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Leaf expansion in *Phaseolus*: transient auxin-induced growth increase

Christopher P. Keller

Department of Biology, Minot State University, 500 University Avenue West, Minot, North Dakota 58707

Abstract

Control of leaf expansion by auxin is not well understood. Evidence from short term exogenous applications and from treatment of excised tissues suggests auxin positively influences growth. Manipulations of endogenous leaf auxin content, however, suggests that, long-term, auxin suppresses leaf expansion. This study attempts to clarify the growth effects of auxin on unifoliate (primary) leaves of the common bean (*Phaseolus vulgaris*) by reexamining the response to auxin treatment of both excised leaf strips and attached leaves. Leaf strips, incubated in culture conditions that promoted steady elongation for up to 48 h, treated with 10 μM NAA responded with an initial surge of elongation growth complete within 10 hours followed by insensitivity. A range of NAA concentrations from 0.1 μM to 300 μM induced increased strip elongation after 24 hours and 48 hours. Increased elongation and epinastic curvature of leaf strips was found specific to active auxins. Expanding attached unifoliates treated once with aqueous auxin α -naphthalene acetic acid (NAA) at 1.0 mM showed both an initial surge in growth lasting 4–6 hours followed by growth inhibition sustained at least as long as 24 hours post treatment. Auxin-induced inhibition of leaf expansion was associated with smaller epidermal cell area. Together the results suggest increasing leaf auxin first increases growth then slows growth through inhibition of cell expansion. Excised leaf strips, retain only the initial increased growth response to auxin and not the subsequent growth inhibition, either as a consequence of wounding or of isolation from the plant.

INTRODUCTION

The morphogenic hormone auxin controls a number of plant developmental and physiological phenomena including embryogenesis, apical dominance, stem elongation, root development, and tropisms (Berleth and Sachs, 2001). Auxin is also important in leaf development controlling initiation of leaf primordia (Reinhardt et al., 2000), vascular development (Sieburth, 1999; Mattsson et al., 2003) and the cell-division phase of leaf expansion (Ljung et al., 2001).

Most final leaf size is produced by the post cell-division cell-enlargement phase of leaf expansion as cell division arrests by the time leaves have reached no more than 20 % of their final surface area (Poethig and Sussex, 1985, Beemster et al., 2005). This leaf expansion is

influenced by other non-auxin plant hormones (Goodwin and Erwee, 1983) and responsive to light (Van Volkenburgh, 1999), but auxin appears to play a role as well.

Excised tobacco (*Nicotiana tabacum* L.) leaf strips from expanding tobacco leaves curve epinastically when incubated in a range of auxin concentrations with the response complete within 24 hours (Keller and Van Volkenburgh, 1997; 1998). This curvature was found to result from an auxin-induced growth increase to the adaxial surface, though this was determined for only one auxin concentration (10 mM IAA). These results contrast, however, with some results that show increasing leaf auxin concentration inhibits leaf growth in intact plants. Transgenic auxin overproducing petunia (*Petunia hybrida*) have smaller epinastic leaves (Klee et al., 1987) and *Arabidopsis* auxin overproducing mutants *sur1* and *sur2* also have reduced leaf expansion (Boerjan et al., 1995). Most recently, Keller et al. (2004) found that aqueous application to expanding leaves of the naturally occurring auxin indole acetic acid (IAA) as well as the synthetic auxin NAA slowed long-term leaf expansion and final leaf size in common bean (*Phaseolus vulgaris*). Inhibition of auxin transport and the resulting elevation of endogenous auxin (approximately double) within individual bean leaves similarly inhibited leaf expansion. This last result, in turn, contrasts with an earlier finding that a well characterized transient hyponastic curvature of bean leaves treated with a range of auxin concentrations (Lippincott and Lippincott, 1971; Hayes and Lippincott, 1976; Hayes, 1977; 1978; 1981; Hayes and Lippincott, 1981) is due to an auxin-induced increase in abaxial surface growth (Hayes and Lippincott, 1976).

The auxin mechanism of action involves binding to the F box containing receptor proteins TIR1 (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005) and AFB1-3 (Dharmasiri et al., 2005b) leading to ubiquitin mediated removal of specific transcription repressor proteins (Leyser, 2002). Auxin perception by expanding leaf strips, however, involves separate signal transduction with auxin interacting with the auxin-binding protein 1 (ABP1) receptor protein (Jones et al., 1998; Chen et al., 2001) at the cell membrane (Steffens et al., 2001) and leading to altered osmoregulation (Yamagami et al., 2004). A hypothetical explanation for the apparent conflicting results concerning the effect of auxin on leaf expansion is that increased leaf auxin first increases growth and later inhibits growth. This current study tests this possibility by examining both the auxin growth response of leaf strips excised from expanding bean leaves as well as, at high resolution, the auxin growth response of expanding intact attached bean leaves. The results might suggest some additional insight into the roles TIR/AFB1-3 and ABP1 mediated signal transduction.

MATERIALS AND METHODS

Plant material

Intact *Phaseolus vulgaris* L cv. Contender (Southern States Cooperative Inc., Richmond, Virginia, USA) seeds of uniform weight (4.25–5.25 g) were imbibed on moist paper towels for 24 hours and grown in vermiculite under green house conditions for 11–13 days as previously described (Keller et al., 2004). Plants selected for experimentation had approximately equal sized unifoliates each with midribs between 40–50 mm in length with the longer at least 45 mm in length.

