Mechanochemical feedback mediates tissue bending required for seedling emergence

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Highlights

- Asymmetric pectin methylesterification is required for differential growth
- Auxin distribution regulates pectin methylesterification during differential growth
- Feedback between pectin and auxin via PIN proteins facilitates growth asymmetry

In Brief

Using hook development as model in Arabidopsis, Jonsson et al. demonstrate a mechanochemical feedback between the plant hormone auxin and pectin methylesterification regulates differential cell elongation to facilitate tissue bending essential for seedling emergence from soil.
Mechanochemical feedback mediates tissue bending required for seedling emergence

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SUMMARY

Tissue bending is vital to plant development, as exemplified by apical hook formation during seedling emergence by bending of the hypocotyl. How tissue bending is coordinated during development remains poorly understood, especially in plants where cells are attached via rigid cell walls. Asymmetric distribution of the plant hormone auxin underlies differential cell elongation during apical hook formation. Yet the underlying mechanism remains unclear. Here, we demonstrate spatial correlation between asymmetric auxin distribution, methylesterified homogalacturonan (HG) pectin, and mechanical properties of the epidermal layer of the hypocotyl in Arabidopsis. Genetic and cell biological approaches show that this mechanochemical asymmetry is essential for differential cell elongation. We show that asymmetric auxin distribution underlies differential HG methylesterification, and conversely changes in HG methylesterification impact the auxin response domain. Our results suggest that a positive feedback loop between auxin distribution and HG methylesterification underpins asymmetric cell wall mecanochemical properties to promote tissue bending and seedling emergence.

INTRODUCTION

During seedling emergence, differential growth causes the hypocotyl to bend into an apical hook, which protects the fragile shoot apical meristem from the soil interface. Hook formation relies primarily on asymmetric cell elongation on two sides of the young hypocotyl, leading to bending of this organ.

The regulation of growth asymmetry, which culminates in a 180° bend, is coordinated by an intrinsic system dominated by the hormone indole-3-acetic acid (IAA/auxin).2 Experiments using the synthetic auxin reporter DR5 have revealed that the transcriptional auxin response is primarily localized to the inner side of the hook.3 Auxin is both a promoter and inhibitor of cell elongation depending on the organ.4 In the Arabidopsis apical hook, high auxin levels on the inner side are associated with a reduction in cell elongation relative to the outer side,5,6 resulting in bending of the hypocotyl. Previous studies in which genetic interference with auxin metabolism, transport, or signaling caused severe apical hook defects further support a critical role of auxin in hook development.5,8 However, the mechanical aspects of cell growth have not been integrated with auxin distribution, and the rapid bending of the hook suggests the existence of an amplification mechanism, which also remains elusive.

Plant cells are enclosed within a rigid cell wall—cell elongation is only possible if the cell wall yields to turgor.9 Thus, cell wall remodeling is at the nexus between auxin activity and tissue bending. In agreement, the promoting effect of auxin on cell elongation described in great detail is thought to involve increased wall extensibility through acidification of the wall and activation of cell wall-loosening proteins as well as through transcriptional control of cell wall-modifying enzymes.10,11 In particular, auxin triggers the de-methylesterification of pectin, resulting in wall softening and organ emergence, at the shoot apical meristem in Arabidopsis.12 In contrast, the downstream events associated with auxin-induced inhibition of cell growth, notably in the root, are less well understood but could involve wall alkalisation.13 Whether this involves pectin modifications is unknown.

The pectic polysaccharide homogalacturonans (HGs) are a major constituent of the cell wall in expanding cells.14 Synthesized in the Golgi,15 HGs are delivered to the wall with a high degree of methylesterification.16 HGs are selectively de-methylesterified through the action of pectin methylesterases (PMEs), which are inhibited by PME inhibitors (PMEIs).17 Previous studies have demonstrated that HG de-methylesterification can enhance cell wall softening, extensibility, and expansion.16,19 Although debated,20 there is evidence from elongating hypocotyls that the loosening of longitudinal walls, which precedes anisotropic growth, depends on the level of HG methylesterification, with a high degree of HG methylesterification stiffening the
(legend on next page)
Consistently, primordium initiation in the apical meristem is preceded by auxin-induced wall loosening through HG de-esterification, with ectopic PMEI expression inhibiting primordia development. Previous findings that the regulation of HG methylesterification is crucial to modifying cell mechanical properties for growth asymmetry at both the cell and tissue level prompted us to study the interplay between HG methylesterification, cell wall mechanics, and auxin in hook development. Our results reveal that a positive feedback loop between HG methylesterification and auxin mediates and amplifies differential cell elongation for tissue bending during hook development.

RESULTS

Auxin modulates cell elongation and mechanical properties

Tissue bending during apical hook development is a result of differential cell elongation on two sides of the hypocotyl. Genetic analyses have shown that auxin plays a key role in hook development. Moreover, auxin response in the apical hook is asymmetric and inversely correlated with cell elongation. In particular, using auxin transport mutants, it has been suggested that the strong auxin response on the inner side reduces cell elongation relative to the outer side and partly explains hypocotyl bending. Here, we addressed the role of auxin distribution and its interaction with the cell wall to understand tissue bending during hook development.

