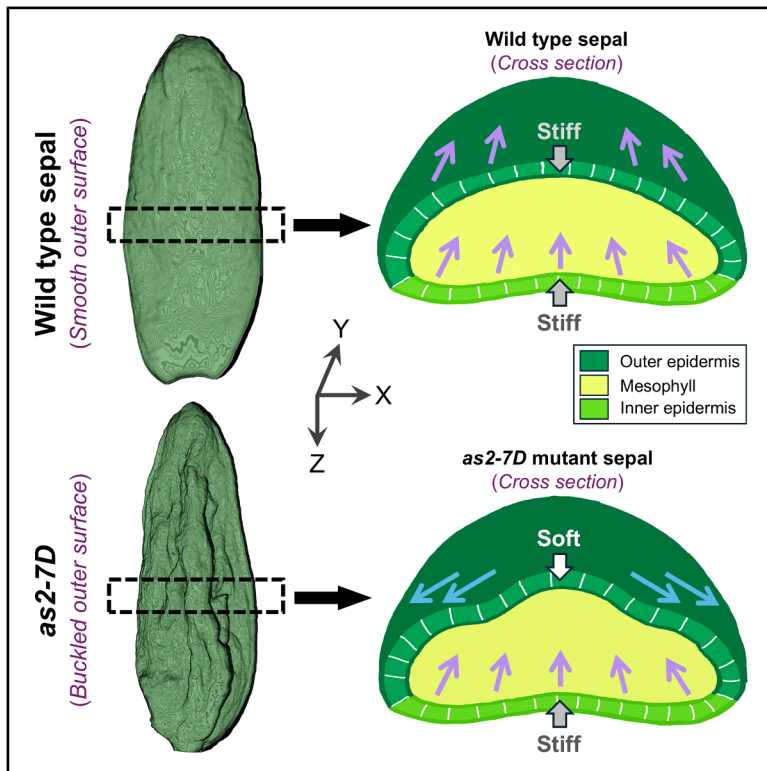


Current Biology

Growth directions and stiffness across cell layers determine whether tissues stay smooth or buckle

Graphical abstract



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In brief

Singh Yadav et al. show that smooth sepal morphogenesis requires aligned longitudinal growth and comparable stiffness across epidermal layers. Ectopic AS2 expression disrupts this balance, causing outer epidermal buckling. Buckling promotes PIN1 convergence at the buckle tips to initiate outgrowths, showing how mechanical and molecular cues interact to shape organs.

Highlights

- Smooth sepal formation requires aligned growth directions and comparable stiffness
- Ectopic expression of AS2 causes buckling of the outer epidermis
- Buckles form due to misaligned growth directions and unequal epidermal stiffness
- Buckling promotes the PIN1 convergence to initiate outgrowths



Article

Growth directions and stiffness across cell layers determine whether tissues stay smooth or buckle

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SUMMARY

Nature exhibits organs of various shapes and forms, ranging from smooth to undulated morphologies. How cells coordinate their growth to produce smoothly shaped leaves and leaf-like organs such as sepals remains unclear. We identified a mutant, *as2-7D*, that exhibits ectopic expression of *ASYMMETRIC LEAVES 2* (*AS2*) on the outer epidermis. Our analysis reveals that ectopic *AS2* expression causes the outer epidermis of *as2-7D* sepals to buckle during early stages of sepal development. Buckling is caused by conflicting cell growth directions and unequal tissue stiffness across the epidermal layers. Overexpression of cyclin-dependent kinase (CDK) inhibitor Kip-related protein 1 (*KRP1*) in *as2-7D* aligns the growth directions of the outer epidermal cells along the longitudinal axis, increases the overall stiffness of the outer epidermis, and restores sepal smoothness. Further experiments suggest that buckling promotes the convergence of auxin efflux transporter protein PIN-FORMED 1 (*PIN1*) to initiate pointed outgrowths. Thus, we show that growth along the longitudinal axis during early developmental stages and comparable stiffness across both epidermal layers of *Arabidopsis thaliana* sepals are essential for smoothness, as seen in the wild type. Our findings suggest that in addition to molecular cues influencing tissue mechanics, tissue mechanics can also modulate molecular signals, giving rise to well-defined shapes.

INTRODUCTION

During development, leaf blades and fly wings form smooth shapes.^{1–6} Conversely, the brain, intestine, and orchid flower petals form complex shapes with undulations, folds, and loops.^{7–12} Folds and undulations frequently form through mechanical buckling, when differential growth generates compressive forces that cause the tissue to fold out of plane.^{8–10,13–15} Buckling causes gyrification of the cerebral cortex to increase neuron content,⁷ looping of the gut to increase contact with food,^{8–10} and initiation of new branches in lung airway epithelia to enhance gas exchange.¹⁴ Similarly, in plants, buckling generates helical twists in roots to increase penetrative forces at the growing tip,^{16,17} triggers lobe deformation in Venus flytraps for rapid energy storage and release to capture prey,¹⁸ and induces nanoscale cuticular ridges on petals to produce iridescence.^{11,12,19} Although the mechanical basis of buckling is well understood, much less is known about how developing tissues regulate cell growth and material properties to generate buckling in some cases or suppress buckling to maintain smooth organ surfaces.

Buckling is not always beneficial. The stem of cereal crops or slender trees can buckle from weight and external forces such as wind or rain, causing structural damage and reduced yield.^{20,21} To counter buckling, plants allocate resources to increase stem thickness and stiffness.^{22,23} Likewise, while buckling generates diverse leaf shapes,^{24–26} leaves generally suppress buckling to generate smooth leaf blades with a relatively flat structure to enhance light absorption and gas exchange for photosynthesis.^{27,28} Sepals, which are the outermost leaf-like floral organs, also exhibit smooth surfaces to ensure that all four sepals close properly to protect developing flower buds.²⁹

Fundamentally, organ shape arises from spatially and temporally regulated cell growth patterns across physically connected and dynamically interacting tissue layers.^{30–32} Such growth patterns emerge from the combined influence of tissue mechanics and the underlying genes that control the developmental programs.^{31,33,34} While transcription factors regulate morphogenesis at the molecular level,³⁵ transcription factors alone cannot drive shape formation without regulating tissue mechanics.^{36,37} A growing body of studies has shown that tissue mechanical



properties are strongly influenced by genetics.^{30,31,38–40} While much is known about how genes influence organ development, how genes influence tissue mechanics to coordinate growth across tissue layers and shape organs remains poorly understood.

The gene regulatory network that establishes the abaxial (outer/lower) and adaxial (inner/upper) identities is a major determinant of smooth and flat leaf lamina.⁴¹ Waites and Hudson proposed that the juxtaposition of adaxial and abaxial identities promotes the lateral growth of the leaf lamina.⁴² In the leaves of *Antirrhinum majus phantastica* (*phan*) mutants, patches of adaxial tissue on the abaxial surface generate ectopic ridges that resemble lamina margins, indicating that juxtaposition of abaxial and adaxial identities can initiate ectopic outgrowths.⁴² Since then, several studies have shown how disruption in the adaxial-abaxial gene regulatory network can generate outgrowths.⁴³

Abaxial identity is specified by *KANADI*, *YABBY*, and *AUXIN RESPONSE FACTOR 3* and *4* (*ARF3* and *ARF4*) gene families, while adaxial identity is established by the HD-ZIP class III genes, *ASYMMETRIC LEAVES 1* (*AS1*), and *ASYMMETRIC LEAVES 2* (*AS2*).^{43–45} The *kan1 kan2* double mutants exhibit radialized organs with ectopic outgrowths on the abaxial side.^{46–48} The outgrowths in *kan1 kan2* double mutants exhibit ectopic *FILAMENTOUS FLOWER* (*FIL*) expression. Removing *FIL* and *YABBY3* (*YAB3*) activity in the *kan1 kan2 fil yab3* quadruple mutants largely suppresses the formation of folds and outgrowths, although the leaves are also radialized and severely reduced in size, indicating that *YABBY*s are required for the development of these outgrowths.⁴⁹ Likewise, removal of *WUSCHEL-RELATED HOMEODOMAIN* (*WOX*) genes *PRESSED FLOWER* (*PRS*)/*WOX3* and *WOX1* suppresses the formation of ectopic outgrowths in the *prs wox1 kan1 kan2* quadruple mutants, although the leaves still exhibit a folded morphology.^{50,51} Conversely, ectopic *WOX1* expression is also sufficient to induce outgrowth formation.^{50,52}

The LATERAL ORGAN BOUNDARIES (*LOB*s) domain encoding transcription factor *AS2* plays an essential role in establishing adaxial identity.^{53–56} While *AS2* loss of function leads to asymmetrically lobed and downwardly curled leaves,^{53,57} overexpression of *AS2* causes the formation of outgrowths on the abaxial surface of the leaves, which is accompanied by the repression of abaxial identity genes (*KAN1*, *KAN2*, *FIL*, and *YAB3*).⁵⁵ The role of *AS2* in regulating the adaxial-abaxial gene regulatory network is well characterized, although it remains unclear how *AS2* influences cell growth and tissue mechanical properties to maintain smooth organ shape.

In this study, using *Arabidopsis thaliana* (henceforth *Arabidopsis*) sepals as a model organ, we investigated the cell growth patterns and tissue mechanical properties that allow the wild-type (WT) sepals to maintain a smooth shape. We identified and characterized the *as2-7D* mutant, a gain-of-function mutant of *AS2*, in which ectopic expression of *AS2* on the outer epidermal layer leads to buckling of the epidermis and, subsequently, ectopic outgrowths. We show that coordinated anisotropic growth of the cells on both the inner and outer epidermal layers along the longitudinal direction, together with the comparable stiffness between the epidermal layers, is critical for preventing buckling and ensuring smooth sepal formation. Finally, we find that outgrowths are mechanically linked to buckling, where outgrowths

form specifically at the end of buckles through PIN-FORMED 1 (*PIN1*) convergence. Thus, we show that molecular cues and tissue mechanics influence one another to determine organ shape.

RESULTS

Ectopic expression of *AS2* disrupts sepal smoothness

To determine how the sepals achieve smooth morphology, we performed a forward genetic screen⁵⁸ and identified an *Arabidopsis* mutant with an irregular morphology, characterized by folds and outgrowths (Figures 1A–1F). The folds comprise ridges and invaginations, whereas the outgrowths appear as pointed epidermal projections at the end of the folds (Figures 1F, S1A, and S1B). These folds and outgrowths are visible only on the outer (abaxial) epidermis, while the inner (adaxial) epidermis is smooth (Figures 1E–1H, S1A, and S1B; Video S1). Positional cloning identified a G-to-A mutation 1,484 bp upstream of the translational start site of *AS2* (Figure 1I). An identical mutation was identified previously⁵⁹; however, the fold and outgrowth phenotype in the sepals was not reported⁵⁹ since the phenotype is subtle in Columbia (Col-0) compared with the Landsberg *erecta* (*Ler-0*) background (hereafter referred to as WT) (Figures 1A–1D and S1C–S1F). Previously, Wu et al.⁵⁹ demonstrated that this mutation in the *AS2* promoter disrupts the binding site of the repressor *KANADI*, leading to ectopic *AS2* expression on the abaxial side. In WT, the *AS2* expression domain is restricted to the adaxial side (Figure 1I). To verify that this mutation also leads to ectopic *AS2* expression in sepals of the *Ler-0* background, we expressed green fluorescent protein (GFP) under the WT *AS2* promoter (*pAS2^{WT}::GFP-GUS*) and the mutant *as2-7D* *AS2* promoter (*pAS2^{as2-7D}::GFP-GUS*). While the *AS2* promoter from WT drives GFP specifically in the inner sepal epidermis (Figures 1J and 1K), the mutant *AS2* promoter drives GFP in both the inner and outer epidermal layers (Figure 1L). The mutant *AS2* promoter drove GFP expression evenly throughout the outer epidermis, and no patchy *AS2* expression patterns were observed on the outer epidermis. In addition, *AS2* is still expressed in its regular expression domain on the inner epidermis in *as2-7D* mutants (Figure 1L). Our analysis confirms that the mutation causes gain of function through ectopic *AS2* expression on the outer epidermis, consistent with the dominant nature of this allele (see method details). Therefore, following the convention of previous reports,^{59,60} we named the mutant allele *as2-7D*.