Leaf strip growth assays

Unifoliate leaf blades 40–50 mm in length were harvested and 10.0×1.5 -mm panels (leaf strips) cut from them using double-bladed cutters. Leaf strips, two from each leaf, were excised approximately mid-leaf from mesophyll regions parallel to secondary veins radiating from the midribs so as to include a minimum of vascular tissue. Leaf strips were immediately placed in 60×15 -mm petri dishes containing 10 mM KCL, 0.1 mM 2-(4-morpholino)-ethane sulfonic acid (Mes)/1, 3-bis[tris(hydroxymethyl)methylamino]propane (Btp) pH 6.0, and either 10 mM sucrose or full strength Murashige and Skoog medium (Murashige and Skoog, 1962) or the same solution also containing either α -naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), NAA, β -naphthalene acetic acid (2-NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,3-dichlorophenoxyacetic acid (2,3-D) or benzoic acid at various concentrations depending on the experiment. The petri dishes were gently agitated on a rotary shaker (30 rpm) under room lights. For leaf strip length measurements at various times depending on the experiment (i.e. 4, 6, 10, 24, and/or 48 hours), the strips were gently removed from solutions and placed on the stage of a dissecting microscope. Curvature was measured as the angle created by the interception of the tangent of the two terminal portions of the leaf strip viewed in profile as previously described (Keller and Van Volkenburgh, 1997). Length was assessed by gently flattening strips against a finely graduated ruler under a dissecting microscope. After measurement strips were placed in fresh solution. No evidence of injury resulting from handling during measurement was evident with any of the strips at subsequent measurements when the strips were again viewed under a dissecting microscope.

Stock solutions of IAA, IBA, NAA, 2-NAA, 2,4-D, 2,3-D and benzoic acid were prepared by dissolving in water with sufficient heating (i.e. briefly to approximately 80°C). Once cool, these solutions were brought to pH 6.0 with Btp. All chemicals were purchased from Sigma Chemical Co., St. Louis MO, USA. Lower concentration solutions were prepared by appropriate dilution of stock solutions.

High resolution leaf growth assays

High resolution measures of leaf growth were accomplished using a set-up similar to ones described by others (Van Volkenburgh and Cleland, 1980, Shackel et al., 1987). Here an attached leaf (initially approximately 45 mm in length) was oriented horizontally and secured between two aluminum hair clips (Goody Products, Peachtree City, GA USA). The first hair clip was immobile (rigidly connected to a ring stand) and was clamped to the leaf along the midrib at mid leaf. The second hair clamp was mobile (attached by a gate hinge to the ring stand) and was initially attached 15 mm towards the leaf margin from the first hair clip. Leaf growth was recorded using a linear displacement transducer (Hewlett-Packard, Palo Alto, CA) connected to a home-built voltage attenuator and a chart recorder (Model 1325; BioRad, Hercules, CA, USA). A small tension was applied to the leaf portion between the hair clips by the weight of the transducer slug (11.8 g) connected to the mobile clamp by a string draped over a teflon coated post. Under incandescent light ($200 \mu\text{mol s}^{-1} \text{ m}^{-2}$) and at room temperature, leaf growth was recorded first for an 8 hour pretreatment period. Initial portions of each recording, starting immediately following mounting between the clips, consistently showed rapid leaf expansion (data not shown) which steadily decayed so that a

more or less steady-state growth rate achieved within 45 to 90 minutes. Treatment consisted of application of either 1 mM NAA or 1 mM benzoic acid as a weak acid control applied to the adaxial blade surface using cotton-tipped applicators as in earlier experiments (Keller et al., 2004). Growth was then recorded for a further 24 hours.

Leaf epidermal cell area assays

One mM NAA was applied as described above to one unifoliate of plants at the desired stage of development (i.e. both unifoliate blade midribs 40–50 mm). Before treatment (Day 0), after 24 hours (Day 1), or after 6 days (Day 6) epidermal peels (or casts) of the adaxial surface of both the treated leaf and of the untreated (control) leaf were prepared from three epidermal regions 5–10 mm lateral to the midrib (opposite the insertion of the petiole [base]; at the midpoint of the midrib; and 10 mm from the leaf tip. The peels consisted of clear nail polish (Hard as Nails, Sally Hansen, Farmingdale, NY) applied with the supplied brush applicator and allowed to air dry several minutes. Peels were mounted in water on microscope slides and digital images of the leaf surface artifacts were prepared using a microscope mounted digital camera (Coolpix8700, Nikon Corporation, Tokyo, Japan). Estimates of mean cell area were determined by counting the number of the ordinary epidermal cells (pavement cells) and determining the area of patches of cells within each peel image using ImageJ software (<http://rsb.info.nih.gov/ij/>). Patches of cells outlined for counting were chosen to avoid trichome complexes and typically contained between 60 and 100 cells. The far smaller and relatively infrequent guard cells were ignored as they would have had a disproportionate impact on mean cell area.

RESULTS

Effect of auxin on excised leaf strip growth

Figure 1A shows the growth response of excised bean (*Phaseolus vulgaris*) leaf strips incubated with or without 10 μ M NAA in media containing 10 mM KCl and 10 mM sucrose as commonly used absorbable osmoticums (Van Volkenburgh and Cleland, 1990; Keller and Van Volkenburgh, 1997). The auxin treated strips (Fig. 1A; ●) were significantly longer after 6 hours than non-NAA treated controls (○) and after 24 hours the NAA treated leaf strips remained longer than the controls. There was no sign of auxin-induced growth inhibition of leaf tissue evident in longer treatments of intact attached bean leaves (Keller et al., 2004). Regardless of treatment, however, leaf strip growth slowed dramatically after 6 h, however, and by 24 h growth of both auxin-treated and untreated leaf strips was essentially complete as there was no significant increase in length between 24 and 48 h. Perhaps auxin-induced growth inhibition was not evident because excised leaf strips were greatly slowing their growth by the time a second growth inhibiting phase of auxin action was taking effect.