First, we examined the effect of auxin on cell elongation in the hypocotyl of dark grown Arabidopsis seedlings, focusing on the region at 0–400 μm from the shoot apical meristem (SAM), where elongation asymmetry was highly pronounced in Col-0 wild-type (WT) (Figure S1; n ≥ 18 cells from each of 6 seedlings), as shown previously. Exogenous IAA application to WT seedlings for 3 h during hook formation significantly reduced cell elongation rates relative to mock-treated WT seedlings (Figures 1A–1E; n ≥ 25 cells from each of 6 seedlings). The most pronounced effect was observed on the outer side, i.e., a 3-fold reduction in the cell elongation rates upon IAA treatment relative to mock (2.4% h⁻¹ ± 0.8% in IAA-treated cells, compared to 8.2% h⁻¹ ± 1.6% in mock-treated cells), while elongation in cells on the inner side reduced from 2.6% h⁻¹ ± 1.0% upon mock to 1.4% h⁻¹ ± 0.5% upon IAA treatment. Conversely, inhibiting auxin response by exogenously applying the auxin antagonist auxinole for 3 h, cell elongation rate was increased 3-fold on the inner side compared with mock treatment (on the inner side, 11.7% h⁻¹ ± 2.2% upon auxinole treatment compared to 2.9% h⁻¹ ± 1.7% upon mock treatment), while auxinole only mildly enhanced elongation on the outer side (12.6% h⁻¹ ± 2.0% upon auxinole treatment compared to 8.0% h⁻¹ ± 1.1% upon mock treatment) (Figures 1F–1J; n ≥ 16 cells from each of 4 seedlings). Taken together, these results formally demonstrate that auxin reduces cell elongation rates during hook formation.

Cell elongation rates correlate with mechanical properties in the apical hook, and, since auxin reduces cell elongation during hook formation as shown above, we investigated the impact of auxin on the mechanical properties of cell walls. We used a previously described approach of modulating turgor pressure by osmotic treatments and measuring elastic cell deformation to gain further insight into cell wall mechanical properties. This approach has been used to analyze mechanical properties of cell wall. This notably revealed that cell wall stiffness correlates with cellular growth patterns in various tissues. To assess the impact of auxin on wall mechanical properties, we performed cell deformation assays following exogenous application of auxin and auxinole. In agreement with previous data, there was a significant difference in cell deformation between the inner and outer side of the hook in the region at 0–400 μm from SAM. Exogenous addition of auxin for 3 h resulted in significantly reduced cell deformation mainly on the outer side (9.3% ± 2.2% upon IAA compared with 14.0% ± 3.1% upon mock treatment) while the inner side was not affected by IAA treatment (3.2% ± 2.6% upon IAA treatment compared with 3.9% ± 3.1% upon mock treatment) (Figures 1K–1L and 1N; n ≥ 13 cells from each of 3 seedlings). In contrast, suppressing the auxin response through auxinole application for 3 h increased cell deformation on the inner side of the hook 3-fold relative to the control (12.0% ± 4.3% upon auxinole treatment compared with 3.9% ± 3.1% upon mock treatment), while auxinole treatment had little effect on the outer side (15.7% ± 1.7% upon auxinole compared with 14.0% ± 3.1% upon mock treatment) (Figures 1M and 1N; n ≥ 13 cells from each of 3 seedlings). These results suggest that asymmetric auxin distribution could mediate differential cell elongation, with high auxin levels promoting wall stiffness and reducing cell elongation on the inner side.

Differential HG methylesterification is required for hook development and seedling emergence

Cell wall mechanical properties are determined by cell wall composition and texture. However, the wall components that could mediate mechanical asymmetry during hook development are not well known. Several recent studies have demonstrated that HG methylesterification levels affect cell wall mechanical properties in the SAM and in the hypocotyl. We therefore...
examined the role of HG methylesterification in differential growth during apical hook development. To this end, we first investigated the spatial pattern of HG methylesterification between 0 and 400 μm from SAM, where elongation asymmetry and differences in cell-wall mechanical properties were most pronounced. We labeled cell walls of WT with the antibodies LM20 and LM19, which preferentially label highly methylesterified and de-methylesterified HG, respectively. At 0–400 μm from SAM, the LM20/LM19 labeling ratio of longitudinal epidermal walls was higher (over 2-fold) in cells on the inner side of the hook (labeling ratio 1.9 ± 1.0) than corresponding cells on the outer side (0.8 ± 0.1) (Figures 2A and S2A; n ≥ 11 cells per seedling, from 8 seedlings). Hence, epidermal cells close to the SAM—the region with the largest asymmetry in cell growth and cell wall mechanical properties—also displayed a pronounced spatial asymmetry in HG methylesterification levels with high HG methylesterification levels correlating with slower growing cells and the opposite trend on the outer side.

We next investigated whether asymmetric HG methylesterification is essential for hook development. We genetically

Figure 2. Asymmetric HG methylesterification is required for proper hook development

(A) Ratio of LM20 and LM19 labeling fluorescence intensity for longitudinal walls of individual epidermal cells in WT and PMEI5oe seedlings at 0–400 μm from SAM. n ≥ 11 cells from each of 8 seedlings.

(B and C) Heatmaps of cell deformation in WT (B) and PMEI5oe (C).

(D) Percentage of cell deformation at 0–400 μm from SAM in WT and PMEI5oe. n ≥ 14 cells from each of 3 seedlings. Cells from individual seedlings are grouped vertically. Black horizontal lines indicate the mean.

(E) Macro-confocal time-lapse images of cells in WT and PMEI5oe at 0 and 3 h during formation.