To test whether ectopic *AS2* expression on the outer epidermal layer is sufficient to generate the folds and outgrowths, we expressed *AS2* under the epidermal-specific *MERISTEM LAYER 1* (*ML1*) promoter (*pML1::AS2*), which ectopically expresses *AS2* in both the inner and outer epidermis of WT. All the transgenic plants form lumps and outgrowths on the outer epidermis of the sepals, similar to *as2-7D*, which suggests that ectopic outer epidermal expression of *AS2* is sufficient to replicate the *as2-7D* phenotype (Figures 1M–1P). Additionally, folds and outgrowths form on the epidermis of the leaves and fruits of the *pML1::AS2* transgenic plants (Figures S1G–S1N), consistent with previous reports,⁵⁵ indicating a stronger effect of constitutive epidermal *AS2* expression in the transgenics than in the *as2-7D* gain-of-function mutant. In summary, the mutation in *as2-7D* induces ectopic *AS2* expression on the outer

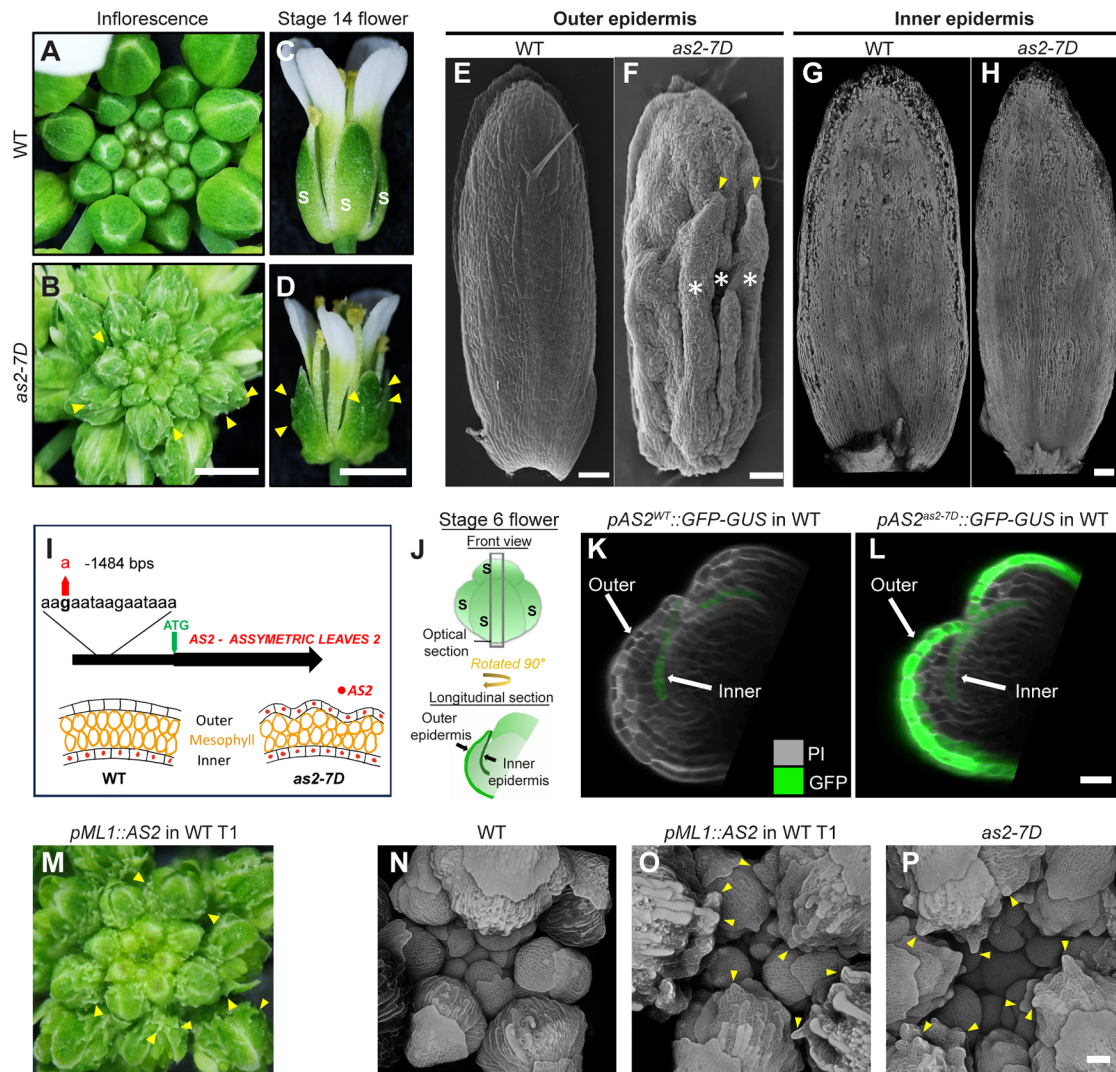


Figure 1. The ectopic expression of AS2 on the outer epidermal layer disrupts sepal smoothness in *as2-7D* mutant

(A and B) Whole inflorescences of (A) wild type (WT, *Ler-0*) and (B) *as2-7D* mutant (in the *Ler-0* background). Yellow arrowheads indicate outgrowths. Scale bar, 1 mm; representative images of $n = 3$ inflorescences for both genotypes.

(C and D) Mature flowers (stage 14⁶¹) of (C) WT and (D) *as2-7D* mutants. WT sepals (green leaf-like organs) are marked with the letter “S” in white (C). Flowers are enclosed within four sepals. Note the outgrowths (yellow arrowheads) on the outer epidermis of *as2-7D* sepals (D). Scale bar, 1 mm; representative images of $n = 3$ flowers.

(E and F) Scanning electron microscopy (SEM) images of the outer epidermis of (E) WT and (F) *as2-7D* mutant sepals. Note the folds (*) and outgrowths (yellow arrowheads) on the *as2-7D* outer epidermis. Scale bar, 100 μ m; representative images of $n = 5$ for WT and $n = 10$ for *as2-7D*.

(G and H) Confocal microscopy images of the inner epidermis of (G) WT and (H) *as2-7D* mutant. The images are representative of T2s harboring pLH13 (35S::mCitrine-RC12A), a yellow fluorescent protein-based cell membrane marker.⁶² Scale bar, 100 μ m; representative images of $n = 3$ for both genotypes.

(I) Schematic diagram representing the genetic mutation in *as2-7D*. Gene body of AS2 showing the G-to-A point mutation (red arrow) at position 1,484 bp upstream of ATG in *as2-7D* (top half). The AS2 expression (red dots) domains in the WT and *as2-7D* sepals are shown (bottom half).

(J) Anatomical diagram representing (top) an early-stage 6 flower and (bottom) an optical longitudinal section through the flower. The letter “S” in black represents sepals. The outer and inner epidermal layers of the outer sepal are marked.

(K and L) Optical longitudinal section of stage 6 flowers of WT. Cell walls were stained with propidium-iodide (PI, grayscale). Images show the GFP expression pattern (green) driven by (K) WT AS2 promoter (*pAS2^{WT}*) and (L) AS2 promoter from *as2-7D* (*pAS2^{as2-7D}*). Arrows indicate the outer and inner epidermal layers of the outer sepal. Scale bar, 20 μ m; $n = 3$.

(M) Whole inflorescence of a representative transgenic (T1) harboring *pML1::AS2* (AS2 expression driven by *ATML1* promoter) transgene in WT. Yellow arrows mark the outgrowths on the sepal epidermis. Independent T1s assessed = 28, all of which show similar levels of outgrowth formation. Representative image of $n = 3$ inflorescences.

(N–P) Confocal images of PI-stained (grayscale) inflorescences of (N) WT, (O) representative transgenic (T1) harboring *pML1::AS2* transgene in WT, and (P) *as2-7D* mutant. WT outer epidermis is smooth, whereas *pML1::AS2* exhibits outgrowths (yellow arrowheads) like *as2-7D*. Scale bar, 50 μ m; $n = 3$ inflorescences per genotype.

See also Figure S1, Video S1, and Table S1.

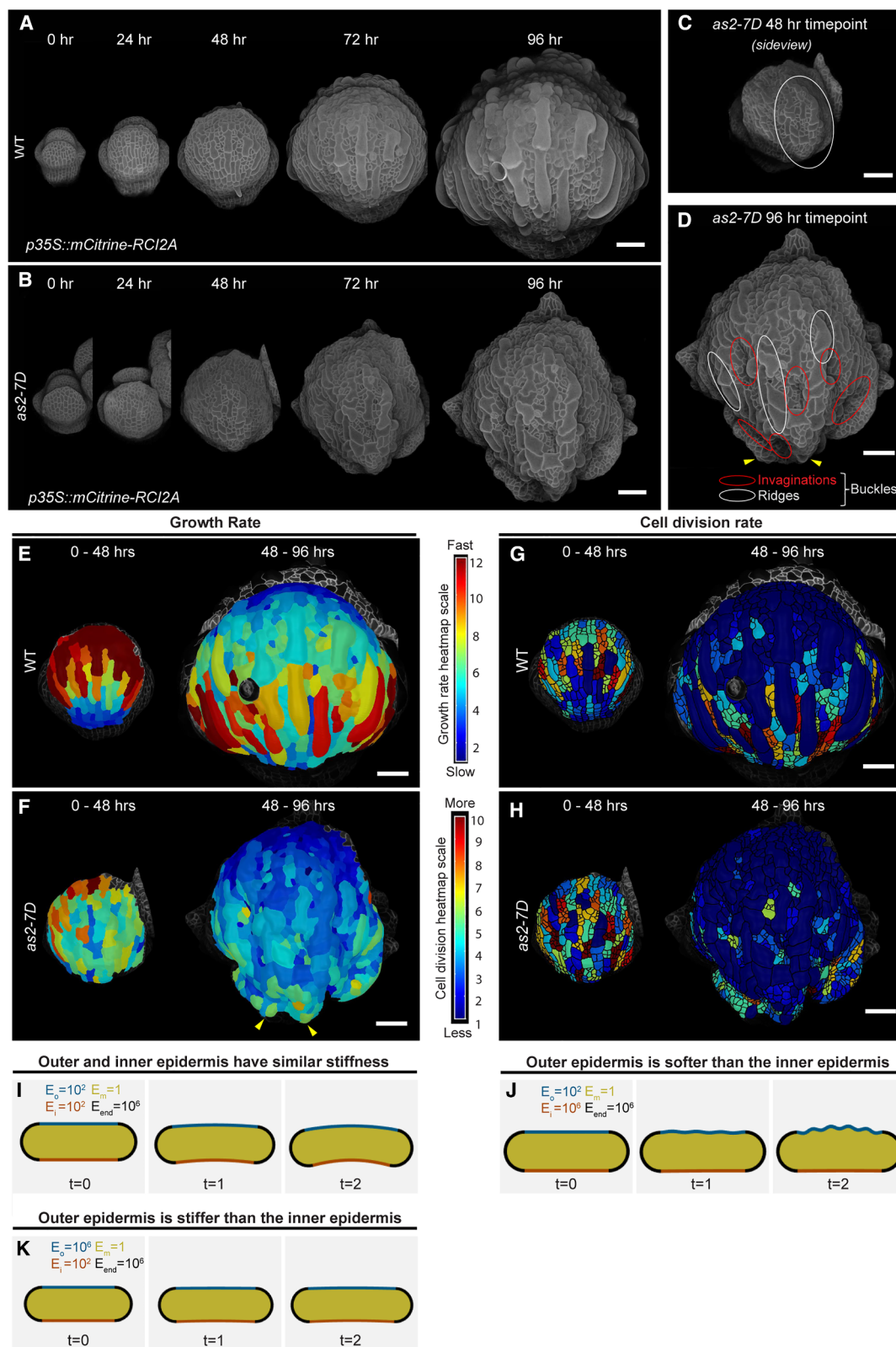


Figure 2. The *as2-7D* sepal outer epidermal morphology transitions from smooth to buckled and subsequently develops outgrowths

(A and B) Representative live-imaging series of (A) a wild-type (WT) flower and (B) an *as2-7D* mutant flower starting from developmental stage 4 over a course of 96 h, imaged at 24-h intervals ($n = 3$). Images capture morphological changes of the outer sepal. Both genotypes harbor the pLH13 (35S::mCitrine-RCI2A) cell line (legend continued on next page)

epidermal layer, which is sufficient to disrupt smooth sepal morphology by generating folds and outgrowths on the outer epidermal layer.