Much more sustained elongation was achieved, however, by incubating leaf strips in a solution containing 10 mM KCl and full strength Murashige and Skoog medium (Murashige and Skoog, 1962) (Fig. 1B; ●, ○). The length of NAA-treated leaf strips (●) and the non-auxin controls (○) was not significantly different when first measured after 4 h, but the auxin treated leaf strips were significantly longer after 10 h. Auxin-induced growth appeared

complete, however, by 10 hours as there was no additional increase in strip length relative to the control strips when measured at 24 and 48 hours in spite of substantial continued growth.

Bean leaf strips incubated in solutions containing 10 mM KCl and full strength Murashige and Skoog media, were found to be significantly longer after 24 hours and after 48 hours when treated with NAA in concentrations ranging from 0.01 μ M to 300 μ M (Fig. 2). Increased leaf strip elongation was found specific to active auxins because leaf strips incubated in known active auxins (i.e. IAA, IBA, NAA, and 2,4-D) at 10 μ M were significantly longer than the control after 24 hours (Fig. 3A). Excised leaf strips treated with the weak auxins 2,3-D and 2-NAA as well as the weak acid control benzoic acid did not exhibit this growth response (Fig. 3A).

Auxin-induced growth of tobacco leaf strips is associated with pronounced epinastic curvature (Keller and Van Volkenburgh, 1997, 1998; Jones et al., 1998; Chen et al., 2001). A similar curvature response is evident when bean leaf strips were treated with active auxins (Figure 3B). As with tobacco leaf strips, the curvature response induced in bean leaf strips appeared complete within 24 hours but the magnitude of epinasty was considerably less than with tobacco. While the curvature of control tobacco leaf strips incubated without auxin did not change, bean leaf strips incubated without auxin developed considerable hyponastic curvature. The weak auxin 2,3-D and the weak acid control benzoic acid had no effect on curvature while the weak auxin 2-NAA significantly decreased hyponastic curvature.

Effects of auxin on growth of intact attached leaves

Figure 4 shows sample leaf tissue elongation rates taken from individual recordings of high resolution leaf growth of the mid-leaf blade regions of rapidly expanding (initially 40 – 50 mm in length) unifoliate (primary) leaves of *Phaseolus* treated at time 0 with either the auxin NAA or a weak acid control, benzoic acid. In all recordings a more or less steady rate of elongation of approximately 0.5 % per hour was soon established. Application of 1 mM NAA invariably resulted in an elongation surge beginning seconds after application (Fig 4, A: detail), complete within 15 to 20 min, and amounting to as much as 0.1 mm of elongation (0.6 % of initial length). This initial transient growth surge was also evident following treatments with 1 mM benzoic acid as well as following a single test of the effect of water alone (data not shown), suggesting it may represent tissue expansion resulting from cuticle and cell wall hydration. An NAA specific increase in growth began about 30 min following treatment (compare Fig 4, A: detail to Fig 4, B: detail) but growth slowed dramatically by 6 h post NAA treatment. In 10 recordings auxin was consistently found to initially accelerate lamina expansion for between 4 and 6 h after which expansion slowed to a rate slower than before auxin treatment so that by 24 h post treatment growth of the benzoic acid treated control leaves caught up to that of the auxin treated leaves (Table I). In some recordings, including the one shown in Figure 1B, application with the weak acid control (benzoic acid) appeared to slightly increase growth after about 1 h but in all recordings this increased growth was small relative to that seen following NAA application and resulted in no significant increase in growth during the 6 h following benzoic acid treatment when compared to the 6 h before application (Table I).

Estimates for mean cell area for ordinary epidermal (pavement) cells at three points (base, middle and tip) along the lamina adjacent to the midrib are reported in Figure 5. Shown are cell areas estimated from leaf surface imprints of rapidly expanding NAA treated unifoliate leaves. Control cell areas were prepared from the opposite similar sized unifoliate of each plant used. Epidermal cell area increased substantially during the initial 24 h at all points along the leaves of both treated and control leaves. While this result does not exclude the possibility of continuing cell division it does suggest increasing cell size rather than increasing cell number may be responsible for the leaf growth recorded in Figure 4. At all three points (base, middle and tip) epidermal cell area was not significantly different between NAA treated and control leaves after 24 h. The NAA treated cells, however, were usually smaller than control cells sampled from the opposite leaf of the same plant and, when between plant variance was removed, in pair-wise analysis (i.e. paired Student's *t* test) the NAA treated cells were significantly smaller than the controls at the middle position after 24 h. The epidermal cells at all three positions of NAA treated leaves were significantly smaller after 6 d.

DISCUSSION

The principle auxin IAA occurs both in active form as free IAA as well as various inactive conjugates of amino acids, sugars, and proteins (Perrot-Rechmann and Napier, 2005; Seidel et al., 2006). While free IAA levels in plant tissues vary greatly with the highest levels in apical meristematic tissue and youngest leaves, expanding leaves contain substantial free IAA which drops as expansion completes (Sitbon et al., 1991). Transforming plants to constitutively overproduce IAA has consistently produced a correlation between increased endogenous free IAA and smaller epinastic leaves in *Petunia* (Klee et al., 1987), tobacco (Sitbon et al., 1991), and *Arabidopsis* (Romano et al., 1993). The *sur* and *yucca* mutants of *Arabidopsis* also have elevated free IAA and smaller leaves (Boerjan et al., 1995; Zhao et al., 2001). The increase in endogenous free IAA associated with reduced leaf growth in intact plants is often not dramatic, however. An approximately 50 % increase in leaf free IAA content was associated with reduced final leaf size in tobacco (Sitbon et al., 1992) while in the *sur1* and *yucca* mutants 50 % to 170 % increases in whole plant free IAA levels produced smaller leaves (Boerjan et al., 1995; Zhao et al., 2001). Experimental application of an auxin transport inhibitor to bean leaf petioles resulted in a near doubling within individual leaf blades of free IAA levels after 24 hours and produced leaves which were shorter than untreated controls within 48 h and which reached much smaller final size (Keller et al. 2004).

