



# Pick a side: Integrating gene expression and mechanical forces to polarize aerial organs

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## Abstract

How organs acquire their shapes is a central question in developmental biology. In plants, aerial lateral organs such as leaves initiate at the flanks of the growing meristem as dome-shaped primordia. These simple structures then grow out along multiple polarity axes to achieve a dizzying array of final shapes. Many of the hormone signaling pathways and genetic interactions that influence growth along these axes have been identified in the past few decades. Open questions include how and when initial gene expression patterns are set in organ primordia, and how these patterns are translated into the physical outcomes observed at the cellular and tissue levels. In this review, we highlight recent studies into the auxin signaling and gene expression dynamics that govern adaxial-abaxial patterning, and the contributions of mechanical forces to the development of flattened structures.

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Organogenesis, Leaf polarity, Adaxial-abaxial pattern, Auxin signaling, Mechanical forces, Cell wall, Cortical microtubules.

## Introduction

Identifying the mechanisms that drive the morphogenesis of organs is a central challenge in developmental biology. In plants, aerial lateral organs such as leaves initiate at auxin maxima localized to the flanks of growing shoot meristems ([Figure 1](#)) [1]. Once specified, primordia emerge as dome-like structures that achieve their final shapes by modulating growth along three

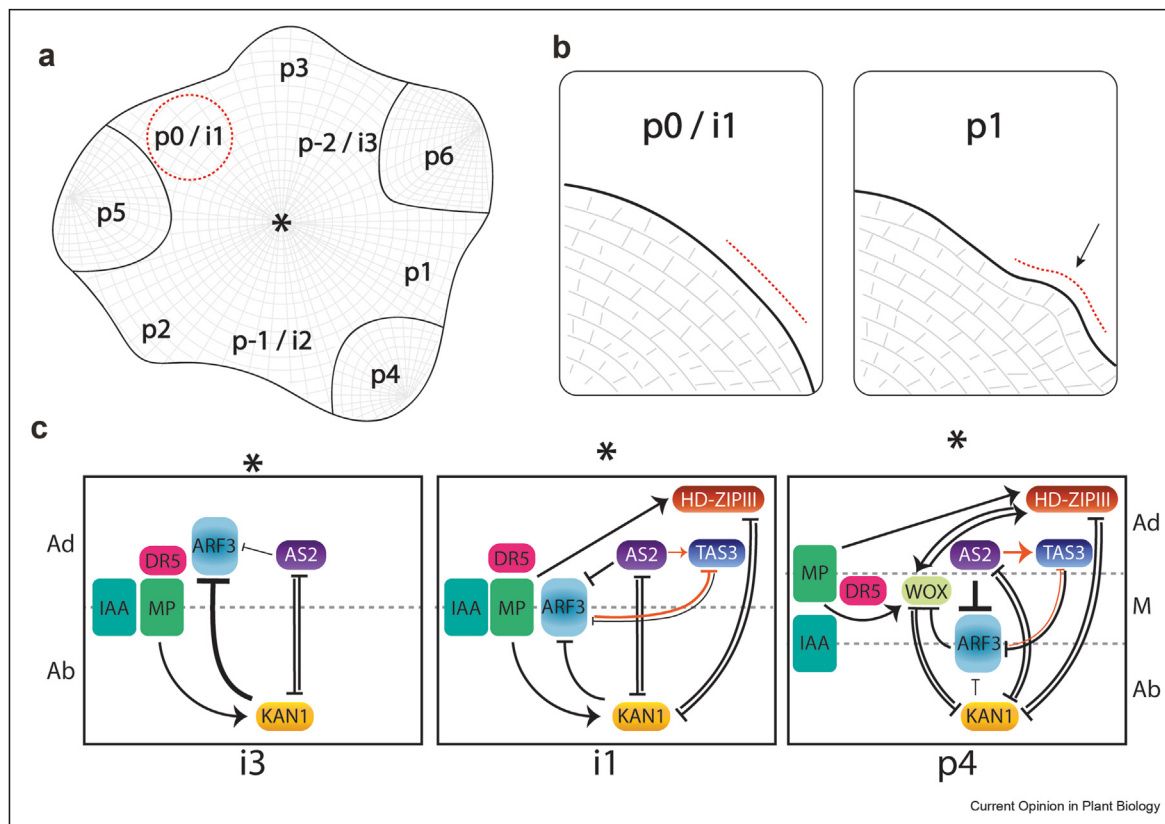
main axes of polarity: 1) adaxial-abaxial (top-bottom), 2) proximodistal (tip-base), and 3) mediolateral (center-to-margin). For instance, the characteristic thin, flat shape of many leaves is achieved by driving growth primarily in the proximodistal and mediolateral directions, while restricting growth in the adaxial-abaxial direction [2]. Adaxial and abaxial layers subsequently become optimized for light capture and gas exchange, respectively, tightly linking adaxial-abaxial patterning to plant fitness [3–5]. A comprehensive review of mechanisms governing plant aerial architecture was recently published [6]. Here, our intent is to highlight recent advances in our understanding of adaxial-abaxial patterning and the acquisition of leaf shape, with a special emphasis on auxin, gene expression dynamics, and mechanical forces.