The *as2-7D* outer epidermis appears to buckle first and then form outgrowths

To understand how ectopic AS2 expression on the outer epidermis disrupts smooth sepal morphology, we live-imaged WT and *as2-7D* flowers from early sepal developmental stages (Figures 2A, 2B, and S2A–S2D; Video S2). Initially, the outer epidermis of both the WT and *as2-7D* sepals is smooth (Figures 2A and 2B; 0–24 h). While the outer epidermis of WT sepals remains smooth throughout development, *as2-7D* develops a fold in the middle of the sepal blade at 48 h (Figures 2B and 2C). The folds intensify as the flower develops, forming several inward invaginations and outward ridges (Figures 2B–2D; Video S2). Outgrowths become apparent only in the later stages, which suggests that formation of the folds precedes the emergence of the outgrowths (Figures 2B–2D, S2C, and S2D).

We considered two hypotheses for how the folds form: (1) due to localized growth at each ridge or (2) due to surface-wide growth disruption, causing mechanical buckling. To test the first hypothesis, we compared the cell growth rates and division rates between WT and *as2-7D* mutant on the outer sepal epidermis (Figures 2E–2H and S2E–S2N; Data S1A and S1B). We do not observe any localized fast growth or cell division in the region where the first fold forms in *as2-7D* at 48 h (Figures 2C, 2F, and 2H). In the initial stages (0–48 h), the WT exhibits the stereotypical basipetal growth pattern,⁶³ where fast cell growth occurs at the distal tip, moderate growth in the center, and slow growth toward the base. By contrast, the cells grow at relatively homogeneous rates throughout the outer epidermis of *as2-7D* (Figures 2E and 2F; 0–48 h). Although the pattern of growth is different, we observe no difference in the average cell growth rates between the WT and *as2-7D* (Figure S2M; Data S1A). This pattern is also reflected in cell division rates, where we observe a basipetal pattern in WT and a more uniform pattern throughout the sepal epidermis in *as2-7D* (Figures 2G and 2H;

0–48 h). Our results suggest that the folds form due to surface-wide disruption of cell growth patterns and not because of localized fast cell growth and division at the location of the ridge. In the later time point (48–96 h), the growth is still relatively uniform; however, we do observe localized growth and division at sites where outgrowths form (Figures 2F, 2H, and S2E–S2L; 48–96 h). We therefore considered the hypothesis that the folds on *as2-7D* outer epidermis are an outcome of mechanical buckling caused by surface-wide growth defects.

Modeling predicts two criteria required for buckling

We formulated a model to determine the conditions that could give rise to buckling (see method details). Our analytical model indicates that buckling, specifically of the outer epidermis, can occur if two conditions are simultaneously met: (1) if the epidermis is under compression, which occurs when the average specified growth rate of the outer and inner epidermis is higher than that of the mesophyll, and (2) if the stiffness of the outer epidermis is significantly lower than the inner epidermis (mesophyll is known to be softer than epidermal layers⁶⁴). The first condition can be met if the outer epidermis overgrows in width relative to the rest of the sepal. This differential growth across tissue layers generates compressive stress (condition 1), while differential stiffness (condition 2) determines how the tissue deforms to accommodate the stress.^{65–67} In our model, the softer outer epidermis is able to accommodate the growth-induced compressive stress by buckling (before the inner epidermis would).

We simulated the model on a two-dimensional cross-section of the developing sepal, comprising the outer epidermis, mesophyll, inner epidermis, and edges (Figures 2I–2K and S3). In these simulations, only the outer epidermis grew in width. When the outer and inner epidermis exhibit similar stiffness, the model tissue curves while maintaining a smooth morphology (Figure 2I). However, when the outer epidermis is softer than the inner epidermis, the outer epidermis buckles while the inner epidermis remains smooth, similar to *as2-7D* (Figure 2J). By contrast, when the outer epidermis is stiffer than the inner epidermis, the inner epidermal morphology remains smooth

membrane marker (grayscale). Trichomes were removed from the outer sepal epidermis by image processing to better visualize the sepal morphology. Scale bar, 50 μ m.

(C) Tilted view of *as2-7D* mutant flower at the 48-h time point. Note the appearance of the out-of-plane protrusion (ridge) in the middle of the sepal, circled in white. Scale bar, 50 μ m.

(D) The *as2-7D* mutant flower at the 96-h time point. Note the dramatic increase in the number and extent of ridges (white circles) and invaginations (red circles), characteristic of buckles, on the outer sepal epidermis. Yellow arrowheads indicate outgrowths. Scale bar, 50 μ m.

(E and F) Cell area growth rate over a 48-h period for the (E) WT and (F) *as2-7D* mutant sepals. Growth rate was calculated as the ratio of cell lineage area at the later time point divided by that of the earlier time point. The heatmap was projected onto the later time point. The red-to-blue gradient shows fast-to-slow growth. The growth rates over 0–48 h (left) and 48–96 h (right) are shown. Arrowheads point to the outgrowths. Scale bar, 50 μ m.

(G and H) Cell division rate over a 48-h period for the (G) WT and (H) *as2-7D*. Cell division rate was calculated based on the number of daughter cells per lineage, represented as a heatmap projected onto the later time point. The red-to-blue gradient shows more to less division. Segmented cells are outlined in black. Representative heatmaps for one of the three replicates ($n = 3$) for WT and *as2-7D* are shown. Scale bar, 50 μ m.

(I–K) Simulations of the model (STAR Methods), providing visual illustrations of the conditions that may favor buckling of the outer epidermis. The simulations illustrate change in tissue morphology along the cross-section when the outer epidermis was allowed to grow in width over two time points. The elastic modulus (E) of the outer epidermis (E_o), inner epidermis (E_i), mesophyll (E_m), and edges (E_{end}) are shown. Initial growth tensors along the width for all the four components were set at 1, with the outer epidermal growth rate set to 0.1, while the growth rates of the other components were set to 0.

(I) Simulation outcomes when the outer and inner epidermis have similar stiffness (E_o and $E_i = 10^2$), causing the tissue to curve.

(J) When the stiffness of the outer epidermis ($E_o = 10^2$) is less than that of the inner epidermis ($E_i = 10^6$), the outer epidermis buckles, while the inner epidermis remains unaffected.

(K) Simulation outcomes wherein the stiffness of the outer epidermis ($E_o = 10^6$) is more than that of the inner epidermis ($E_i = 10^2$), leading to a slight increase in tissue curvature over time.

See also Figures S2 and S3, Video S2, and Data S1A and S1B.

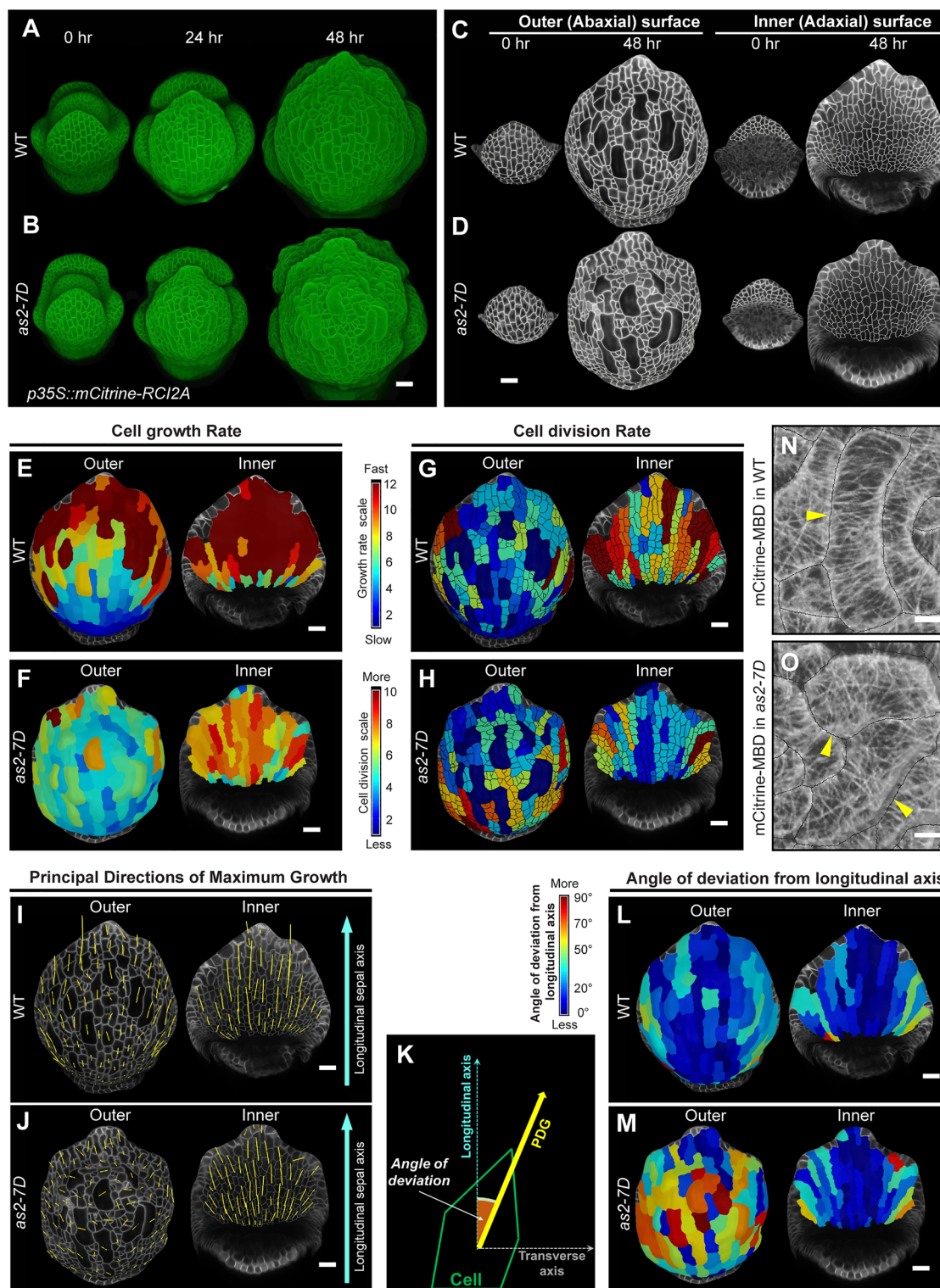


Figure 3. Aligned growth in both epidermal layers is essential for smooth sepal development

(A and B) Representative confocal images of the (A) wild type (WT) and (B) *as2-7D* mutant live-imaged with a high laser intensity (~16%–20%) every 24 h. Both genotypes harbor pLH13 (*35S::mCitrine-RCI2A*) cell membrane marker (green). Scale bar, 20 μ m. One of the three representative replicates ($n = 3$) is shown. (C and D) Segmented cells in gray superimposed onto the plasma membrane signal (also in gray) projected on mesh surfaces generated using MorphoGraphX.⁶⁹ Mesh surfaces for 0 and 48-h time points of the outer (left) and inner (right) epidermis of the (C) WT and (D) *as2-7D* mutant are shown. Note the strikingly reduced number of cells on the inner versus outer surface at the 0-h time point. Scale bar, 20 μ m; $n = 3$.