In experiments reported here, excised leaf strips responded to treatment with NAA (Fig. 1, 2) as well as other auxins (Fig. 3A) with an initial surge in growth after which growth was insensitive to auxin (Fig. 1). It is possible this loss of sensitivity represents an exhaustion of a capacity to respond to auxin, that after an initial accelerated growth response the tissues are no longer capable of such rapid growth. Auxin treatment has, however, been found to increase subsequent auxin growth-responsiveness of excised *Avena* coleoptile sections (Cleland, 1983).

Following an initial auxin-induced surge in growth, excised leaf strips exhibited no evidence of subsequent auxin-induced growth inhibition even when strip growth was substantial through 48 h (Fig 1, 2). A range of NAA concentrations from 0.1 μM to 300 μM failed to produce growth inhibition (Fig. 3). The free IAA levels in expanding leaves ranges from 10 to 20 ng IAA per g fresh weight (ng/g) in tobacco (Sitbon et al., 1991; Thornburg and Li, 1991) to less than 0.1 ng/g in *Arabidopsis* (Ljung et al., 2001). The free IAA content of untreated intact expanding bean unifoliate leaves is about 29 ng/g (Keller et al., 2004). Assuming that bean leaf tissue is 95 % water, this last value represents an average leaf tissue concentration of about 0.17 μM free IAA.

In tobacco, wounding has been shown to induce a drop in leaf free IAA concentration of two- to threefold within 6 h following wounding (Thornburg and Li, 1991). Thus the exogenous auxin bathing excised leaf strips might initially be expected to maintain growth in excised strips by replacing diminishing endogenous IAA. Given, however, the low endogenous leaf auxin concentration in intact expanding leaves and the relatively small auxin increases in auxin overproducing mutants and transformants that produces leaf growth inhibition (discussed above), it is remarkable that incubating leaf strips in a range of NAA concentrations up to 300 μM for 48 h (Fig. 2) did not inhibit growth relative to the untreated controls.

Incubation of bean leaf strips in solutions containing full strength Murashige and Skoog medium and 10 mM KCl with or without NAA appeared to result in slower strip elongation than incubation in the KCL and sucrose (compare ● and ○ at 4 hours in Fig. 1B to ● and ○ at 6 hours in Fig. 1A). This is likely due to the high osmolarity of the solution (~100 mOsM) initially reducing the cell turgor essential to cell expansion (Lockhart, 1965). Auxin treatment of excised coleoptile or stem sections induces increased elongation growth after a lag of only about 10 min (Ray and Ruesink, 1962; Yamagata and Masuda, 1975) coincident with increased cellular proton efflux (Cleland 1976) resulting in cell wall loosening and turgor-driven cell expansion (Rayle and Cleland, 1992). The auxin-induced growth of tobacco leaf strips, incubated in 10 mM KCl and 10 mM, sucrose, initiates only after an hour and does not appear associated with the acid-growth mechanism (Keller and Van Volkenburgh, 1998). Remarkably, no NAA –induced growth was evident with bean leaf strips incubated in Murashige and Skoog medium and 10 mM KCl after 4 hours (Fig 1B, ● and ○) though the auxin induced surge in growth appeared complete by 10 h. It is not clear from these experiments how, or if, the probable initial drop in cell turgor resulting from incubation in the high osmolarity solution disrupts the onset of auxin-induced growth.

Applied to expanding leaves of intact common bean, auxin results in a short term transient hyponastic curvature response (Lippincott and Lippincott, 1971; Hayes and Lippincott, 1976; Hayes, 1977; 1978; 1981; Hayes and Lippincott, 1981). Results presented here (Fig. 4; Table I) confirm that the curvature response is due to a transient auxin-induced growth acceleration (Hayes and Lippincott, 1976).

Unlike auxin treatment of bean leaf strips, however, application of NAA to intact leaves was found here to result in inhibition of growth following an initial growth surge (Fig. 4; Table I). This confirms an earlier finding that exogenous application of auxin results in long-term

growth inhibition of intact attached bean leaves (Keller et al. 2004), at least in part, as a result of auxin-induced inhibition of cell expansion (Fig. 5).