(F) Cell-elongation rates of epidermal cells at 0–400 μm from SAM in WT and PMEI5oe seedlings during formation. n ≥ 13 cells from each of 8 seedlings. Cells from individual seedlings are grouped vertically. Black horizontal lines indicate the mean.

(G) Cell lengths of epidermal cells in WT and PMEI5oe seedlings at early hook maintenance phase, 30 h after germination. n ≥ 80 cells from each of 6 seedlings.

(H) Apical hook development in WT and PMEI5oe seedlings. n = 16 seedlings. Inset depicts hook angle was obtained.

(I) Macro-confocal time-lapse images of cells in WT and PMEI5oe at 0 and 3 h after germination.

(J) Apical hook development in WT and PMEI5oe seedlings. n = 13 cells from each of 8 seedlings. Cells from individual seedlings are grouped vertically. Black horizontal lines indicate the mean.

(K) Quantification of soil emergence based on (I) and (J). n = 30 seeds, repeated 3 times.

In (A) and (K), error bars represent standard deviation of the mean. In (H), error bars represent standard error of the mean. Statistical significance was determined by paired, two-tailed Student’s t test, where **p < 0.005, ***p < 0.0005. White asterisks in (B), (C), and (E) mark the position of SAM. All scale bars, 100 μm. See also Figure S2.
Auxin promotes HG methylesterification to mediate cell-wall composition asymmetry during hook development

The finding that asymmetric HG methylesterification is important for the differential growth observed during hook development prompted us to investigate which factors mediate this asymmetry. The domains of high HG methylesterification and auxin response overlap spatially during hook development and previously, exogenous auxin application has been shown to affect HG methylesterification during organ initiation in Arabidopsis. Therefore, we analyzed whether auxin mediates differential HG methylesterification in the hook. We used a genetic approach to investigate the role of auxin in HG methylesterification. More specifically, we examined HG methylesterification in yuc1D mutants—which show enhanced auxin levels due to increased YUCCA1, a rate-limiting enzyme in the indole-3-pyruvate pathway of auxin biosynthesis. yuc1D seedlings displayed an over 2-fold and a 4-fold increase in HG methylesterification on the inner and outer sides, respectively, relative to WT seedlings (in yuc1D, LM20/LM19 ratio: 3.7 ± 1.7 on outer side and 4.3 ± 1.1 on inner side, compared to WT: 0.8 ± 0.1 on outer side and 1.9 ± 1.0 on inner side), which severely attenuated HG methylesterification asymmetry (Figures 3A and 3B; n ≥ 10 cells from each of 6 seedlings). Thus, the enhanced HG methylesterification observed in yuc1D seedlings phenocopied PMEI5oe seedlings. Conversely, suppressing auxin response by expression of dominant-negative tir1E12K-GUS (tir1E12K) resulted in an almost 2-fold reduction in HG methylesterification.
levels on the inner side of the hook compared to WT seedlings (on inner side, LM20/LM19 ratio in tirE12K 1.0 ± 0.1, compared to WT with 1.7 ± 0.2), while outer side HG methylesterification levels in tirE12K did not differ from WT (on outer side LM20/LM19 ratio in tirE12K 1.0 ± 0.1, compared to WT 0.9 ± 0.1) (Figures 3B and S3B; n ≥ 6 cells from each of 4 seedlings). We then examined cell elongation rates in yuc1D and tirE12K mutants. Compared with WT, yuc1D seedlings exhibited a markedly decreased cell elongation rate on the outer side of the hook (3.7% h⁻¹ ± 1.2% in yuc1D compared to 6.6% h⁻¹ ± 1.3% in WT), while cell elongation rates on the inner side did not differ significantly between yuc1D and WT (2.9% h⁻¹ ± 0.9% in yuc1D compared to 3.6% h⁻¹ ± 1.2% in WT) (Figures 3C and S3C; n ≥ 10 cells from each of 8 seedlings). Conversely, tirE12K seedlings exhibited elevated cell elongation rates on the inner side compared to WT (5.2% h⁻¹ ± 1.7% compared to 3.6% h⁻¹ ± 1.2% in WT), while the elongation rate of cells on the outer side did not differ significantly between tirE12K and WT (5.2% h⁻¹ ± 0.6% in tirE12K compared to 6.6% h⁻¹ ± 1.3% in WT) (Figures 3C and S3C; n ≥ 10 cells from each of 8 seedlings). Taken together, the enhanced HG methylesterification in yuc1D mutants and the converse in tirE12K mutants, the resultant alterations in elongation rates, together with the spatial correlation between high auxin response and HG methylesterification in WT seedlings, suggest that auxin levels are positively associated with HG methylesterification.

**HG methylesterification feeds back onto auxin response**

Cell wall composition can impact the auxin response domain during hook development. For example, reduced xyloglucan levels attenuates the auxin response domain on the inner side of the hook and adversely affects hook development.22 These observations, along with defects in hook development in PMEI5oe seedlings, prompted us to investigate whether HG methylesterification impacts the auxin response. Whereas high auxin response domain is restricted to the inner side of the hook in WT seedlings, PMEI5oe seedlings exhibited a broad auxin response domain that extended along both sides of the hook, increasing DR5 intensity on the outer side more than 4-fold (in PMEI5oe, outer DR5-Venus relative intensity 0.53 ± 0.18 compared with WT 0.12 ± 0.02). Thus, the loss of asymmetric HG methylesterification also perturbs auxin response domain asymmetry (Figures 4A and S4A; n ≥ 11 cells from each of inner and outer side of 9 seedlings). These results suggest a positive feedback loop between auxin and HG methylesterification during apical hook development.