## Auxin and gene expression dynamics

The plant hormone auxin (indole-3-acetic acid, IAA) is a well-known regulator of plant growth [reviewed in 7]. Auxin maxima play a key role in determining sites of organ initiation, and upon emergence, auxin contributes to outgrowth and leaf flattening through its concentrated activity in the middle domain ([Figure 1](#)). Nearly thirty years of molecular genetic analyses have identified many other components involved in promoting the adaxial, abaxial, and middle (or marginal) domains that comprise the adaxial-abaxial axis. A subset of adaxializing factors include the *CLASS III HOMEODOMAIN LEUCINE ZIPPER (HD-ZIP III)* transcription factors *REVOLUTA (REV)*, *PHABULOSA (PHB)*, and *PHAVOLUTA (PHV)* [8–10], the *LOB DOMAIN* transcription factor *ASYMMETRIC LEAVES2 (AS2)* [11–13], as well as the mobile trans-acting siRNAs derived from the *TAS3A* locus (tasiR-ARFs) [14]. Factors promoting abaxial fate include the transcription factors *KANADI1 (KAN1)* and *AUXIN RESPONSE FACTOR3 (ARF3)* [15–17], as well as the mobile microRNA miR166 [10,18,19]. Finally, the middle domain is specified by members of the *WUSCHEL-RELATED HOMEBOX (WOX)* family which includes the transcription factors *WOX1* and *PRESSED FLOWER (PRS/WOX3)*. For in-depth reviews of these factors, see Refs. [6,7,20].

Organogenesis is dynamic, integrating changes in gene expression and auxin concentrations over space and time. The adaxial-abaxial axis is already polarized by the

Figure 1



**Primordia stages and gene expression dynamics.** (a) Top-down representation of a shoot apical meristem containing primordia of varying developmental stages (asterisk indicates tip of meristem). Primordial stages are assigned relative to the earliest-detectable bulging primordium (p1) which emerges at the site of an auxin maximum in the peripheral zone (red dashed circle). Primordia which are pre-emergence are “incipient” primordia and are referred to interchangeably as i3, i2, i1 or p-2, p-1, p0, ordered from youngest to oldest. Incipient primordia are pronounced as ‘p minus 2’, and so on. (b) Side view of a pre-emergence (left) and post-emergence primordium (right) at site of auxin maximum. Auxin maximum is denoted by a red dashed line. (c) Gene expression dynamics at three stages of leaf primordium development (i3, i1, p4). The asterisk indicates relative position of meristem and increased distance represents displacement from the meristem as primordium develops. At the i3 stage (*left*), the adaxial-abaxial pre-pattern is defined by mutually repressive AS2 and KAN1. Auxin input (IAA) is uniform while auxin response (DR5) is specific to the adaxial side. ARF3 is restricted to the adaxial side via strong KAN1 repression (strength of regulation indicated by arrow thickness), and KAN1 expression is promoted by MP. At the i1 stage (*middle*), the HD-ZIP III transcription factor, REV, accumulates on the adaxial side and ARF3 expression begins to shift towards the abaxial domain. Expression of HD-ZIP III and KAN1 is promoted by MP, and KAN1 and REV are mutually repressive. A speculative ‘protective mechanism’ may lead to increased TAS3A expression which in turn begins to exclude ARF3 from the adaxial side (speculative regulatory relationship indicated with orange arrows). By the p4 stage (*right*), WOX genes mark the middle domain, IAA shifts down to span the middle domain, MP becomes more adaxial, and the auxin response (DR5) moves to the middle domain. ARF3 expression shifts further to span the middle and abaxial domains possibly in response to easing of KAN1 repression and increased AS2 repression. The speculative ‘protective mechanism’ would now be fully in place (orange arrows).