(legend continued on next page)

(Figure 2K). Although our simple model does not account for all the complex dynamics of tissue growth and buckling in 3D, it guided our investigation toward testing whether differences in growth and tissue stiffness between the outer and inner epidermal layers contribute to buckling in *as2-7D*.

Aligned cell growth direction between epidermal layers is essential for smooth sepal formation

To test the first of our model predictions, we extracted the inner and outer epidermal layers of a growing sepal by image processing⁶⁸ (Figures 3A–3D). We found that the WT inner epidermal cells grow significantly faster than the outer epidermal cells (Figures 3E, S4A, S4B, and S4E; Data S1C). We also observe fast cell divisions on the inner epidermis (Figures 3G, S4F, S4G, and S4J; Data S1D). The fast growth of the inner epidermis was surprising, since one would intuitively expect the cells on both epidermal layers to grow at similar rates to maintain flatness. However, sepals are initially curved. Hence, to flatten, the inner epidermis must grow faster than the outer epidermis. Like WT, the inner epidermal cells of *as2-7D* mutant also grow faster than outer epidermal cells; however, the inner epidermal cells of *as2-7D* do not grow as fast as WT (Figures 3E, 3F, and S4A–S4E; Data S1C). The cell division rate is also lower on the inner epidermis of *as2-7D* compared with WT (Figures 3G, 3H, and S4F–S4J; Data S1D).

Since buckling is a surface-wide phenomenon in our model, we analyzed the differences in whole inner versus outer epidermal growth rates. On an epidermis-wide scale, we found that the outer epidermis of WT and *as2-7D* grows at the same rate (Figures S4E, S5A, S5B, and S5E; Data S1C and S1F). However, the inner epidermis of *as2-7D* grows significantly slower than WT (Figures S4E and S5C–S5E; Data S1C and S1F). Our findings indicate that the rate at which the inner epidermis of *as2-7D* grows is insufficient to catch up with the increasing outer epidermal surface area (Figure S5F; Data S1G).

We then asked whether outer epidermal ridges in *as2-7D* could potentially be attributed to local hypergrowth of the mesophyll cells underneath the outer epidermis. We found that the spatial growth patterns of the outer layer of mesophyll cells of both WT and *as2-7D* mimic their respective outer epidermal cell growth patterns (Figure S6; Data S1H and S1I). Notably, there was no evidence of local hypergrowth in the central region of the sepal blade, where the initial buckle forms (Figure S6).

Since cell growth directions are critical for determining organ shapes,^{71–73} we assessed the principal direction of maximal cell growth (PDG_{max}) in the WT and *as2-7D* mutant. The WT cells on both epidermal layers grow in a highly aligned manner along the sepal longitudinal axis (Figures 3I, 3K, 3L, S4K, S4L, and S4O; Data S1E). In *as2-7D*, although inner epidermal cells grow parallel to the sepal longitudinal axis, the outer epidermal cells exhibit misaligned growth, with the majority of PDG_{max} tensors oriented in the transverse direction (Figures 3J, 3K, 3M, and S4M–S4O; Data S1E). Since microtubules orient cellulose deposition and thereby the maximal cell growth direction,^{74–76} we then tested the microtubule orientation pattern in comparable regions of the WT and *as2-7D* outer epidermis (Figures 3N and 3O). For WT, the microtubules show the typical transverse alignment pattern, which is perpendicular to the sepal longitudinal growth direction (Figure 3N). Conversely, the microtubules are largely misoriented in *as2-7D* and, in certain instances, mirror the transverse cell growth pattern (yellow arrowheads, Figure 3O).

Taken together, overgrowth of the *as2-7D* outer epidermis in width compared with the inner epidermis contributes to the buckling of the outer epidermis. Our observations align with the prediction of our model simulations, which indicates that the transverse expansion of the outer epidermis, constrained along the edges by the inner epidermis, would favor buckling. We did attempt to test whether the outer sepal epidermis was under compression using laser ablations, although the results were inconclusive (Figure S7). Overall, our data suggest that anisotropic cell growth aligned in the same direction on both epidermal layers is important for maintaining smooth sepal morphology.

Since we hypothesized that the transverse growth orientation of the outer epidermal cells causes the outer epidermis to buckle, we tested whether reorienting cell growth along the longitudinal direction can rescue the *as2-7D* phenotype. Overexpressing cyclin-dependent kinase (CDK) inhibitor Kip-related protein 1 (KRP1) throughout the epidermis (*pML1::KRP1*) at moderate levels triggers the epidermal cells to skip division and undergo endoreduplication, resulting in giant cell formation.^{77,78} Giant cells, which are exclusive to the outer epidermis, grow anisotropically⁷⁷ along the longitudinal sepal axis (Figures 4E, 4G, S8A, and S8C; Video S3). While we did not observe any obvious association between the location of giant cells, small cells, and the

(E and F) Cell growth rates over a 48-h period for the (E) WT and (F) *as2-7D* mutants are shown. The heatmap has been projected onto the 48-h time point surface. In each panel, the growth rate of cells on the outer surface (left) and inner surface (right) is shown. The red-to-blue heatmap gradient shows fast-to-slow growth. Scale bar, 20 μ m; $n = 3$.

(G and H) Cell division rates over a 48-h period for the (G) WT and (H) *as2-7D* mutant on the outer surface (left) and inner surface (right) are shown. Segmented cells are outlined in black. The red-to-blue gradient shows more to less/no cell division, respectively. Scale bar, 20 μ m; $n = 3$.

(I and J) Principal directions of maximal growth (PDG_{max} , yellow) of the cells on the outer (left) and inner (right) epidermis of (I) WT and (J) *as2-7D*. Segmented cells in gray are superimposed on the plasma membrane signal (also in gray). The length of any given PDG_{max} tensor is directly proportional to the cell growth rate in that direction. A blue arrow indicates the longitudinal sepal axis. Note the misoriented PDG_{max} tensors on the *as2-7D* outer epidermis. Scale bar, 20 μ m; $n = 3$.

(K) A schematic diagram depicting the calculation of the angle of deviation between PDG_{max} tensor and the longitudinal axis to obtain a quantitative measure of the deviation.

(L and M) Heatmaps show the angle of deviation of the PDG_{max} tensor from the sepal longitudinal axis for the outer (left) and inner (right) epidermal cells of the (L) WT and (M) *as2-7D* mutant. Scale for the angle of deviation ranges from 90° (highly deviated, red) to 0° (no deviation, blue). Scale bar, 20 μ m; $n = 3$.

(N and O) Cortical microtubule orientation (grayscale) from comparable regions of late-stage 6 flower outer epidermis for the (N) WT and (O) *as2-7D* mutant is shown. These plants harbor *pPDF1::mCitrine-MBD*, a YFP-based microtubule marker.⁷⁰ The cell outlines are shown in black. In (N), the yellow arrowhead shows that the WT microtubules are oriented perpendicular to the sepal longitudinal axis. In (O), the yellow arrowheads show that the *as2-7D* microtubules are not only misoriented relative to the longitudinal axis, but their alignment matches the transverse growth orientation of the cells. Scale bar, 10 μ m; $n = 3$.

See also Figures S4–S7 and Data S1C–S1I.

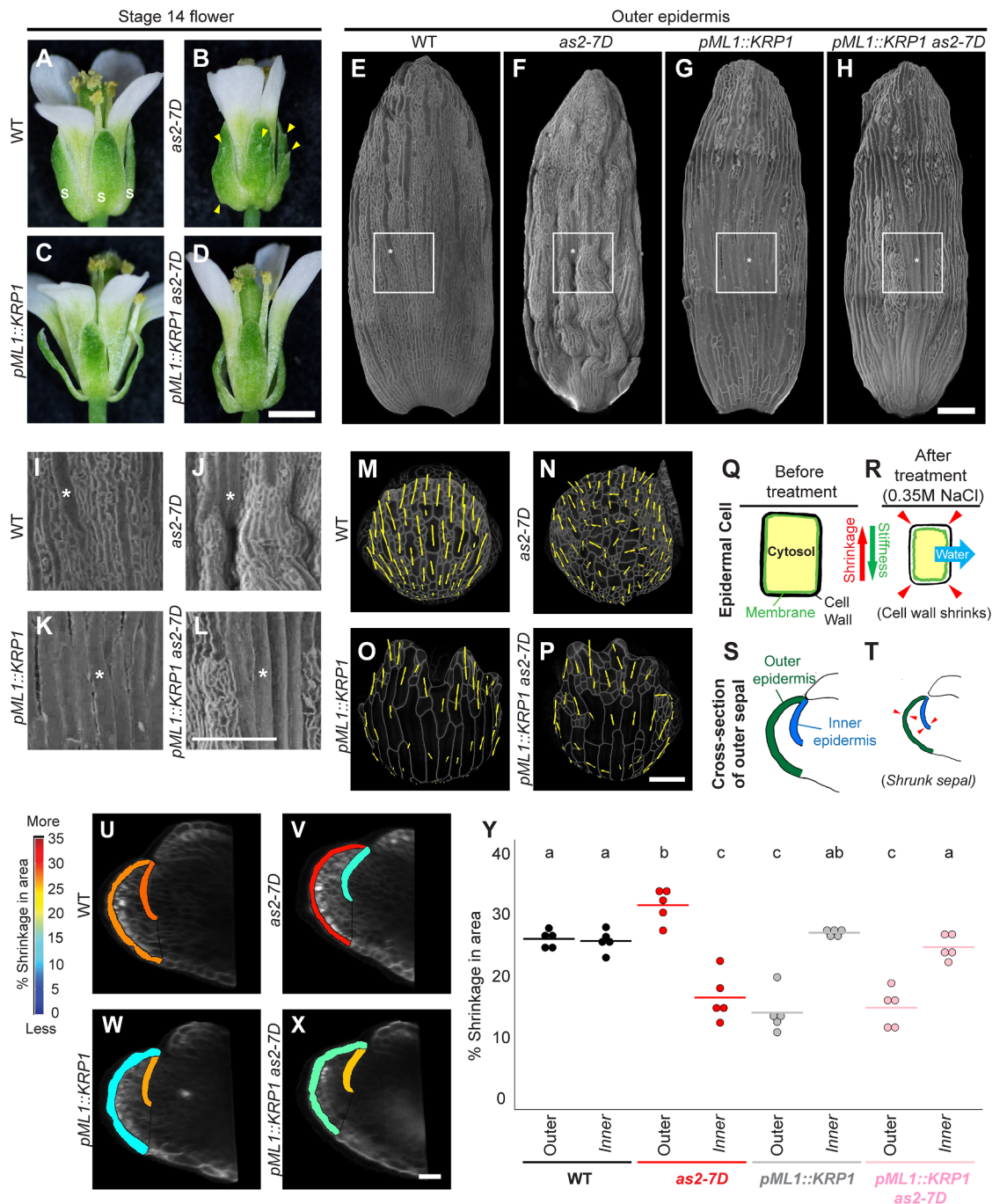


Figure 4. Aligning growth along the longitudinal direction and increasing outer epidermal stiffness is sufficient to rescue buckling in *as2-7D*
(A–D) Representative stage 14 flowers of (A) wild type (WT), (B) *as2-7D* mutant, (C) *pML1::KRP1*, and (D) *pML1::KRP1 as2-7D*. The WT sepals are marked with the letter “S” in white (A). Yellow arrowheads point to outgrowths in *as2-7D* (B). Note the smooth sepal of *pML1::KRP1 as2-7D* compared with *as2-7D*. Scale bar, 1 mm; *n* = 3 flowers for all genotypes.