**Attenuation of auxin response rescues hook defects from enhanced HG methylesterification**

The perturbation of asymmetric HG methylesterification in PMEI5oe seedlings negatively affects the differential growth necessary for hook formation. Interestingly, PMEI5oe seedlings display a broader auxin response domain than WT seedlings. We reasoned that this enhanced auxin response on the outer side in PMEI5oe (in contrast to WT) may repress cell elongation on this side, thereby attenuating differential growth across the hypocotyl. We addressed this hypothesis by suppressing the auxin response in PMEI5oe seedlings via auxinole treatment. Cell elongation rates were strongly enhanced by short-term auxinole application (50 μM over 3 h) to PMEI5oe seedlings relative to mock-treated control (upon auxinole 10.9% h⁻¹ ± 2.3% and 13.8% h⁻¹ ± 1.9% on outer and inner sides, respectively, compared to 4.3% h⁻¹ ± 1.0% and 3.3% h⁻¹ ± 1.6% on outer and inner sides upon mock treatment) with the elongation rates reaching levels similar to what was observed for auxinole-treated WT seedlings (12.6% h⁻¹ ± 2.0% and 11.7% h⁻¹ ± 2.2% on outer and inner sides, respectively) (Figures 4B and S4B; n ≥ 11 cells from each of 8 seedlings), causing hook development disruption.

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**Figure 4. HG methylesterification affects auxin response**

(A) Average nuclear DR5-Venus intensity on inner and outer sides of the hook in WT and PMEI5oe seedlings. n ≥ 11 cells from each of inner and outer side of 9 seedlings.

(B) Individual cell-elongation rates (% h⁻¹) at 0–400 μM upon 3 h mock and 50 μM auxinole treatment in WT and PMEI5oe seedlings. n ≥ 12 seedlings.

(C) Apical hook development in WT and PMEI5oe upon mock and 5 μM auxinole treatment. n ≥ 12 seedlings.

(D) Quantification of PM signal of AUX1-YFP, PIN3-GFP, PIN4-GFP, and PIN7-GFP in epidermal cells at 0–400 μM from SAM during hook formation. For AUX1-YFP and PIN3-GFP, n = 5 cells from each of 10 seedlings. For PIN4-GFP and PIN7-GFP, n = 5 cells each of 9 seedlings. Statistical significance was determined by paired, two-tailed Student’s t test, where “p < 0.05,” “p < 0.005,” and “p < 0.0005.”

For (A), (B), and (D), error bars represent standard deviation of the mean. For (C), error bars represent standard error of the mean. See also Figure S4.
transport machinery. Therefore, we examined the behavior of the auxin response domain in PMEI5oe seedlings, with PMEI5oe seedlings reaching an average angle of 163° upon auxinol treatment compared with 132° upon mock treatment (Figure 4C; n ≥ 12 seedlings).

**HG methylesterification mediates control of asymmetric auxin response via polar auxin transport**

The asymmetric auxin response witnessed during hook development is established through the concerted action of the auxin transport machinery. Therefore, we examined the behavior of specific auxin influx and efflux transporters to determine whether the broad auxin response domain in PMEI5oe seedlings was caused by perturbations in the auxin transport machinery. We observed a 25% increase in fluorescence levels for the auxin influx carrier AUX1-YFP at the plasma membrane (PM) in PMEI5oe seedlings relative to WT seedlings (Figures 4D and S4D). In contrast, the fluorescence signal for the auxin efflux carrier PIN1-GFP was absent from the PM, almost exclusively localized to intracellular structures in PMEI5oe seedlings. In comparison, the PIN1-GFP signal was localized to the PM of cells on the inner side of the hook in WT seedlings. Furthermore, in PMEI5oe seedlings, PM intensity of efflux carriers PIN3-GFP, PIN4-GFP, and PIN7-GFP were reduced to 74%, 67%, and 55% of WT levels, respectively (Figures 4E and S4D, for AUX1-YFP and PIN3-GFP, n = 10 cells from each of 10 seedlings, for PIN1-GFP, n ≥ 8 seedlings, for PIN4-GFP and PIN7-GFP, n = 5 cells from each of 9 seedlings). These results suggest that alterations in HG methylesterification could impact the polar auxin transport machinery and broaden the auxin response domain. Hence, our results suggest that, while auxin levels affect HG methylesterification, this methylesterification can also influence factors that drive auxin distribution patterns. Such a positive feedback loop would promote hypocotyl bending during apical hook formation.

**DISCUSSION**

Apical hook development is crucial to the survival and establishment of germinating seedlings, as it protects the fragile shoot apical meristem from the soil interface. Here, we show that differential growth during hook formation is mediated by a HG methylesterification gradient that depends on auxin distribution. Moreover, we discovered that HG methylesterification affects the transport machinery that controls auxin distribution in the hook. Our results thus reveal that tissue bending during hook formation is regulated by a positive mechanochemical feedback loop.