i1 primordium stage [21,22]. However, key questions remain about this process. For instance, when does this polarization occur and how do auxin and the polarity determinants behave during this rapid and dynamic patterning process? A recent study combining live-imaging and cell-lineage analyses begins to address these questions [23]. Tracing cells from the adaxial, abaxial, and middle domains across multiple plastochrons revealed i3 primordia are comprised of non-overlapping domains of AS2 and KAN1, corresponding to future adaxial and abaxial domains, respectively (Figure 1c, left). This indicates that adaxial-abaxial polarity is in place by the i3 stage, a full 3 plastochrons before primordium emergence (Figure 1a). As HD-

ZIP III gene expression does not occur until the i2 or i1 stage, these important adaxializing factors thus likely respond to, rather than establish, the adaxial-abaxial axis. i1 stage primordia, defined by adaxial AS2/HD-ZIP III and abaxial KAN separated by a small gap, are then triggered to emerge from the meristem by an auxin maximum (Figure 1c, middle) [1,21,23]. Finally, WOX1 and PRS, localized to the middle domain, drive growth perpendicular to adaxial-abaxial axis, flattening the developing leaf (Figure 1c, right) [3,24]. Imaging markers of auxin distribution and auxin signaling provided additional surprising insights into its dynamic behavior during organogenesis. In i3 primordia, auxin is uniformly distributed throughout the adaxial and abaxial

domains. As primordia develop, auxin is depleted first from the adaxial side, then from the abaxial side, with its accumulation eventually restricted solely to the middle domain [23,25]. Interestingly, auxin signaling does not simply mirror these dynamics. Despite the uniform accumulation of auxin across i3, i2, i1, and p1 primordia, auxin signaling is only detected in the adaxial domain. Surprisingly, this binary readout appears to be mediated by *ARF3*, a transcription factor best known for its abaxializing properties [15]. *ARF3* accumulates on the adaxial side of i3 primordia; then, its accumulation shifts across the adaxial-abaxial axis as primordia develop transiting through the middle domain before adopting abaxial-specific accumulation by p6. The mechanism driving this shift is not clear but is proposed to involve *KAN1* and *AS2* whose repression of *ARF3* weakens or strengthens, respectively, over developmental time. These new insights also highlight the utility of time-lapse imaging and cell-lineage analyses in developmental biology.

Another recent study investigating the role of auxin in adaxial-abaxial polarity took a computational modeling approach [26]. Using a “seesaw” model of *REV* and *KAN* mutual repression as an initial framework, the authors found that auxin promotes the expression of both genes via *ARF5/MONOPTEROUS* (MP). The simultaneous activation of these two antagonistic factors was sufficient to recapitulate a robust adaxial-abaxial boundary during outgrowth. Iterative simulations then tested the impact of additional regulatory relationships which were validated using molecular genetic approaches. Interestingly, a positive feedback loop was identified between *REV* and both *WOX1* and *PRS*, while a negative feedback loop was shown for *KAN1* and both *WOX1* and *PRS*. Upregulation of *KAN1* by MP could explain why *KAN1* is not downregulated by auxin in incipient primordia but would seem to contrast with observations that auxin depletion at wounding sites leads to increased *KAN1* expression [21,27]. One potential explanation is that the increase in *KAN1* expression in low auxin environments stems from loss of repression by *REV*, *WOX1*, or *PRS*, all of which decrease in the absence of auxin [21,27].