(E–H) Confocal images of the outer epidermis of (E) WT, (F) *as2-7D* mutant, (G) *pML1::KRP1*, and (H) *pML1::KRP1 as2-7D* stage 14 sepals. The sepals were stained with PI (gray, cell walls). Note the increased number of highly elongated giant cells in *pML1::KRP1* and *pML1::KRP1 as2-7D* sepal, and the smooth outer epidermis of *pML1::KRP1 as2-7D* compared with the highly rugose morphology of *as2-7D*. Scale bar, 200 μ m; *n* = 3.

(I–L) Magnified insets of the (I) WT, (J) *as2-7D* mutant, (K) *pML1::KRP1*, and (L) *pML1::KRP1 as2-7D* sepal for the regions marked by white boxes in (E)–(H), respectively. Asterisks denote a typical giant cell. Scale bar, 200 μ m; *n* = 3.

(M–P) The principal direction of growth (PDG_{max}) for the outer epidermal cells of the (M) WT, (N) *as2-7D*, (O) *pML1::KRP1*, and (P) *pML1::KRP1 as2-7D* sepals. Yellow lines mark the PDG_{max} tensors calculated over a 48-h period, projected onto the later time point. For WT and *as2-7D*, the flower images used are the same as in Figure 2. Segmented cells in gray are superimposed onto the plasma membrane signal (also in gray). Scale bar, 20 μ m; *n* = 3.

(legend continued on next page)

buckles in the *as2-7D* mutant, we wondered whether increasing giant cells can restore directional growth in *as2-7D* and, consequently, reduce buckling. Indeed, overexpressing *KRP1* in *as2-7D* (*pML1::KRP1 as2-7D*) largely suppresses buckling of the outer sepal epidermis (Figures 4A–4L and S8A–S8D; Video S3), without altering the two-dimensional outline of the sepal shape (Figures S8E–S8L). The principal growth directions of *pML1::KRP1 as2-7D* outer epidermal cells are largely reoriented longitudinally (Figures 4M–4P). In addition to reduced buckling, the characteristic outgrowths of the *as2-7D* outer epidermis are also nearly absent in *pML1::KRP1 as2-7D* (Figures 4E–4H), suggesting that buckling is a prerequisite for outgrowth formation.

Of note, the longitudinal growth of the giant cells is one of several phenotypic effects of *KRP1* overexpression. Other effects of *KRP1* overexpression, such as altered stiffness (verified below), increased outer epidermal thickness, and reduced cell division in *pML1::KRP1 as2-7D*, may also contribute to the suppression of buckling. However, recent work shows that removing *JAGGED* (*JAG*) activity from *as2-7D* in the *jag as2-7D* double mutant realigns growth along the longitudinal axis and restores sepal smoothness,⁸⁰ confirming the importance of aligned cell growth directions.

Reduced stiffness promotes buckling of *as2-7D* outer epidermis

The second condition of our 2D model predicts that buckling can be restricted to the outer epidermis if it is softer than the inner epidermis. To test the relative stiffness of the outer and the inner epidermal layers, we performed osmotic treatments^{79,81} and compared how the epidermal layers of *as2-7D* shrink relative to WT (Figures 4Q–4T and S8M–S8P; see also method details). Briefly, when exposed to a hyperosmotic solution, cells undergo plasmolysis. The protoplast retracts and thereby fails to exert turgor pressure on the cell wall,⁷⁹ causing the cell wall to contract (Figures 4Q–4T). The extent of cell wall shrinkage gives a proxy measure for cell wall stiffness, with low shrinkage indicating the wall is stiff (high Young's modulus) and high shrinkage indicating the wall is soft (low Young's modulus).⁷⁹ We found that the WT inner and outer epidermal layers exhibit similar shrinkage, indicating similar stiffness (Figures 4U and 4Y; Data S1J). By contrast, the outer epidermis of *as2-7D* shrank more than the inner epidermis, indicating that the *as2-7D* outer epidermis is softer (Figures 4V and 4Y; Data S1J), consistent with our model (Figures 2I and 2J). To then test whether increasing giant cell

formation affects outer epidermal stiffness, we performed osmotic treatments on *pML1::KRP1* and *pML1::KRP1 as2-7D*. We found a significant increase in the stiffness of the outer epidermis of *pML1::KRP1 as2-7D* relative to *as2-7D* (Figures 4W–4Y; Data S1J). Quantification of outer epidermal stiffness at a cellular resolution confirmed that cells throughout the outer epidermis of *as2-7D* are significantly softer than the WT, while *KRP1* overexpression increases the stiffness of the *as2-7D* outer epidermal cells (Figures S8Q–S8U; Data S1K). Although we cannot rule out other pleiotropic effects of *KRP1* overexpression such as accelerated differentiation, these observations align with our simulation prediction that increasing outer epidermal stiffness restores smooth epidermal morphology (Figure 2K).

Taken together, our results suggest that aligning growth along the longitudinal direction, decreasing cell division, and increasing stiffness not only rescues the outer epidermal buckling but also suppresses subsequent outgrowth formation in *as2-7D*. Our findings collectively suggest that growth along the same direction and comparable stiffness across both epidermal layers are essential for smooth sepal formation, as observed in the WT.

Buckles in early *as2-7D* sepals occur without obvious disruptions in the abaxial-adaxial patterning network

Substantial published evidence shows that outgrowths can form due to ectopic juxtaposition of adaxial/inner and abaxial/outer identities.^{42,47,49,50,82–84} In this study, we observed two phenotypically distinct disruptions of the smooth sepal surface in *as2-7D* mutants, namely (1) buckles/folds and (2) outgrowths.

Recent work shows that removing *PRS*⁵⁰ activity from *as2-7D* in the *prs as2-7D* double mutant suppresses outgrowth formation, but buckles still form,⁸⁵ confirming buckles and outgrowths are distinct. Although our evidence suggests the folds on *as2-7D* sepals form through mechanical buckling, since *AS2* promotes adaxial epidermal identity,^{53–55} we tested the alternative hypothesis that the folds, like the outgrowths, result from ectopic juxtaposition of adaxial and abaxial identity. Therefore, we examined the expression pattern of *REVOLUTA* (*REV*), an adaxial identity transcription factor,⁸⁶ and *FIL*, an abaxial identity transcription factor.⁸⁷ We observed no difference in *REV* and *FIL* expression patterns between WT and *as2-7D*, and particularly, no ectopic juxtaposition between adaxial and abaxial gene expression (Figures 5A–5H). Since *REV* promotes adaxial identity in conjunction with *AS2*, we tested whether *REV* is required for the *as2-7D* phenotype. Ectopic *AS2* expression in the *rev-6*

(Q and R) Schematic diagrams demonstrating the concept of cell wall stiffness assessment by osmotic treatment assays.⁷⁹

(Q) A typical plant epidermal cell before osmotic treatment. The cell wall and cell membrane are shown in black and green, respectively.

(R) A cell that has undergone plasmolysis in a hyperosmotic solution (0.35 M NaCl).

(S and T) Anatomical diagrams demonstrate the use of osmotic treatments to estimate epidermal stiffness. Longitudinal sections of an early-stage 6 flower (S) before and (T) after osmotic treatment are shown. The outer (green) and inner (blue) sepal epidermis are marked. Arrowheads (red) demonstrate aerial shrinkage.

(U–X) Heatmaps show the aerial shrinkage (in percentage) of a longitudinal sepal cross-section for the inner and outer epidermis of (U) WT, (V) *as2-7D* mutant, (W) *pML1::KRP1*, and (X) *pML1::KRP1 as2-7D*, post-osmotic treatment in 0.35 M NaCl solution. Shrinkage was computed as the decrease in area of a comparable cross-sectional region from an early-stage 6 flower before and after osmotic treatment. Heatmaps are projected on the post-treatment surface. More shrinkage is represented in red and less in blue. Scale bar, 20 μ m. Heatmaps for each genotype are representative of 5 independent replicates ($n = 5$).

(Y) Graph showing the percentage aerial shrinkage post osmotic treatment. Data across 5 independent replicates each for the WT (black dots), *as2-7D* (red dots), *pML1::KRP1* (gray dots), and *pML1::KRP1 as2-7D* (pink dots) for both the outer and inner epidermis are shown. The letters above the plots represent significant differences based on one-way analysis of variance (ANOVA) with Tukey's honest significant difference (HSD) post hoc correction ($p < 0.05$). Similar letters indicate no significant difference.

See also Figure S8, Video S3, and Data S1J and S1K.

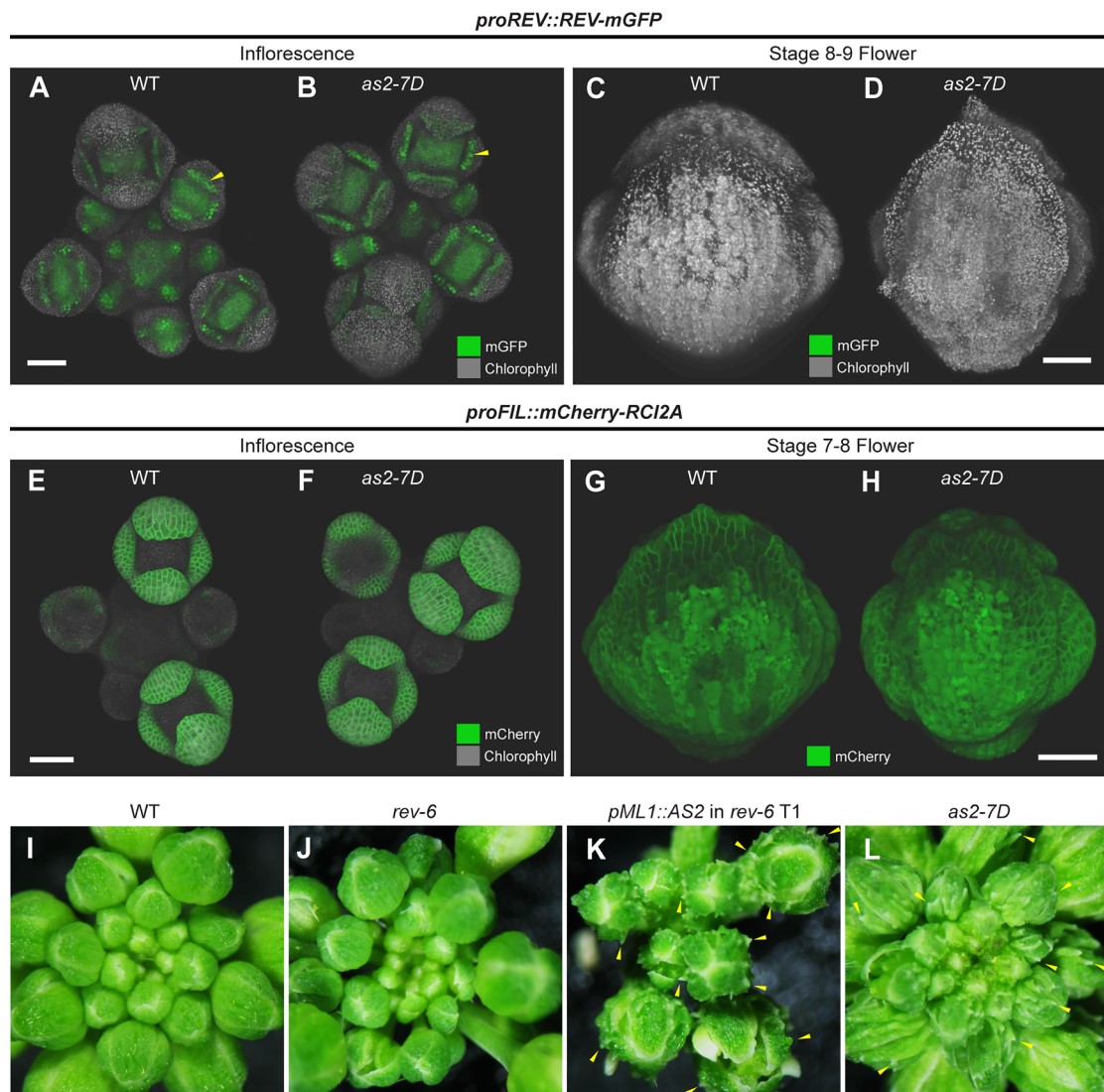


Figure 5. Buckling in *as2-7D* is not associated with an obvious disruption of outer (abaxial)-inner (adaxial) polarity