**Auxin mediates differential cell elongation by influencing cell-wall stiffness**

Several studies had previously demonstrated that the asymmetric auxin response across the hypocotyl is essential for proper hook development. Auxin can either promote or repress cell elongation. Based on our data, the high auxin levels on the inner side, when compared to low levels on the outer side, repress cell elongation during apical hook formation. Nevertheless, how this asymmetric auxin response translates into differential cell elongation remains poorly understood. Interestingly, the auxin response domain spatially overlaps with pronounced differences in cell wall mechanical properties and reduced cell elongation on the inner side. Therefore, it is plausible that the auxin repression of cell elongation may be mediated via modulation of cell wall mechanics. Notably, exogenous IAA application can reduce cell elongation on the outer side, which usually shows low auxin response (Figures 1A–1E). Conversely, suppressing auxin response through auxinole application enhances elongation on the inner side of the hook (Figures 1F–1J). Hence, auxin can influence cell wall mechanical properties, as increased auxin levels correspond with significant differences in cell-wall properties and elongation, on the outer side of the hook (Figures 1K–1L and 1N), and the converse on the inner side of the hook upon auxin inhibition (Figures 1K, 1M, and 1N). These data suggest that the auxin-mediated modulation of cell wall mechanical properties may regulate differential cell elongation. Importantly, our results highlight that auxin effects on cell elongation are presumably concentration and context dependent and provide a plausible mechanism for organizing tissue-scale control of differential growth and mechanical properties via asymmetric distribution of auxin.

Our data suggest that asymmetric auxin distribution establishes differential cell elongation regions by affecting mechanical properties, and more specifically, wall stiffness. However, the pathways through which auxin alters mechanical properties in the apical hook have remained unknown. Furthermore, the link through which asymmetric auxin distribution translates to differences in mechanical properties and elongation rates across the hypocotyl remains unclear. Several recent studies have suggested that HG methylesterification influences wall structure, mechanical properties, growth anisotropy, and tissue patterning. Our analysis of HG methylesterification showed that the inner side of the hook—characterized by low cell elongation and elasticity—exhibited longitudinal walls with highly methylesterified HG relative to the outer side (Figure 2A). Moreover, we found the perturbation of HG methylesterification asymmetry to severely affect mechanical properties (Figures 2B–2D) and differential cell elongation (Figures 2E and 2F), which resulted in defective hook formation (Figure 2H). Our results thus highlight the importance of mechanochemical asymmetry of the cell wall across the hypocotyl during hook formation.

Auxin response maxima and lower rates of cell elongation overlap with regions of high HG methylesterification in the hook. Furthermore, increased auxin levels (yuc1D mutant) and a suppressed auxin response (tir1E12K) showed opposite effects on HG methylesterification (Figures 3A and 3B), suggesting that asymmetric auxin distribution is linked to HG methylesterification. Thus, the auxin-mediated gradient in HG methylesterification could regulate the differential mechanical properties that are critical for the tissue-level differences in cell elongation that induce hypocotyl bending. Currently, the mechanism underlying auxin-promoted HG methylesterification remains unclear. Auxin is known to positively regulate the expression of several PMEIs, although post-transcriptional mechanisms may also be involved in HG methylesterification. For example, auxin can alter apoplastic pH by activating PM-localized proton pumps, which may modulate the activity of several wall-modifying enzymes that can subsequently affect HG
methylesterification. Intriguingly, cell wall sensors such as FERONIA have been shown to bind pectin and also mediate in auxin response and pH changes and thus could provide a link between auxin and cell wall compositional changes critical for differential growth. The plethora of PME and PMEI genes in Arabidopsis, assumptions of genetic redundancy, and the paucity of knowledge regarding their enzymatic properties all make it difficult to pinpoint the exact mechanism for how auxin mediates HG methylsterification, although immunohistochemical and genetic data clearly indicate a link between auxin and HG methylsterification with high auxin correlating with methylsterified HG. Previously, a link between auxin and HG methylsterification has been suggested during auxin-mediated organ initiation at the SAM. For example, exogenous auxin application results in increase in de-methylsterified HG during organ initiation. In contrast, our results show that during hook development, high auxin levels on the inner side favor high methylsterified HG. These differential effects of auxin on HG methylsterification could reflect the different roles of auxin in organ initiation and differential growth. Whereas during organ initiation, auxin promotes tissue softening by favoring de-methylsterification, whereas, during hook development, high auxin levels on inner side restrict growth by mediating high methylsterification relative to outer side in the hook.

Cell-wall properties influence the auxin response domain in the apical hook

Intriguingly, PMEI5oe seedlings exhibited a strongly altered auxin response domain. In contrast with the WT seedlings—in which the auxin response was distinctly localized to the inner side—PMEI5oe seedlings exhibited significant DR5 expression in cells on the outer side as well (Figures 4A and S4A). AUX/LAX and PIN family proteins, which mediate polar auxin transport, have been shown to establish the asymmetric auxin response domain. Our data from PMEI5oe seedlings reveal alterations in the abundance of auxin transporters (AUX1, PIN1, PIN3, PIN4, and PIN7) in the PM, which could disrupt polar auxin transport (Figures 4E and S4C). Thus, a wider auxin response domain is most likely a consequence of the perturbed polar auxin transport associated with enhanced HG methylsterification that impacts both influx and efflux carriers at the PM. Previously, enhancing HG methylsterification was shown to cause altered PIN1 polarity that is critical for organ initiation. Whereas pin1 mutant exhibits only minor defects in hook, PIN3, PIN4, and PIN7 play critical roles in hook development: the pin3 pin4 as well as pin4 pin7 double mutants exhibit severe hook defects. Importantly, the levels of PIN3, PIN4 and PIN7 are all significantly reduced at the PM in PMEI5oe. Thus, our data now connect HG methylsterification with abundance of PIN3, PIN4 and PIN7 proteins that are critical during hook development.