A possible explanation for the difference between the longer term effect of NAA on leaf strips versus intact leaves is that a non-physiologically high concentration of auxin was applied to the intact leaves and that this higher concentration is toxic. While this explanation can not be ruled out, there are reasons to think that, longer term, the growth of intact plant leaves responds differently to auxin than does the growth of excised leaf strips or excised stem and coleoptile sections. First, there are the reports discussed above showing that small increases in endogenous IAA within expanding leaves achieved either through transformation, mutation or petiolar application of NPA results in slower growth and smaller leaves. Secondly, except for root elongation where auxins inhibit growth (Scott, 1972), the effect of auxin in general is to stimulate growth of excised susceptible non-leaf tissues even at high concentration (Nissen, 1985). For example, though IAA induced growth of *Avena* coleoptile sections peaks at 10 μM , concentrations as high as 1 mM steadily induce growth relative to controls when monitored up to 10 h (Kefford and Bonner, 1961). Thirdly, lower concentrations of NAA inhibit intact bean leaf growth. In the high resolution recordings described here 1 mM NAA and 1 mM benzoic acid treated leaves, tensioned by the weight of an 11 g linear displacement transducer slug, had similar total growth after 24 h (Fig. 4; Table I). Previously application of both 1.0 mM and 0.1 mM NAA was found to reduce final bean leaf length and weight after 6 days (Keller et al., 2004). In those experiments, where leaf length was measured daily, NAA treatment significantly reduced leaf length 24 hours after application with as little as 10 μM NAA when between plant variance is removed. Finally, only a fraction of the auxin contained within a single aqueous application applied to an intact leaf can be expected to enter the leaf. Though transient leaf elongation apparently associated with cuticle and tissue hydration is evident upon solution application to intact leaves (Fig. 4), the cuticle is a potent barrier to aqueous solutions (Dowler et al., 1974). Thus, the effective concentration of NAA in cells of treated leaves would seem likely to fall within the range of those applied to the excised leaf strips incubated in solution. Furthermore, the growth of excised leaf strips, the cells of which are exposed through the cut surfaces to a range of high auxin concentrations well above levels inhibitory to the expansion of intact leaves (discussed above), shows no signs of auxin-induced growth inhibition (Fig. 1, 2, 3).

The synthetic auxin NAA was used in preference to IAA in the experiments described here because NAA had been shown earlier (Keller et al., 2004) to be more effective than IAA at inducing long-term growth inhibition of intact leaves presumably due to the greater metabolic and chemical stability of NAA compared to IAA (Epstein et al., 1980). Although the growth of intact attached leaves (measured as increased midrib length) was inhibited by IAA concentrations as low as 1.0 μM after 24 hours (Keller, unpublished data), single applications of high concentration IAA (1.0 mM) had no effect on final leaf size after 6 d. IAA is chemically and metabolically unstable (Epstein et al., 1980) so the effect of a pulse of the compound on the growth of treated leaves could be expected to be of short duration. NAA accumulating in tobacco protoplasts is glycosylated to some extent but otherwise remains unmetabolized (Delbarre et al., 1994) though NAA and IAA have been shown to have identical gene expression effects in an *Arabidopsis* microarray study (Pufky et al.,

2003). Thus single applications of NAA would appear provide the best picture of the effects of sustained auxin increases.

The auxin-induced growth response of excised leaf strips from bean (Fig. 3B) and from tobacco (Keller and Van Volkenburgh, 1997; Jones et al., 1998) involves epinastic curvature resulting from relatively greater auxin-induced expansion of the adaxial cell layers (Keller and Van Volkenburgh, 1997). Auxin is known to induce ethylene production (Abeles et al., 1992) and ethylene exposure can result in leaf (usually petiole) epinasty. The possibility, however, that auxin-induced growth of tobacco leaf strips involves or requires ethylene is unlikely because of a poor correlation between inhibition of auxin-induced strip epinasty and inhibition of ethylene production and the inability of ethylene alone to induce strip epinasty (Keller and Van Volkenburgh, 1997). Auxin-induced growth inhibition of intact leaves also does not appear to result from auxin-induced ethylene synthesis as growth is not rescued by inhibition of ethylene synthesis in beans and leaf auxin increases in the ethylene insensitive mutant *ein4* also resulted in smaller epinastic leaves (Keller et al., 2004). Further, crossing of an *Arabidopsis* transgenic auxin overproducer with ethylene insensitive *ein1* and *ein2* mutants also found that elevated auxin-induced leaf epinasty is not controlled by ethylene (Romano et al., 1993).

Auxin perception by responding tobacco leaf strips clearly involves the auxin receptor auxin-binding protein 1 (ABP1). Ectopic expression of Arabidopsis ABP1 in tobacco resulted in leaves exhibiting premature auxin sensitivity in leaf strip assays (Jones et al., 1998; Chen et al., 2001). Auxin binding to ABP1 at the cell membrane is associated with ion movements and protoplast swelling (Thiel et al., 1993; LeBlanc et al., 1999; Steffens et al., 2001; Yamagami et al., 2004). The F box protein TIR1 is also an auxin receptor (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005) as are three closely related proteins; AFB1-3 (Dharmasiri et al., 2005b). Binding of auxin to these proteins leads to degradation of specific transcription repressor proteins with a range of developmental consequences (for reviews see Badescu and Napier, 2006; Leyser, 2002). The possibility that two distinct auxin signaling pathways exist, one dependent on ABP1 and the other dependent on TIR1/AFB1-3, is suggested by recent experiments testing the effects of anti-ABP1 antibodies on auxin-induced swelling of pea protoplasts (Yamagami et al., 2004). In the experiments reported here (Fig. 4) increased auxin levels within intact expanding bean leaves initially increases growth while the same results, as well results from other reports, indicates higher auxin levels subsequently inhibits growth of intact attached leaves. Excised bean strips, however, only respond to auxin with a short lived growth response with no subsequent growth inhibition. It is tempting to suggest that the two auxin-inducible signal transduction pathways are separately responsible for auxin-induced growth and auxin-induced growth inhibition in leaves. First, the ABP1 mediated pathway would lead to growth induction. The second pathway mediated by TIR1/AFB1-3 would control growth inhibition. Perhaps tissue excision, either as a wound response or through isolation from other growth controlling influences supplied by the rest of the plant, results in disruption or alternation of critical elements within TIR1/AFB1-3 pathway.