(A–D) REVOLUTA (REV) protein reporter (*proREV::REV-mGFP*) shows nuclear localization of REV, restricted exclusively to the inner epidermis of the wild type (WT) and *as2-7D* sepals.

(A and B) Confocal images of inflorescence meristems show adaxial-specific REV accumulation (yellow arrowheads) in the young sepals of (A) WT and (B) *as2-7D* mutant.

(C and D) Representative middle-stage (stages 8 and 9) flowers of (C) WT and (D) *as2-7D* show no detectable REV accumulation on the outer epidermis of the sepals in the folds or outgrowths. Images of representative T3s ($n = 3$) show mGFP in green and chlorophyll in grayscale. Scale bar, 50 μ m.

(E–H) FILAMENTOUS FLOWER (FIL) promoter reporter (*proFIL::mCherry-RCI2A*) expression pattern is restricted to the outer sepal epidermis and shows no difference between the WT and *as2-7D* mutant.

(E and F) Confocal images of inflorescence meristems show *FIL* expression pattern in the early floral developmental stages of (E) WT and (F) *as2-7D* mutant. The membrane-localized mCherry is shown in green and chlorophyll in grayscale.

(G and H) Representative middle-stage (stages 7 and 8) flowers of (G) WT and (H) *as2-7D* mutant showing the *FIL* promoter reporter signal (mCherry, green). Images are representative of three T3s ($n = 3$). Scale bar, 50 μ m.

(I–L) Whole inflorescence images of (I) WT, (J) *rev-6* mutant, (K) a representative transgenic (T1) harboring *pML1::AS2* transgene in *rev-6*, and (L) *as2-7D* mutant. Note the outgrowths (yellow arrows) on the sepal outer epidermis of *pML1::AS2 rev-6* are similar to those of *as2-7D* and *pML1::AS2* in WT (Figure 1M). Multiple independent T1s for *pML1::AS2* in *rev-6* were assayed ($n = 19$), all of which show similar outgrowth formation on the sepal outer epidermis in the *rev-6* background. See also Figures S9 and S10 and Table S1.

mutant (*pML1::AS2 rev-6*) leads to buckles and outgrowths similar to *pML1::AS2* in WT (Figures 1M–1P, 5I–5L, S1G–S1N, and S9A–S9I), indicating REV is not necessary for either the buckles or the outgrowths. Conversely, ectopic expression of

REV in the WT sepals is insufficient to generate either buckles or outgrowths (Figures S9J and S9K). Further, ectopic REV expression in *as2-7D* also does not enhance the phenotype (Figures S9L and S9M).

Since abaxial identity-promoting KANADI transcription factors directly repress AS2, and the disruption of a KANADI binding site leads to ectopic AS2 expression in *as2-7D*,⁵⁹ we also assessed the sepal phenotypes of *kanadi* loss-of-function mutants (*kan1-2 kan2-1*). Consistent with previous reports,^{46,47,54} *kan1-2 kan2-1* displayed severe floral development and phyllotaxy defects, characterized by irregular initiation and arrangement of sepals, petals, and stamens and conspicuously enlarged carpels (Figures S10A, S10B, and S10E–S10L). In particular, the *kan1-2 kan2-1* sepals from early developmental stages are strongly radialized with little sepal lamina (Figures S10F–S10L). While the mature *kan1-2 kan2-1* sepals are curved downward, indicative of strong adaxialization (predominantly inner identity), we do not observe the strong folds and associated outgrowths on the outer epidermis of *kan1-2 kan2-1* sepals, unlike *as2-7D* (Figures 1E, 1F, S10C, and S10D). However, as reported previously,^{46,47,53,54,57} we do observe the folds and outgrowths on the outer epidermis of the *kan1-2 kan2-1* leaves, similar to AS2 constitutive overexpression phenotypes in the leaves (Figures S10M–S10O). The positions of outgrowths typically overlap folds (Figure S10O), which suggests a potential link between buckling and ectopic outgrowth in the *kan1-2 kan2-1* mutant as well.

Overall, based on the key polarity-associated genes such as *REV*, *FIL*, and *KAN* that we could test, the evidence so far suggests that the outer epidermal buckling exhibited by *as2-7D* sepals may not be associated with an obvious ectopic inner-outer polarity boundary, unlike the outgrowths. However, our investigations do not rule out the possibility that additional polarity-associated genes may also be disrupted in *as2-7D*.

Epidermal buckling in *as2-7D* promotes ectopic PIN convergence and local auxin maxima at the site of outgrowth formation

The polar localization of auxin efflux transporter PIN1 in the plasma membrane determines the directionality of auxin flow. When PIN1 polarity from multiple neighboring cells converges to the same location, auxin accumulates at that site to create an auxin maximum.⁸⁸ PIN1 convergence sites form at the boundary between the *REV* and *KAN1* expression domains, which coincides with the site of leaf initiation.⁴⁴ In the context of the ectopic outgrowths, Izhaki and colleagues first showed that the site of outgrowth formation in *kan1 kan2 kan4* triple mutants coincides with the site of PIN1 convergence, indicating that outgrowth formation involves PIN1.⁴⁸ This was also later confirmed by Abley and colleagues, who showed that PIN1 convergence sites form before the outgrowth initiates in *kan1 kan2* mutant leaves.⁸⁹ Localized auxin import, auxin biosynthesis, and boundary genes are also involved in driving outgrowth formation.⁸⁹

We consistently observe that *as2-7D* outgrowths typically form at the tip of the epidermal buckles (Figures 1F and 2B–2D). To test whether *as2-7D* outgrowths also involve PIN1 convergence, we analyzed PIN1-GFP localization. In the inflorescence and early-stage flowers, the PIN1-GFP accumulation pattern in *as2-7D* is like the WT (Figures 6A and 6B). However, in the later stages, PIN1-GFP converges specifically at the outgrowth formation sites (Figures 6C, 6D, S11A, and S11B). By contrast, no PIN convergence sites are observed in the middle of WT sepals (Figures 6C, 6D, S11A, and S11B). The auxin-

responsive promoter DR5 is also specifically expressed where outgrowths form in *as2-7D* (Figures S11C and S11D), consistent with PIN1 convergence creating auxin maxima.⁹⁰

As discussed earlier, the genetics underlying PIN1 convergence and how auxin drives the subsequent progression of the outgrowths are well known.^{48,89} However, what determines the precise location of PIN1 convergence in the sepal blade, thereby marking the site of outgrowth, remains elusive. From a biomechanical perspective, PIN1 polarity is known to respond to mechanical stress.^{92–94} Since buckling inherently alters mechanical stress^{95,96} and buckling precedes outgrowths, we hypothesized that buckling-induced mechanical stress may influence PIN1 convergence to generate outgrowths in *as2-7D*. To test our hypothesis, we live-imaged growing *pPIN1::PIN1-GFP as2-7D* sepals from pre-buckling stages through the stages where the outer epidermis buckles and outgrowths are evident (Figure 7). Initially, when the sepal is smooth, no ectopic PIN1 convergence is observed on the sepal blade (Figures 7A–7D). As the sepal grows and forms a buckle in the middle of the sepal blade, PIN1 converges at the tip of the buckle, marking the site of outgrowth initiation (Figures 7E–7H). PIN convergence occurs only at the tip of the buckle and does not extend throughout the buckle (Figures 7E–7H). In the following time point, an outgrowth emerges right at the PIN1 convergence site (Figures 7I–7L). As the outgrowth progresses, PIN1 continues to converge at the tip, while PIN1 is absent elsewhere (Figures 7M–7P). Collectively, our observations suggest that the tissue buckles first, then PIN1 converges at the end of a buckle, where the outgrowth finally forms. Our data are consistent with the hypothesis that buckling-induced mechanical stress influences PIN1 to converge and initiate an outgrowth.

We next tested whether PIN-mediated polar auxin transport is required specifically for the formation of outgrowths and not buckles. We treated growing *as2-7D* inflorescences with polar auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) and compared the phenotype with mock-treated samples. While NPA-treated *as2-7D* developed characteristic buckles, the formation of outgrowths was attenuated (Figures 6E, 6F, and S11E–S11H; Video S4). Buckling continues to occur since the characteristic growth patterns and misdirected (sideways) PDG_{max} tensors remain unaltered with NPA treatment (Figures 6G–6J). However, the localized fast growth leading to outgrowth formation was largely reduced in the NPA-treated samples (Figures 6K and 6L). Therefore, consistent with prior studies,^{48,89} our investigations also show that polar auxin transport is required for the formation of outgrowths. However, our results suggest that PIN1-mediated polar auxin transport is not required for the formation of the buckles, consistent with growth and tissue mechanics being the primary drivers of buckling. Overall, our results are consistent with our hypothesis that mechanical buckling promotes ectopic PIN1 convergence, generating local auxin maxima and fast growth, leading to outgrowth formation.