A long-standing mystery in organ polarity has been the theoretical meristem-derived adaxializing molecule termed the Sussex signal [28]. In brief, surgical incisions isolating primordia from the meristem led to abaxialization of organs in *Solanum tuberosum* (potato), suggesting signals from the meristem are required to establish stable adaxial-abaxial patterning [29,30]. Recent studies in *Arabidopsis* and *Solanum lycopersicum* (tomato) suggest an alternate explanation for the original observations. Confocal imaging of tomato meristems identified two small points of auxin convergence in the lateral regions of incipient primordia [27,31]. Disrupting these convergence points by incisions [29–31] or blocking of

polar auxin transport [27] led to a sharp reduction in the expression of *SIWOX1* and *SIREV*, an expansion of *SIKAN2C* expression throughout the leaf primordia, and the formation of abaxialized organs [27,31]. Thus, an alternate hypothesis explaining Sussex’s observations is the loss of an auxin-induced positive feedback loop between the adaxial and middle domain determinants [21,26,27,31]. Interestingly, similar surgical experiments in *Arabidopsis* did not yield abaxialized organs [21,22], despite the presence of ectopic *KAN* and the loss of *REV* [21]. One possibility is that tomato primordia rely on externally derived auxin for longer than *Arabidopsis* primordia [27]. This notion is supported by computational modeling; however, the mechanism underlying these species-specific responses remains unclear.

### Mechanobiology and organ polarity

Growth of plant cells is driven by high internal turgor pressure which is actively resisted by rigid cell walls comprised of cellulose, pectin, and other carbohydrates. To allow growth, cell walls must plastically deform, a process which is influenced by the elasticity of wall matrix components and the orientation of cellulose microfibrils [32]. For instance, the emergence of primordia from the meristem is preceded by auxin-induced cell wall loosening and a reorientation of the cortical microtubules (CMTs) that guide cellulose microfibril deposition [reviewed in Ref. [33]]. Thus, directional growth of cells, tissues, and organs involves the integration of multiple biomechanical pathways [reviewed in Refs. [6,32,34]]. Here, we highlight recent studies into how CMT dynamics and cell wall composition guide the anisotropic growth that underlies flattened architecture, as well as the possible influence of polarity factors on this process.

Anisotropic arrangement of CMTs and cellulose microfibrils parallel to the direction of principal stress provides structural reinforcement to growing organ primordia while positioning cell division planes [35]. A recent study explored the relationship between stress and CMT anisotropy, and its impact on organ flattening [36]. First, principal stress axes of young organ primordia were computationally modeled in a three-dimensional ellipsoid under turgor pressure. In the model, CMTs aligned along the adaxial-abaxial axis, matching experimental observations in early leaf and sepal primordia (Figure 2a, left). However, the model deviated from experimental observations of later-stage primordia which have isotropically arranged epidermal CMTs (Figure 2a, right) [36]. To explain this discrepancy, the authors considered whether CMTs could be responding to stress-mediated mechanical feedback, as proposed in Ref. [37]. Simulating this mechanical feedback throughout the entire developing ellipsoid was sufficient to promote leaf flattening but also generated significant mechanical conflict between internal and

epidermal layers. Removal of the feedback mechanism from the epidermal layer resolved this conflict and enhanced primordia flattening. Importantly, CMTs in internal layers were anisotropically oriented along the adaxial-abaxial axis while CMTs in the epidermal layer were isotropically arranged, matching *in vivo* observations. Further support for this stress-mediated feedback mechanism comes from genetic and chemical manipulations of epidermal CMTs which found a positive correlation between isotropic CMTs in the epidermal layer and degree of organ flattening [36]. Finally, as leaves of *wox1 prs* double mutants had wildtype CMT arrangements, CMT coordination appears to be independent of the marginal growth promoting *WOX* genes. Taken together, these results support a model in which adaxial-abaxial polarization sets up the initial asymmetry of primordia which is then amplified by CMT-dependent growth anisotropy [36].