There are two plausible routes through which changes in HG methylsterification affect the polar auxin transport machinery. First, an increase in HG methylsterification stimulates ectopic brassinosteroid (BR) signaling. BR has been shown to stimulate PIN expression in shoots and to enhance PIN protein sorting at the PM. However, our data show that enhanced HG methylsterification had an opposite effect on PIN levels at the PM. Alternatively, HG methylsterification can cause changes in cell wall mechanical properties that influence PIN PM levels as has been shown for PIN1. While we cannot exclude the role of altered BR response, our data support that changes in cell wall mechanical properties influence the PM levels of auxin transporters.

Importantly, our data showing the effect of altering HG methylsterification on auxin response domain are indicative of a potential feedback whereby changes in cell wall composition may modulate auxin distribution to alter growth patterns. Intriguingly, changes in cell wall composition can significantly affect the polar auxin transport machinery. For example, the loss of xyloglucan in xxt1 xxt2 mutant perturbed polar auxin transport machinery, similar to what was observed in PMEI5oe seedlings with increased HG methylsterification. However, the effect of altered HG methylsterification on polar auxin transport is distinct from defects in the xxt1 xxt2 mutant. For example, PIN1 was transcriptionally upregulated in xyloglucan mutants, whereas PIN1 was excluded from the PM and instead localized intracellularly in PMEI5oe seedlings. Hence, qualitative changes in cell wall composition can have distinct effects on the components of polar auxin transport machinery.

Based on our results, we suggest the following model for hook development. Local auxin response maxima favor a high degree of HG methylsterification on the inner side of the hypocotyl relative to the outer side (Figure 5). Peaucelle et al. have shown that high HG methylsterification results in stiffer cell walls. Our results in the hook are consistent with this finding: our cell deformation assays indicate low and high deformation on the inner and outer side that overlap these differential HG methylsterification patterns. Thus, high HG methylsterification on the inner side of the hook results in stiffer walls on the inner side and asymmetric cell pliancy across the hypocotyl. In other words, spatial differences in wall mechanical properties translate into differential growth rates, which lead to hypocotyl bending. Enhanced HG methylsterification interferes with the auxin response pattern across the hypocotyl and perturbs spatial control of mechanical asymmetry, with increased auxin inhibiting growth on the outer side and resulting in defective hook development. We thus propose that HG methylsterification plays an instructive role on auxin pattern in a positive feedback loop, to amplify the asymmetry and further promote tissue bending. In support of this, we observed that the attenuation of auxin response by auxinole treatment largely mitigated the repressed cell elongation caused by PMEI5 overexpression (Figure 4B) and, furthermore, could reverse the defective hook development observed in PMEI5oe seedlings (Figure 4C). These results extend the finding in the shoot apical meristem, linking auxin to PME activity. However, whereas in meristem, auxin-mediated wall softening, results in promotion of cell elongation and organ outgrowth; we show here that auxin can also repress cell elongation, through induced wall stiffening and HG methylsterification, leading to tissue bending.

Hook formation requires the dynamic control of spatial cell elongation gradients. Previous research has suggested that feedback loops between tissue mechanics and auxin distribution could enable continuous fine-tuning of growth, e.g., cell wall strain patterns instruct the polarity and abundance of PIN1 protein at the PM in the apical meristem. During organ
initiation, a feedback loop involves auxin-mediated tissue softening that feeds back into PIN1 polarity to coordinate local accumulation of auxin with organ outgrowth. In contrast, our data reveal asymmetric auxin distribution mediates tissue-level differential ratio of methylesterified to demethylestrified HG. The resulting spatial differences in mechanical properties with a rigid inner side and relatively softer outer side in turn act on auxin transport machinery components PIN3, PIN4, PIN7, and AUX1 that are known to play a key role in hook development, to generate distinct domains of cell elongation to achieve bending. Hence, auxin distribution dynamically responds to asymmetries in mechanical properties to reinforce growth patterns. Thus, it is conceivable that asymmetric auxin distribution and cell wall composition result from interdependent mechanisms in which feedback between mecanochemical and hormonal signals reinforces growth asymmetry. Such interaction may reflect a general mechanism that operates also during apical hook formation as suggested by our results.