DISCUSSION

Buckling generates complex organ shapes that are required for specialized functional roles.^{7–12,14,16} However, buckling also needs to be circumvented to generate smooth organs. Using *Arabidopsis* sepals as the model, our study shows that aligned

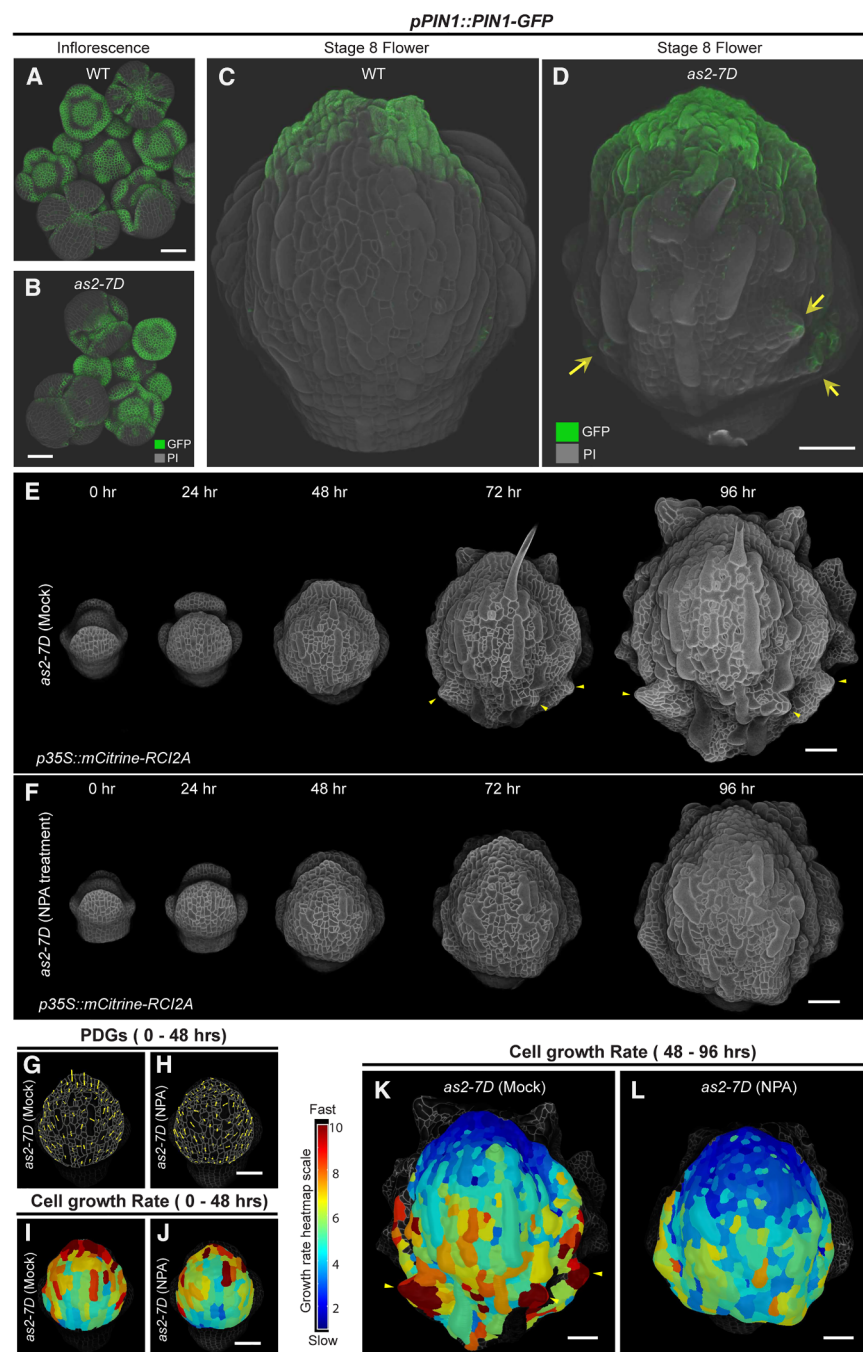


Figure 6. Mechanical buckling drives ectopic PIN convergence, creating auxin maxima, causing fast local growth and subsequent outgrowth formation

(A–D) Confocal stack images showing the expression pattern of auxin efflux carrier protein PINFORMED 1 (PIN1) in the wild type (WT) and *as2-7D*. (A and B) Expression pattern of PIN1 protein reporter (*pPIN1::PIN1-GFP⁹¹*) in the inflorescence meristem of (A) the WT and (B) *as2-7D* mutant. The PIN1 expression pattern in *as2-7D* is identical to WT in the young floral development stages, wherein PIN1 polarity is directed toward the insipient primordia and growing sepal edges, as described previously.⁹¹ Scale bar, 50 μ m.

(C and D) Expression pattern of PIN1 in a representative middle-stage (stage 8) flower of the (C) WT and (D) *as2-7D* mutant. Note the expression of PIN1 at the growing tip of the outgrowths (yellow arrows) in *as2-7D*, whereas no PIN1 expression is detected in the blade region where the epidermis buckles. In (A)–(D), the images are representative of three *pPIN1::PIN1-GFP* and *pPIN1::PIN1-GFP as2-7D* plants ($n = 3$). PI is shown in grayscale and GFP in green. Trichomes were removed from the outer sepal epidermis by image processing. Scale bar, 50 μ m.

(E and F) Representative live-imaging series of (E) mock treated *as2-7D* and (F) 10 μ M NPA treated *as2-7D* developing flowers over 96 h, imaged at every 24-h interval (representative of $n = 3$ flowers). Note the development of outgrowths from the 72-h time point onward (yellow arrowheads) in the mock-treated *as2-7D*, which are absent in NPA-treated *as2-7D* flowers. Plants harbor pH13 (*35S::mCitrine-RCI2A*) cell membrane marker (grayscale). Scale bar, 50 μ m.

(G and H) The PDG_{max} tensors (yellow) of the cells on the outer epidermis of (G) mock-treated and (H) 10 μ M NPA treated *as2-7D* over the first 48-h growth period for the representative samples shown in (E) and (F). Characteristic misoriented PDG_{max} tensors (relative to the longitudinal sepal axis) on the outer epidermis of *as2-7D* remain unaffected in the NPA-treated samples, thus driving buckling in later stages. Scale bar, 50 μ m.

(I–L) Outer epidermal cell growth rates over (I and J) 0–48-h growth period and (K and L) 48–96 h growth period of the mock and 10 μ M NPA treated *as2-7D* flowers, respectively. Heatmaps are projected onto the later time point. Fast (red) to slow (blue) growth rates are shown. In (K), note the fast cell growth driving outgrowth formation (yellow arrowheads), which is absent in the NPA-treated sample (L). Scale bar, 50 μ m.

See also Figure S11 and Video S4.

growth of both the outer and inner epidermal cells and similar tissue stiffness across the epidermal layers are key drivers of smooth sepal formation in WT. Conversely, the outer epidermis of *as2-7D* mutants buckles due to the imbalance of growth direction and tissue stiffness across the outer and inner epidermal layers.

Previous studies document that overexpression of AS2 leads to ectopic outgrowths.^{53,55,97} However, we identify buckling as an upstream morphological deformity that occurs before the

outgrowths are even evident in the sepals. PIN1 convergence sites form specifically at the tip of buckles, marking the site of outgrowth initiation. Since the Waite-Hudson model was proposed in 1995,⁴² the initiation of ectopic outgrowths has been attributed primarily to juxtaposition of abaxial-adaxial identities. Here we show that mechanics can also promote outgrowth initiation. Our results suggest that mechanical stress arising from tissue buckling can promote PIN convergence and drive the formation of auxin maxima, thereby initiating outgrowths. We

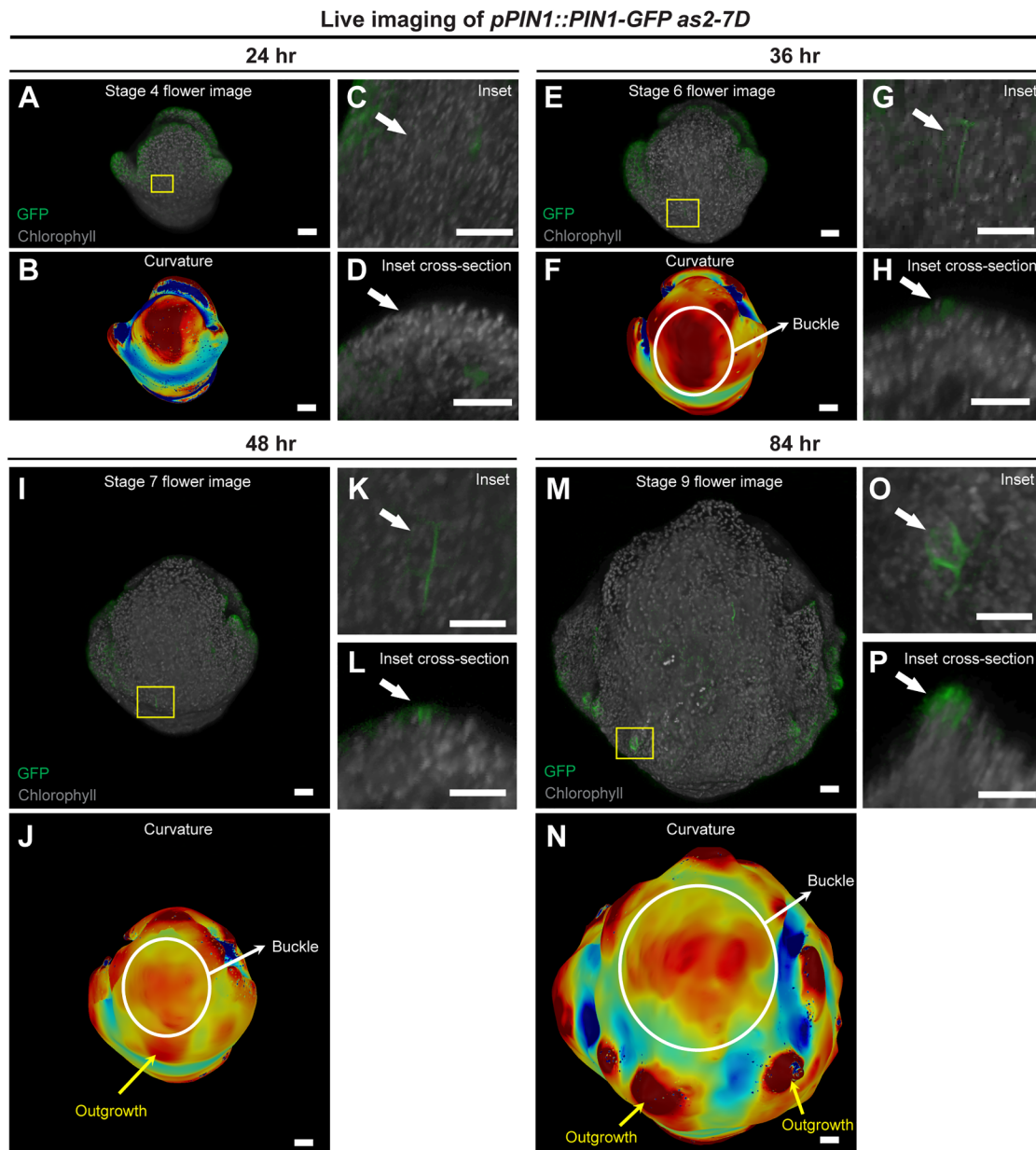


Figure 7. PIN1-GFP converges at the end of a buckle where an outgrowth subsequently forms in *as2-7D* mutants

Confocal live imaging series of a *pPIN1::PIN1-GFP as2-7D* flower, imaged every 12 h starting from stage 3 (representative of $n = 3$). PIN1-GFP (green) and chlorophyll autofluorescence (gray) are shown. We analyzed the curvature (minimal curvature), in which warm colors denote out-of-plane (bulging), and cool colors represent inward (saddling) deformations. Scale bars, 20 μ m.

(A–D) Confocal image of *pPIN1::PIN1-GFP as2-7D* stage 4 flower at the 24-h time point (A) and the analysis of its minimal curvature (B) is shown. At this stage, the sepal exhibits uniform outward curvature. The inset (C) and the cross-section through the inset (D) for the region highlighted in (A) (yellow box) depict the site where an outgrowth will form in the later stages. Note the absence of PIN1-GFP expression at this site (white arrow).

(E–H) The *pPIN1::PIN1-GFP as2-7D* flower at the 36-h time point as it transitions into stage 6 (E) and its minimal curvature (F) is shown. At this stage, the initial buckle (marked by a white circle) appears as an out-of-plane fold in the center of the sepal blade (deep red on the curvature). The inset (G) and the cross-section through the inset (H) for the region highlighted in (E) (yellow box) are shown. As the outer layer buckles, PIN1 expression begins to converge in the highlighted region (white arrow), marking the site of the later outgrowth.