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Abbreviations

2,3-D	2,3-dichlorophenoxyacetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
IAA	indole acetic acid
IBA	indole butyric acid
Mes	2-(4-morpholino)-ethane sulfonic acid
NAA	α -naphthalene acetic
2-NAA	β -naphthalene acetic acid
NPA	1-N-naphthylphthalamic acid

LITERATURE CITED

- Abeles, FB., Morgan, PW., Saltveit, ME, Jr. Ethylene in Plant Biology. 2. Academic Press; San Diego, CA: 1992.
- Badescu GO, Napier RM. Receptors for auxin: will it all end in TIRs? Trends Plant Sci. 2006; 11:217–223. [PubMed: 16564202]
- Beemster GTS, De Veylder L, Vercruysse S, West G, Rombaut D, Van Hummelen P, Galichet A, Gruissem W, Inzé D. Genome-wide analysis of gene expression profiles associated with cell cycle transitions in growing organs of Arabidopsis. Plant Physiol. 2005; 138:734–743. [PubMed: 15863702]
- Berleth T, Sachs T. Plant morphogenesis: long-distance coordination and local patterning. Curr Opin Plant Biol. 2001; 4:57–62. [PubMed: 11163169]
- Boerjan W, Cervera MT, Delarue M, Beeckman T, Dewitte W, Bellini C, Caboche M, Onckelen HV, Montagu MV, Inzé D. *superroot*, a recessive mutation in *Arabidopsis*, confers auxin overproduction. Plant Cell. 1995; 7:1405–1419. [PubMed: 8589625]
- Chen J-G, Shimomura S, Sitbon F, Sandberg G, Jones AM. The role of auxin-binding protein 1 in the expansion of tobacco leaf cells. Plant J. 2001; 28:607–617. [PubMed: 11851907]
- Cleland RE. Kinetics of hormone-induced H⁺ excretion. Planta. 1976; 58:210–213.
- Cleland RE. The capacity for acid-induced wall loosening as a factor in the control of *Avena* coleoptile cell elongation. J Expt Bot. 1983; 34:676–680.
- Delbarre A, Muller P, IMhoff V, Morgat JL, Barbier-Brygoo H. Uptake, accumulation and metabolism of auxins in tobacco leaf protoplasts. Planta. 1994; 195:159–167.
- Dharmasiri N, Dharmasiri S, Estelle M. The F-box protein TIR1 is an auxin receptor. Nature. 2005a; 435:441–445. [PubMed: 15917797]
- Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jürgens G, Estelle M. Plant development is regulated by a family of auxin receptor F box proteins. Dev Cell. 2005b; 9:109–119. [PubMed: 15992545]
- Dowler MJ, Rayle DL, Cande WZ, Ray PM, Durand H, Zenk MH. Auxin does not alter the permeability of pea segments to tritium-labeled water. Plant Physiol. 1974; 53:229–232. [PubMed: 16658681]

- Epstein E, Cohen JD, Bandurski RS. Concentration and metabolic turnover of indoles in germinating kernels of *Zea mays* L. *Plant Physiol.* 1980; 65:415–421. [PubMed: 16661204]
- Goodwin, PB., Erwee, MG. Hormonal influences on leaf growth. In: Dale, JE., Milthorpe, FL., editors. *The Growth and Functioning of Leaves*. Cambridge University; Cambridge, UK: 1983. p. 207-232.
- Hayes AB. Developmental aspects of leaf blade hyponasty. *Bot Gaz.* 1977; 138:52–55.
- Hayes AB. Auxin-cytokinin effects in leaf blade hyponasty. *Bot Gaz.* 1978; 139:385–389.
- Hayes AB. The interaction of auxin and ethylene in the maintenance of leaf blade form in *Phaseolus vulgaris* L var. Pinto *Amer J Bot.* 1981; 68:733–740.
- Hayes AM, Lippincott JA. Growth and gravitational response in the development of leaf blade hyponasty. *Amer J Bot.* 1976; 63:383–387.
- Hayes AM, Lippincott JA. The timing of and effect of temperature on auxin-induced hyponastic curvature of the bean primary leaf blade. *Amer J Bot.* 1981; 68:305–311.
- Jones AM, Im K-H, Savka MA, Wu M-J, DeWitt G, Shillito R, Binns AN. Auxin-dependent cell expansion mediated by overexpressed auxin-binding protein 1. *Science.* 1998; 282:1114–1117. [PubMed: 9804548]
- Kefford NP, Bonner J. Steady state growth of *Avena* coleoptile sections in high auxin concentrations. *Plant Physiol.* 1961; 36:323–325. [PubMed: 16655513]
- Keller CP, Stahlberg R, Barkawi LS, Cohen JD. Long-term inhibition by auxin of leaf blade expansion in bean and *Arabidopsis*. *Plant Physiol.* 2004; 134:1217–1226. [PubMed: 14988474]
- Keller CP, Van Volkenburgh E. Auxin-induced epinasty of tobacco leaf tissues. A non-ethylene mediated response. *Plant Physiol.* 1997; 113:603–610. [PubMed: 12223629]
- Keller CP, Van Volkenburgh E. Evidence that auxin-induced growth of tobacco leaf tissues does not involve cell wall acidification. *Plant Physiol.* 1998; 118:557–564. [PubMed: 9765541]
- Kepinski S, Leyser O. The *Arabidopsis* TIR1 protein is an auxin receptor. *Nature.* 2005; 435:446–451. [PubMed: 15917798]
- Klee HJ, Horsch RB, Hinchey MA, Hein MB, Hoffman NL. The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. *Genes Dev.* 1987; 1:86–96.
- LeBlanc N, David K, Grosclaude J, Pradier JM, Barbier-Brygoo H, Labiau S, Perrot-Rechenmann C. A novel immunological approach establishes that the auxin-binding protein Nt-abp, is an element involved in auxin signaling at the plasma membrane. *J Biol Chem.* 1999; 274:28314–28320. [PubMed: 10497189]
- Leyser O. Molecular genetics of auxin signaling. *Annu Rev Plant Biol.* 2002; 53:377–398. [PubMed: 12221981]
- Ljung K, Bhalerao RP, Sandberg G. Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *Plant J.* 2001; 28:465–474. [PubMed: 11737783]
- Lippincott BB, Lippincott JA. Auxin-induced hyponasty of the leaf blade of *Phaseolus vulgaris*. *Amer J Bot.* 1971; 58:817–826.
- Lockhart, JA. Cell extension. In: Bonner, J., Varner, JE., editors. *Plant Biochemistry*. Academic Press; NY: 1965. p. 826-849.
- Mattsson J, Ckurshumova W, Berleth T. Auxin signaling in *Arabidopsis* leaf vascular development. *Plant Physiol.* 2003; 131:1327–1339. [PubMed: 12644682]
- Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco cultures. *Physiol Plant.* 1962; 15:473–479.
- Nissen P. Dose responses of auxins. *Physiol Plant.* 1985; 65:357–374.
- Perrot-Rechenmann, C., Napier, RM. Auxins. In: Litwack, G., editor. *Plant hormones, Vitamins and Hormones*. Vol. 72. Academic press; Elsevier St Louis: 2005. p. 203-233.
- Poethig, Rs, Sussex, AW. The cellular parameters of leaf development in tobacco: a clonal analysis. *Planta.* 1985; 165:170–184. [PubMed: 24241041]
- Pufky J, Qiu Y, Rao MV, Hurban P, Jones AM. The auxin-induced transcriptome for etiolated *Arabidopsis* seedlings using a structure/function approach. *Funct Integr Genomics.* 2003; 3:135–143. [PubMed: 14648238]