Another study aimed to identify the cellular growth patterns that underlie bilaterally symmetric leaves versus axisymmetric floral primordia [38]. Live imaging revealed leaf primordia are characterized by faster abaxial growth rates while floral primordia are characterized by faster adaxial growth rates. Transgenic manipulation of these growth rates altered the geometry of organ primordia, generating axisymmetric leaves and asymmetric floral primordia, supporting their biological relevance. Consistent with prior work [36], computational modeling supports anisotropic CMT alignment along the adaxial-abaxial axis as one mechanism underlying these differential growth rates. Another mechanism appears to be the modulation of cell wall composition. Increased cell wall elasticity is correlated with faster growth and decreased methyl-esterified pectic homogalacturonan (HG) [39,40]. In leaves, demethyl-esterified HG is detected first in the abaxial domain of p1/p2 primordia, then the adaxial domain of p3 primordia, and is largely absent from the middle domain in p5 primordia (Figure 2b) [41]. This dynamic process creates mechanical heterogeneity — softer adaxial and abaxial domains flanking a stiffer middle domain — that computational modeling predicts is essential for developing bilateral symmetry in leaves (Figure 2b, right) [38]. By contrast, floral primordia have homogenous levels of demethyl-esterified HG and cell wall elasticity is uniformly high across the primordia. Thus, bilateral symmetry is proposed to depend on the juxtaposition of cells with differential elasticities, although how the middle domain drives growth if its cells are relatively stiffer remains unclear.

Pectin can also be modified by the activity of polygalacturonases (PGs) which cleave its main HG domain and promote cell wall loosening [reviewed in Ref. [42]]. A recent reverse genetic screen in *Arabidopsis* identified *PG45* as a factor influencing leaf flatness [43]. In addition to floral and branching phenotypes, *pg45* loss-of-

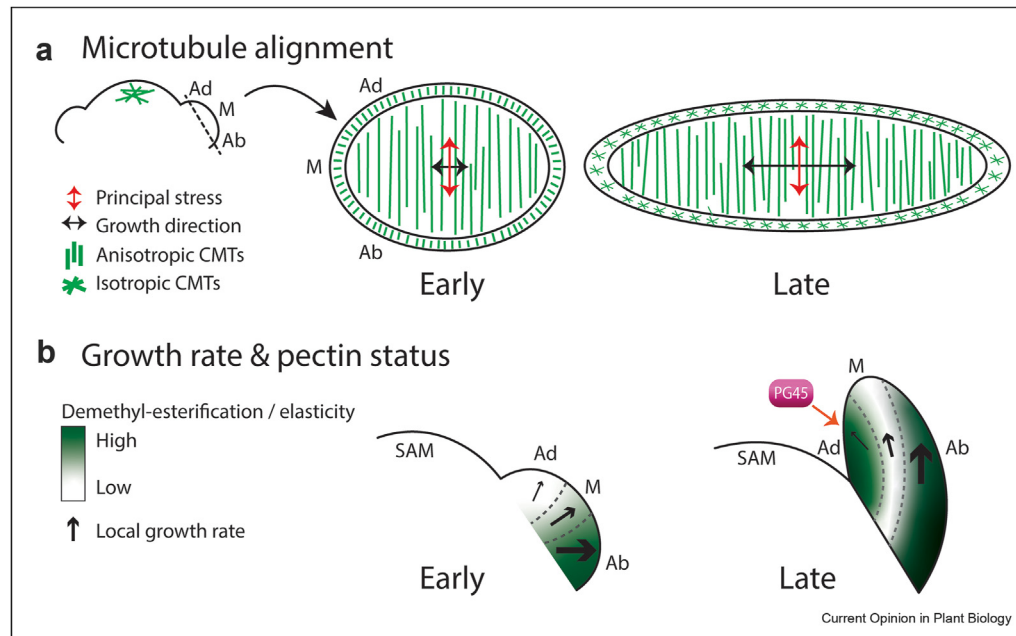
function mutants have dramatically upward curling leaves with disorganized cell layers and abnormal cell shape. Interestingly, analyses of cell division patterns reveal *pg45* leaves have lost coordination of their cell proliferation windows across the adaxial-abaxial axis. Specifically, cells in the adaxial epidermis stop dividing earlier than cells in the abaxial epidermis, leading to overgrowth from the bottom side and thus upward curling. Cell expansion rates, however, are unaffected by mutation of *PG45*. These findings demonstrate that adaxial and abaxial cell division rates can be uncoupled and present the intriguing possibility that these rates may be under differential regulatory control. The mechanism by which *PG45* modulates cell division rates is unknown. However, pectin cleavage by PGs produces oligogalacturonides (OGs) which inhibit auxin responses in a dose-dependent manner [44]. *PG45* may thus impact adaxial-abaxial polarity by modulating ARF activity. Consistent with this possibility, the leaves of *arf3 arf4* double mutants show similar upward curling. Alternatively, *PG45* might be involved in the switch from low to high adaxial demethyl-esterification observed during primordial outgrowth (Figure 2b, right) [38].

