratio 189 and 195 for the corresponding molecular ions.

Statistical analysis. Analysis of within plant leaf and leaf strip growth and curvature data was by means of Student paired t-tests. Between plant growth and curvature data analysis and endogenous leaf IAA content data analysis were by means of one-way analysis of variance with means separated using Duncan's multiple range test. All analyses were conducted using SPSS 17.0 (SPSS Inc.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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