- Ray PM, Ruesink AW. Kinetic experiments on the nature of the growth mechanism in oat coleoptile cells. *Dev Biol.* 1962; 4:377–397.
- Rayle DL, Cleland RE. The acid growth theory of auxin-induced cell elongation is alive and well. *Plant Physiol.* 1992; 99:1271–1274. [PubMed: 11537886]
- Reinhardt D, Mandel T, Kuhlemeier C. Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell.* 2000; 12:507–518. [PubMed: 10760240]
- Romano CP, Cooper ML, Klee HJ. Uncoupling auxin and ethylene effects in transgenic tobacco and *Arabidopsis* plants. *Plant Cell.* 1993; 5:181–189. [PubMed: 12271061]
- Scott TK. Auxins and roots. *Ann Rev Plant Physiol.* 1972; 23:235–258.
- Seidel C, Walz A, Park S, Cohen JD, Ludwig-Müller J. Indole-3-acetic acid protein conjugates: novel players in auxin homeostasis. *Plant Biol.* 2006; 8:340–345. [PubMed: 16807826]
- Shackel KA, Matthews MA, Morrison JC. Dynamic relationship between expansion and cellular turgor in growing grape (*Vitis vinifera* L.) leaves. *Plant Physiol.* 1987; 84:1166–1177. [PubMed: 16665579]
- Sieburth LE. Auxin is required for leaf vein pattern in *Arabidopsis*. *Plant Physiol.* 1999; 121:1179–1190. [PubMed: 10594105]
- Sitbon F, Sundberg B, Olsson O, Sandberg G. Free and conjugated indoleacetic acid (IAA) contents in transgenic tobacco plants expressing the *iaaM* and *iaaH* IAA biosynthesis genes from *Agrobacterium tumefaciens*. *Plant Physiol.* 1991; 95:480–485. [PubMed: 16668009]
- Sitbon F, Hennion S, Sundberg B, Little CHA, Olsson O, Sandberg G. Transgenic tobacco plants coexpressing the *Agrobacterium tumefaciens iaaM* and *iaaH* genes display altered growth and indoleacetic acid metabolism. *Plant Physiol.* 1992; 99:1062–1069. [PubMed: 16668972]
- Steffens B, Feckler C, Palme K, Christian M, Bottger M, Lüthen H. The signal for protoplast swelling is perceived by extracellular ABP1. *Plant J.* 2001; 27:591–599. [PubMed: 11576442]
- Thornberg RW, Li X. Wounding *Nicotiana tabacum* leaves causes a decline in endogenous indole-3-acetic acid. *Plant Physiol.* 1991; 96:802–805. [PubMed: 16668256]
- Thiel G, Fricker MR, White IR, Millner P. Modulation of K⁺ channels in Vicia stomatal guard cells by peptide homologs to the auxin-binding protein C terminus. *Proc Natl Acad Sci USA.* 1993; 90:11493–11497. [PubMed: 8265579]
- Van Volkenburgh E. Leaf expansion — an integrating plant behavior. *Plant Cell Environ.* 1999; 22:1463–1473.
- Van Volkenburgh E, Cleland RE. Proton excretion and cell expansion in bean leaves. *Planta.* 1980; 148:273–278. [PubMed: 24309830]
- Van Volkenburgh E, Cleland RE. Light-stimulated cell expansion in bean (*Phaseolus vulgaris* L.) leaves I. Growth can occur without photosynthesis. *Planta.* 1990; 182:72–76. [PubMed: 11538275]
- Yamagami M, Haga K, Napier RM, Iino M. Two distinct signaling pathways participate in auxin-induced swelling of pea epidermal protoplasts. *Plant Physiol.* 2004; 134:735–747. [PubMed: 14764902]
- Yamagata Y, Masuda Y. Comparative studies on auxin and fusicoccin actions on plant growth. *Plant Cell Physiol.* 1975; 16:41–52.
- Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J. A role for flavin monooxygenase-like enzymes in auxin biosynthesis. *Science.* 2001; 291:306–309. [PubMed: 11209081]

Figure 1.