### Computational modeling and robustness

There has been increasing appreciation for the power of computational modeling in biology, and most of the studies discussed in this review contain at least some modeling. Models generating novel hypotheses which can be tested experimentally are particularly powerful. For instance, the “seesaw” model of polarity maintenance was seeded with mutual inhibition of *HD-ZIPIII* and *KAN* genes [26]. From this core assumption, multiple new insights into adaxial-abaxial patterning were derived, including the simultaneous activation of antagonistic genes, and positive and negative feedback loops linking the adaxial-middle and abaxial-middle domains, respectively (Figure 1c, right) [26]. From the mechanobiology perspective, one prediction to emerge from recent computational modeling was the promotive effect on organ flattening of isotropically arranged CMTs in the epidermal layer of older leaf primordia (Figure 2a, right) [36]. Consistent with this hypothesis, a microtubule severing mutant with constitutively isotropic epidermal CMTs had flatter leaves and sepals. A second prediction was that properties of the cell wall influence growth rates across the adaxial-abaxial axis and contribute to the flattening of developing leaves [38,41]. This was tested using genetic and chemical manipulations which confirmed that differential stiffness between the adaxial, abaxial, and middle domains contributes to leaf flatness (Figure 2b, right), although the relationship between stiffness and components in the adaxial-abaxial network remains unclear.

Finally, models can propose parameters or regulatory dynamics that might be important for robustness of the biological system. For instance, sensitivity analyses of the

Figure 2



**Dynamic mechanical properties in leaf primordium outgrowth.** (a) Microtubule dynamics across the adaxial-abaxial axis. At early stages of outgrowth (*left*), cortical microtubules (CMTs) in both internal and epidermal cell layers are highly anisotropic and align mostly along the principal stress direction (red arrow). Stress-based mechanical feedback promotes outgrowth (black arrow) orthogonal to the direction of stress, resulting in a flattened, asymmetric structure. In later stages (*right*), epidermal CMTs must switch to an isotropic arrangement to maintain this stress-based feedback mechanism. (b) Growth rates and pectin composition across the adaxial-abaxial axis. In early primordia (*left*), highest growth rates are in the abaxial domain, followed by the middle domain, then the adaxial domain (growth rate corresponds with arrow thickness). Growth rates positively correlate with elasticity and amount of demethyl-esterified pectin. In later stages (*right*), demethyl-esterification in the adaxial domain increases to match the abaxial domain while the middle domain has the least amount of demethyl-esterified pectin and is the least elastic. A speculative mechanism to explain an adaxial-specific increase in demethyl-esterified pectin is the activity of PG45 (orange arrow). Ad = adaxial domain, M = middle domain, Ab = abaxial domain.

“seesaw” model suggest the stability of the *REV-KAN* pattern tolerates stronger inductions of *REV* by MP but is highly sensitive to reductions in activation, with the inverse pattern observed for *KAN1* [26]. In another example, the effects of noise on a one-dimensional model of adaxial-abaxial patterning suggests a positive correlation between degradation rates and the robustness of boundary positioning [45]. Importantly, these testable hypotheses were not immediately obvious from experimental data.