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- **QUANTIFICATION AND STATISTICAL ANALYSIS**

**SUPPLEMENTAL INFORMATION**

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**AUTHOR CONTRIBUTIONS**

K.J. and R.P.B. designed research; K.J., R.S.L., and D.K. performed research; K.J., R.S.L., D.K., A.-L.R.-K., O.H., and R.P.B. analyzed data; K.J., D.K., A.-L.R.-K., O.H., and R.P.B. wrote the paper.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| LM19 (Rat IgM)      | Plantprobes | Cat. No. LM19; RRID: AB_2734788 |
| LM20 (Rat IgM)      | Plantprobes | Cat. No. LM20; RRID: AB_2734789 |
| Cy5 anti-Rat (Rabbit IgG) | Jackson ImmunoResearch | RRID: AB_2338263 |
| Biological Samples  |        |            |
| 35S::PMEI5          | 27     | N/A        |
| yuc1D               | 24     | N/A        |
| pTIR1::tir1E12K-GUS | 28     | N/A        |
| LT16a-GFP           | 41     | N/A        |
| DR5::Venus          | 42     | N/A        |
| pPIN1::PIN1-GFP     | 6      | N/A        |
| pPIN3::PIN3-GFP     | 6      | N/A        |
| pPIN4::PIN4-GFP     | 43     | N/A        |
| pPIN7::PIN7-GFP     | 43     | N/A        |
| pAUX1::AUX-GFP      | 44     | N/A        |
| Chemicals, peptides, and recombinant proteins |        |            |
| Murashige and Skoog (MS) medium | Duchefa | M0222 |
| LR White Medium Grade | TAAB UK | L012 |
| Indole-3-acetic acid (IAA) | Sigma-Aldrich | 1003530010 |
| Auxinole            | MedChemExpress | HY-111444 |
| Plant Agar          | Duchefa Biochemie | P1001 |
| MES hydrate         | Sigma-Aldrich | M5287 |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich | D8418-500ML |
| Paraformaldehyde 16% (w/v) | ThermoFisher | 28908 |
| Experimental models: organisms/strains |        |            |
| Arabidopsis thaliana | NASC   | N1092      |
| Software and algorithms |        |            |
| Microsoft Excel (Office Professional Plus 2016) | Microsoft | N/A |
| ImageJ 1.50e        | N/A    | https://imagej.net/Fiji/Downloads |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Rishikesh P. Bhalerao (rishi.bhalerao@slu.se).

Materials availability
This study has not generated any new reagents.

Data and code availability
This study did not generate any unique dataset or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant material
The Arabidopsis thaliana ecotype Columbia-0 wild-type and the following transgenic lines were used in this study: 35S::PMEI5 (PMEI5oe), 27 yuc1D, 24 pTIR1::tir1E12K-GUS, 28 LT16a-GFP, 41 DR5::Venus, 42 pPIN1::PIN1-GFP, pPIN3::PIN3-GFP, 6 pPIN4::PIN4-GFP, pPIN7::PIN7-GFP, 43 pAUX1::AUX1-YFP 44
Growth conditions

Plants were grown on square Petri dishes supplied with 3/4 × 3/4 × 3/4 MS (2.2 g/l Murashige & Skoog nutrient mix (Duchefa), 0.8% (w/v) plant agar (Duchefa), 0.5% (w/v) sucrose, 2.5 mM 2-Morpholinoethanesulfonic acid (MES) (Sigma-Aldrich) buffered to pH 5.8 with KOH. For confocal microscopy and macro-confocal analysis, seeds were stratified for 2 days at 4°C, given a 6 hours light treatment and subsequently grown in darkness on vertically oriented agar Petri dishes at 21°C for the required time length. For time-lapse analysis of apical hook development, pharmacological treatment using auxinole (Hölzel Biotech), 5 μM auxinole dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) from a stock solution of 50 mM auxinole was added to the medium, and the equivalent amount of solvent was added to the mock treatment.

METHOD DETAILS

Immunohistochemistry

Seedlings were fixed using 4% paraformaldehyde in PBS buffer for 45 minutes, and subsequently washed with PBS buffer 4 times. Samples were then sequentially dehydrated in 30 minute increments at 30%, 50%, 70%, 90% and 100% ethanol in PBS. LR White Medium grade (TAAB, UK) was added to samples dropwise to 10%, and incubated at 4°C for 6h. Solution was then exchanged for 50% LR White in PBS and incubated overnight at 4°C. Solution was then exchanged for 100% LR White in 3 sequential 12h incubations at 4°C. All LR White incubations were performed on a shaker table at 120 rpm. Samples were then cured at 60°C for 36 hours. Samples were sectioned at 2.5 μm thickness using a Reichert Ultracut S Wild M3Z microtome mounted with a Diatome Diamond Knife (8.0mm 45° angle). Sections were placed on glass slides. Immunolabeling was performed on sections using antibodies with the following dilutions: Primary antibodies Rat-LM19 and Rat-LM20 (PlantProbes, UK) were diluted 1:50 with PBS buffer. Secondary antibody anti-Rat Cy5 (Jackson ImmunoResearch, UK) was diluted 1:200. Imaging was performed using a Carl Zeiss LSM780 using the segmented line tool. From this, cell elongation percentage per hour was calculated. For pharmacological treatments used with 500 nM IAA (Sigma-Aldrich), or 50 μM auxinole. Auxinole was diluted from a 50 mM stock solution dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) from a stock solution of 50 mM auxinole was added to the medium, and the equivalent amount of solvent was added to the mock treatment.