Effect of 10 mM NAA elongation of mid-leaf interveinal bean leaf strips incubated in sucrose containing media (A) and complete media (B). The leaf strips were incubated in 10 mM KCL, and 0.1 mM Mes/Btp pH 6.0 and either 10 mM sucrose (A) or full strength Murishige and Skoog medium (B), without (○) or with (●) 10 μ M NAA. Errors bars indicate the standard error of the mean percent increase in leaf length. From 6 hours onward the NAA treated strips were significantly longer ($p < 0.05$; $n = 20$) than the respective controls.

Figure 2.

Dose response effect of NAA on elongation of interveinal bean leaf strips. Leaf strips excised mid-leaf were incubated 48 hours in 10 mM KCL, full strength Murishige and Skoog medium, 0.1 mM Mes/Btp pH 6.0, and with the indicated concentrations of NAA. Length measurements were made after 24 hours (open bars) and after 48 hours (striped bars). Errors bars indicate the standard error of the mean percent increase in leaf length. After 24 hours leaf strips treated with all NAA concentrations tested were significantly longer ($p < 0.05$; $n = 24$) than the 0 mM NAA control (far left). After 48 hours leaf strips treated with all NAA concentrations tested except 0.1 and 0.3 μM were significantly longer than the 0 mM NAA control.

Figure 3.

Effect of different active and weak auxins and of a non-auxin weak acid on strip elongation (A) and on strip curvature (B). Leaf strips excised mid-leaf were incubated 24 hours in 10 mM KCL, full strength Murishige and Skoog medium, 0.1 mM Mes/Btp pH 6.0, and with the indicated auxin or benzoic acid at 10 μ M. Length and curvature was determined after 24 h. Curvature is displayed with degrees of hyponastic curvature as negative and degrees of epinastic curvature as positive. Errors bars indicate the standard error of the mean percent increase in leaf length (A) and degrees of curvature (B). Significant differences ($p < 0.05$) between the length and curvature of the variously treated leaf strips are indicate by the letters a-d.

Figure 4.

Growth rate from typical examples of high resolution recordings of the effects of NAA (A) and of a weak acid control (B) on bean leaf expansion. Expanding attached unifoliate leaves (40–50 mm in length) were connected as described in the Materials and Methods to a linear displacement transducer so that elongation of an initially 15 mm long midleaf region between the midrib and the leaf margin was recorded on a chart recorder. Leaf growth was recorded first for an 8 hour pretreatment period and treatment began at time 0 with a one time application in A of 1 mM NAA (pH 6.0 with Btp) and, in B, of a weak acid control (1 mM Benzoic acid, also pH 6.0). Growth rate is reported as successive elongation increments over 0.1 hour periods in A and B and from elongation increments over 0.033 hours for A:detail and B:detail. A: detail and B:detail show enlarged views of the growth rate from 0.5 hours before to 1.0 hours after application.

Figure 5.

Effect of NAA on leaf epidermal cell expansion. On day 0, one attached expanding unifoliate leaf from 22 bean plants each with an approximately equal sized pairs of unifoliates, each 40–50 mm long, was treated with 1 mM NAA. The opposite leaf was left untreated. Pretreatment (6 additional plants; $n = 12$ leaves), after day 1 ($n = 14$), and after day 6 ($n = 8$) mean surface area of ordinary epidermal (pavement) cells of both the treated leaves (●) and the opposite untreated leaves (○) of each plant was determined from nail polish peels at three points along the length of growing leaves 5–10 mm distal to the midrib adjacent to the petiole insertion (base), at the mid point of the midrib (mid-leaf), and 5 – 10 mm from the leaf tip (tip). Error bars indicate standard error of the mean cellular area. Significant differences ($p < 0.05$) between the cell surface area of treated versus untreated leaves (i.e. after 1 day at mid-leaf and after 6 days at base, mid-leaf and tip) are indicate by asterisks.

Table I

The effect of 1 mM NAA on primary bean leaf expansion measured at high resolution. The elongation of initially 15 mm long regions of leaf tissues was recorded as for Figure 4. Following an 8 hour pretreatment period, at time 0, 1 mM NAA (pH 6.0 with Btp) or a weak acid control (1 mM Benzoic acid) was applied once. Growth was then recorded for a further 24 hours. Values shown in parenthesis below each mean indicate increase in length as a percent of the initial tissue length. Errors indicate the standard error of the mean (n = 10). Asterisks indicate significantly more ($p < 0.05$) leaf expansion resulting from one of the treatments versus the other over the same time period.

Treatment	Elongation (mm)		
	<i>before application</i>	<i>after application</i>	
	–6 to 0 hours	0 to 6 hours	6 to 24 hours
1.0 mM NAA	0.35 $\pm$ 0.08 (2.3 %)	*1.02 $\pm$ 0.14 (6.8 %)	0.63 $\pm$ 0.09 (4.2 %)
1.0 mM benzoic acid	0.45 $\pm$ 0.10 (3.0 %)	0.47 $\pm$ 0.06 (3.1 %)	*1.26 $\pm$ 0.16 (8.4 %)