## Perspectives

Morphogenesis involves the integration of gene expression dynamics and mechanical forces. The studies highlighted in this review have yielded new insights into the adaxial-abaxial polarity gene network and the mechanical forces that enable the formation of flattened organs from axisymmetric primordia. These studies have also sparked new questions and research directions.

For instance, adaxial-abaxial polarity is established much earlier than previously thought with an adaxial-abaxial

prepattern evident in i3 primordium (Figure 1c, left) [23]. This prepattern is defined by mutual antagonism between adaxially-expressed *AS2* and abaxially-expressed *KAN1*. However, *as2* mutants still produce leaves with relatively normal adaxial-abaxial polarity [46], and the *KAN1* prepattern is unchanged by loss of *AS2* [23]. This suggests additional unknown factor(s) act redundantly with *AS2*. Identifying these factors is an important next step. The underlying mechanism driving the remarkable dynamism of *ARF3* accumulation also remains unclear (Figure 1c). A possible explanation is suggested by two observations of *TAS3A*, whose transcripts are processed into the mobile tasiR-ARFs that target *ARF3* [14]. First, *AS2* binds the *TAS3A* promoter directly adjacent to an auxin response element [47]. Second, *TAS3A* is strongly repressed by *ARF3*, but only in the absence of *AS2* [47]. These observations suggest *AS2* protects *TAS3A* from direct repression by *ARF3* (Figure 1c, middle & right). A slow acquisition of this ‘protective mechanism’ on the adaxial side of primordia could help create the adaxial-to-middle and middle-to-abaxial dynamics of *ARF3* observed during primordium development.

Computational modeling and experimental data support the idea that mechanical forces alone are insufficient to break morphological symmetry [6,27,36,48]. Initial asymmetry of the primordium is instead likely set by auxin and adaxial-abaxial polarity determinants, which is then amplified through mechanical feedback [6,21,23,27,36]. Nevertheless, it is interesting to consider whether these polarity determinants may also provide instructive cues for mechanobiology factors,

wall properties that coordinate the production of flat leaves.

### Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Aman Y. Husbands reports financial support was provided by National Science Foundation.

## Glossary

**Primordium.** An organ in its earliest morphologically recognizable stage of development.

**Incipient primordium.** Cells that give rise to a primordium, but which are currently morphologically indistinguishable from the meristem.

**Adaxial.** Refers to upper or dorsal side of an organ; nearest to the meristem.

**Abaxial.** Refers to lower or ventral side of an organ; further from the meristem.

**Axisymmetric.** Symmetric around a single axis; also referred to as radially symmetric.

**Bilateral symmetry.** Divisible into two symmetrical halves across a given plane.

**Isotropic microtubules.** Microtubules displaying random organization; microtubules with even constraint against growth forces that contribute to non-directional growth.

**Anisotropic microtubules.** Microtubules displaying directional organization; microtubules with direction-dependent constraint that contribute to directional growth.

**Demethyl-esterified pectin.** Pectin with relatively low amounts of methyl-esterification of its homogalacturonan domain; is associated with increased cell wall elasticity due to reduced stiffness.

perhaps later in primordium development. Adaxial and abaxial CMT arrangements are not obviously different [36]. Demethyl-esterified HG and cell wall elasticity, however, do differ across the adaxial-abaxial axis of leaves, and these properties are altered in polarity mutants [27,41]. It is therefore plausible that enzymes driving the spatiotemporal dynamics of cell wall composition are downstream of the canonical polarity determinants. For instance, *pg45* mutants have attenuated cell division on the adaxial side of leaves leading to an upward-curved, adaxialized phenotype [43]. Is this polarized response a consequence of polar localization of PG45? Does PG45 coordinate the activity of polarity determinants, such as *ARF3* and *ARF4*, across the adaxial-abaxial axis? Localization experiments, possibly in conjunction with single-cell approaches, will be essential in addressing these outstanding questions. Finally, computational modeling will continue to greatly aid our progress in understanding the complex and dynamic interactions between hormone signaling, gene expression, and cell

### Data availability

No data was used for the research described in the article.

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- \* of special interest
- \*\* of outstanding interest

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