Time-lapse imaging of cell elongation

Seeds were given 6 h light treatment and subsequently grown on vertical agar Petri dishes in darkness and imaging was performed 16-20 h post-germination, corresponding to the hook formation phase. Seedlings were imaged on vertically oriented agar Petri dishes using a Nikon AZ-C2 vertical macro-confocal equipped with 5x/0.5 WD 15 mm macro-objective at 3-hour intervals in a dark room. Seedlings expressing LT16a-GFP were excited at 488 nm and emission was detected using spectral detector. Between laser exposures, Petri dishes were wrapped in aluminum foil to minimize exposure to light. Under these conditions, hypocotyl elongation and hook development were not significantly different from dark-grown seedlings over the course of 24 hours subsequent to initial excitation. Cell elongation was measured using ImageJ, where cell length for each cell was measured at two time points, using the segmented line tool. From this, cell elongation percentage per hour was calculated. For pharmacological treatments using indole-3-acetic acid (IAA) or auxinole, seedlings were imaged and subsequently submerged in 1/2 MS medium supplemented with 500 nM IAA (Sigma-Aldrich), or 50 μM auxinole. Auxinole was diluted from a 50 mM stock solution dissolved in DMSO, and IAA was diluted from a 500 μM stock solution dissolved in DMSO. Mock treatments were supplied with the equivalent amount of solvent. In order to investigate the effect of suppressing auxin response on HG methylesterification, WT and tirE12K, not expressing a plasma-membrane marker, seedlings were incubated in darkness in liquid 1/2 MS medium supplemented with propidium iodide (10 mg/mL) for 1 h prior to imaging.

Cell length measurements

Seeds were grown on vertical agar Petri dishes in darkness and imaging was performed 24-28 h post-germination, corresponding to the early hook maintenance phase. Seedlings were imaged by confocal microscopy using a Carl Zeiss LSM780 equipped with a 25x lens (Zeiss Plan-Neofluar 25x/0.8 W Corr DIC M27). Cell lengths were obtained using ImageJ by drawing a segmented line along the length of each cell in the region of 0-400 μm from SAM.

Time-lapse analysis of apical hook development

For time-lapse analysis of apical hook development seedlings were grown vertically on Petri dishes in a dark room at 21°C illuminated only with far-infrared light source. Seedlings were photographed at 4h intervals using a Canon D50 camera without infrared filter. Hook curvature was measured using the angle tool in the software ImageJ, whereby the angle between the hypocotyl axis vector and cotyledons was measured (See Figure 2H inset). For each time-lapse analysis n ≥ 12 seedlings per treatment. For all treatments, seedlings were germinated and grown on medium supplemented with the respective chemical dissolved in DMSO. Mock treatments were supplemented with an equivalent amount of DMSO.
Quantitative analysis of plasma membrane intensity
For analysis of AUX1-YFP, PIN3-GFP, PIN4-GFP and PIN7-GFP fluorescence intensity at the PM, confocal images were acquired using identical acquisition parameters (resolution, laser power, photomultiplier, offset and zoom factor) between WT and PMEI5oe seedlings. Intensity was measured by outlining the PM with a segmented line along the whole membrane and calculating the average intensity using ImageJ. For AUX1-YFP and PIN3-GFP 10 cells from each of 10 seedlings were analyzed. For PIN4-GFP and PIN7-GFP, 5 cells from each of 9 seedlings were analyzed.

Quantitative analysis of DR5::Venus intensity
Z stacks of seedlings expressing DR5::Venus were imaged by confocal microscopy under non-saturating imaging conditions, where identical acquisition parameters (resolution, laser power, photomultiplier, offset and zoom factor) were used between WT and PMEI5oe seedlings. Fluorescence signal between 0-400 μm from SAM was segmented longitudinally into a inner and outer dataset. For each dataset, the ImageJ plugin 3D Objects Counter was used to identify Venus-expressing objects (nuclei), and to obtain mean fluorescence intensity. For all sample analyses, identical threshold parameters (size and signal intensity) were used. For each genotype and dataset ≥15 cells from each of 9 seedlings were analyzed.

Analysis of cell deformation
Seedlings expressing PM marker LTI6a-GFP were first inflated by immersion in H2O for 30 minutes. Subsequently Z stacks of seedlings were obtained by confocal microscopy. Following imaging, seedlings were then transferred to medium with 0.35 M NaCl for 30 minutes to deflate cells. Epidermal cell surface area from Z stacks was calculated using the software MorphographX as described previously.26,45 The change in cell surface area from H2O treatment to 0.35 M NaCl treatment was calculated using MorphographX. Heatmaps in Figures 1K–1M, 2B, and 2C were generated by averaging cell size change for each cell with its direct neighbors, to emphasize tissue-level patterns. Analysis of the elastic deformation in WT and the effect of altering HG methylesterification on elastic deformation was performed by comparing WT and PMEI5oe seedlings at the same time. The effect of auxin or suppressing auxin response on elastic deformation was performed in separate experiment by adding IAA and auxinole or mock treatments of WT.

Soil emergence assay
Sterilized seeds were stratified in sterile H2O at 4°C for 2 days and subsequently pipetted onto the soil surface in pots. For germination inside soil, a 5 mm layer of soil was added on top of seeds. Pots were then placed in a growth chamber at 22°C with 16h light/8h dark cycle for 5 days. Seedlings emerging at the soil surface were then counted. For each treatment, 30 seeds per genotype were sown.

Definition of apical hook outer and inner side
Data obtained from confocal imaging of the hook was divided into four equally sized radial quadrants, the outer quadrant, two lateral quadrants and one inner quadrant. For comparison of cells on the outer versus inner side, we only considered data from the outer and inner quadrants, and disregarded the lateral quadrants.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical significance was determined using Microsoft Excel, employing a paired, two-tailed Student’s t test, on averages of biological replicates. For all analyses, *p < 0.05, **p < 0.005, ***p < 0.0005, as indicated in figures. Error bars in figures represent either standard deviation of the mean or standard error of the mean, as indicated in figure legends. Replicate numbers are indicated in figure legends.