(I–L) The *pPIN1::PIN1-GFP as2-7D* flower at the 48-h time point (I) and its minimal curvature (J) are shown. At this stage, the outgrowth begins to form (yellow arrow pointing toward the red bulge) at the end of the buckle. The inset (K) and its corresponding cross-section (L) for the region highlighted in (E) (yellow box) show the PIN1 expression associated with the insipient outgrowth (white arrow).

(M–P) The *pPIN1::PIN1-GFP as2-7D* flower at the 84-h time point as it transitions into stage 9 (M) and its minimal curvature (N) are shown. The inset (O) and the cross-section through the inset (P) for the region highlighted in (M) (yellow box) where the outgrowth has already formed are shown. By this stage, PIN1 expression (white arrow) converges toward the growing tip of the outgrowth.

See also Figure S11.

speculate that auxin maxima influence the gene regulatory networks involved in outgrowth formation that have been extensively characterized by other studies.^{48,89}

Our study does have limitations. First, since *as2-7D* is a gain-of-function mutant, we refrain from extrapolating the native role of AS2 based on our findings. Second, our model is based on a cross-sectional 2D representation of a sepal. Hence, the Young's moduli values chosen to achieve outer epidermal buckling are not comparable to experimental observations. The model guided our experimental trajectory, rather than validating our findings. Third, we used osmotic treatments^{58,79,81,98,99} to assess the stiffness of the outer and inner epidermal layers, because the inner epidermis at early stages of sepal development is inaccessible.⁶⁸ Alternative methods like atomic force microscopy¹⁰⁰ are impractical because they require the tissue to be accessible. Fourth, there are currently very limited genetic tools available to tune growth and change tissue mechanics. Although a crude tool, we chose to overexpress *KRP1*, since *KRP1* is known to cause anisotropic growth⁷⁷ and is also unrelated to the core polarity-associated genetic network. While *KRP1* inhibits cell division, the evidence suggests it is not the inhibition of cell division that suppresses buckling. The *pML1::KRP1 as2-7D* sepals still have dividing cells that could generate buckles and outgrowths, but we do not observe them. Likewise, overexpression of *KRP1* could potentially suppress buckling in *as2-7D* by accelerating differentiation, although live imaging shows that the stomatal differentiation is not accelerated in *pML1::KRP1 as2-7D*. Finally, we presented negative data showing no evidence that buckling is caused by ectopic juxtaposed adaxial-abaxial identity. Since AS2 influences a plethora of genes, we cannot exclude the possibility that untested genes contribute to buckling.

We found that the microtubules on the outer epidermis of *as2-7D* mutants are not only misoriented, but they also reflect the transverse growth direction of the cells. The cortical microtubules constrain the direction of cell growth by guiding cellulose microfibril deposition.¹⁰¹ Whether the disorganized microtubule orientations in *as2-7D* are the primary cause of transverse cell growth directions or a downstream effect of altered tissue-wide mechanics^{75,102} remains unclear. Molecular evidence also suggests that AS2 may influence cell wall stiffness via the downstream genes and molecular interactions with *JAG*,^{80,103} *ARF3*,^{104–106} and class-1 KNOTTED1-like homeobox (*KNOX*) genes,^{57,107,108} all of which regulate cell wall genes. Investigating how AS2 directly regulates growth and wall stiffness will be an interesting avenue for future investigations.

Intuitively, one would expect AS2 ectopic expression to mainly affect the outer epidermis. However, we find the growth rate and stiffness of the inner epidermis are affected as well in *as2-7D*. This observation may be explained by considering the epidermis as a continuous sheet of tissue,¹⁰⁹ where the inner and outer epidermis are physically connected at the margins (sepal edges) and loosely via the mesophyll layer. Therefore, the two epidermal layers cannot grow independently.¹¹⁰ In *as2-7D*, the transverse overgrowth of the outer epidermis imposes spatial and mechanical constraints on the inner epidermis to limit growth of the inner epidermis indirectly. Simultaneously, transverse expansion of the spatially constrained outer epidermis generates in-plane compressive stress within the outer epidermis. Since the outer epidermis of *as2-7D* is also soft, this allows the outer epidermis

to relieve the stress through buckling (out-of-plane deformation), consistent with studies showing how growth-induced compressive stress in layered tissues is relieved through buckling.^{111,112} This conceptually resembles trying to fit a cloth inside a box. If the cloth fits perfectly along the edges, it remains smooth. Otherwise, the cloth must wrinkle (buckle) to fit inside the box. Our results show how the growth of an epidermal cell layer (or speculatively an animal epithelium¹¹³) relative to the tissue to which it is attached drives organ shape.

While how molecular cues influence tissue mechanics has been relatively well studied,^{30,114–116} how tissue mechanics inform molecular signals remains elusive. Our study suggests that not only does AS2 influence tissue mechanics, generating buckles, but also that buckles feed back into molecular signals. Our results suggest that buckling promotes convergence of the PIN1-auxin efflux transporter, supporting previous observations that mechanical stress affects PIN1 polarity.^{92,94,117} We find an association between the tips of buckles and the establishment of PIN1 polarity convergence sites, causing the formation of auxin maxima to subsequently trigger the initiation of epidermal outgrowths. In 1992, Paul Green proposed that buckling influences the initiation of primordia in the shoot apical meristem.¹¹⁸ However, in *Arabidopsis*, PIN1 polarizes before primordia bumps are visible, indicating that PIN1 can converge without buckling.¹¹⁹ Our collective evidence suggests that in certain contexts, buckling promotes convergence of PIN1 and generation of outgrowths.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to, and will be fulfilled by, the lead contact, Adrienne Roeder (ahr75@cornell.edu).

Materials availability

The transgenic lines generated in this study have been deposited with the Arabidopsis Biological Resource Center (ABRC), associated with the following accession numbers: CS73559 (pLH13 35S::mCitrine-RCI2A Ler-0), CS73560 (pLH13 35S::mCitrine-RCI2A *as2-7D*), CS73561 (pLH13 35S::mCitrine-RCI2A *pML1::KRP1* Ler-0), and CS73562 (pLH13 35S::mCitrine-RCI2A *pML1::KRP1 as2-7D*).

All other unique/stable reagents generated in this study are available from the lead contact with a completed material transfer agreement.

Data and code availability

- The primers used for this study are provided in Table S1. The raw datasets used for making the plots in this article are provided in Data S1.
- The meshes and images are publicly available at [osf.io](https://doi.org/10.17605/OSF.IO/P5Q39) (Project doi: <https://doi.org/10.17605/OSF.IO/P5Q39>).
- The R codes used for generating the plots are available at GitHub (https://github.com/Avilash1993/Growth_directions_and_stiffness_across_cell_layers_determine_whether_tissues_stay_smooth_or_buckle) under GNU General Public License v3.0.
- Numerical model implementation and the codes for exploration of the parameter space are available under the LGPL license at https://gitlab.inria.fr/mosaic/publications/growth_buckling.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.H.K.R., A.S.Y., and L.H.; experimentation, A.S.Y., L.H., P.M.K., X.H., and M.H.; imaging, A.S.Y., L.H., M.H., and P.M.K.; image processing, A.S.Y., L.H., P.M.K., I.M.B., A.M.D.G., and R.S.S.; data analysis, A.S.Y., L.H., and A.K.; analytical modeling, A.K. and A.B.; simulations, A.K., M.P., and A.B.; funding acquisition, A.H.K.R., A.S.Y., A.B., and L.H.; project administration, A.H.K.R. and A.B.; supervision, A.H.K.R., A.B., and L.H.; writing – original draft, A.S.Y., L.H., and A.H.K.R.; writing – review & editing, A.S.Y., L.H., P.M.K., A.K., X.H., I.M.B., M.H., A.M.D.G., R.S.S., A.B., and A.H.K.R.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
<i>Escherichia coli</i> (Top10)	N/A	N/A
<i>Agrobacterium tumefaciens</i> (GV3101)	N/A	N/A
Biological samples		
pLH13 35S::mCitrine-RCI2A Ler-0	This study	CS73559
as2-5D	Wu et al. ⁵⁹	CS67863
as2-7D	This study	N/A
pLH13 35S::mCitrine-RCI2A as2-7D	This study	CS73560
pML1::KRP1 Ler-0	Bemis and Torii ⁷⁸	N/A
pLH13 35S::mCitrine-RCI2A pML1::KRP1 Ler-0	This study	CS73561
pML1::KRP1 as2-7D	This study	N/A
pLH13 35S::mCitrine-RCI2A pML1::KRP1 as2-7D	This study	CS73562
pPIN1::PIN1-GFP pin1-4	Heisler et al. ⁹¹	N/A
pPIN1::PIN1-GFP as2-7D	This study	N/A
rev-6	Otsuga et al. ⁸⁶	CS6962
kan1-2 kan2-1/+	Eshed et al. ⁴⁷	N/A
Chemicals, peptides, and recombinant proteins		
Propidium Iodide	VWR	CAS# 89139-064
200 Proof Ethanol	VWR	CAS# 64-17-5
Triton X-100	Amresco	CAS# 9002-93-1
Murashige & Skoog Medium (Finer & Nagasawa Modification) w/ Macroelements & Microelements; w/o Vitamins, Sucrose & Agar	HIMEDIA	REF# PT051-5L
MES hydrate	Sigma-Aldrich	CAS# 1266615-59-1
Gamborg B5 Vitamin Mix, 1000X	plantMedia	SKU# 30630027-1
Plant Preservative Mixture (PPM)	Plant Cell Technology	N/A
Phytoagar	plantMedia	CAS# 9002-18-0
Agarose RA	VWR	CAS# 9012-36-6
Sucrose	Macron Fine Chemicals	CAS# 57-50-1
Sodium Chloride	Fisher chemical	CAS# 7647-14-5
Tryptone	Fisher BioReagents	CAS# 91079-40-2
Yeast Extract	Sigma-Aldrich	CAS# 8013-01-2
Bacto Agar	BD	REF# 214030
Tris base	Fisher bioreagents	CAS# 77-86-1
Cetyl Trimethyl Ammonium Bromide (CTAB)	Amresco	CAS# 57-09-0
Ethylenediaminetetraacetic Acid (EDTA)	VWR	CAT# 97061-018
Hygromycin B	GoldBio	CAS# 31282-04-9
Spectinomycin Dihydrochloride	GoldBio	CAS# 22189-32-8
Basta (Glufosinate Ammonium)	Fisher Scientific	CAS# 77182-82-2
Kanamycin	Goldbio	CAS# 25389-94-0
Rifampicin	Goldbio	CAS# 13292-46-1
Gentamycin	Goldbio	CAS# 1405-41-0
Gateway LR Clonase II Enzyme mix	ThermoFisher Scientific	CAT# 11791020
pENTR/D-TOPO Cloning Kit	ThermoFisher Scientific	CAT# K240020
Phusion High-Fidelity DNA Polymerase (2 U/μL)	ThermoFisher Scientific	CAT# F530S
N-(1-Naphthyl)phthalamic acid	Krackeler (Sigma)	CAS# 132-66-1

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Raw datasets	This study	Data S1
Meshes and images	This study	osf.io (Project doi: https://doi.org/10.17605/OSF.IO/P5Q39)
Recombinant DNA		
p35S::mCitrine-RC12A	Robinson et al. ⁶²	pLH13
pPDF1::mCitrine-MBD	Armezzani et al. ⁷⁰	N/A
pML1::AS2	This study	pLH79
pML1::REV	This study	pASY04
pAS2 ^{WT} ::GFP-GUS	This study	pLH138
pAS2 ^{as2-7D} ::GFP-GUS	This study	PLH140
proREV::REV-GFP	This study	pXH27
proFIL::mCherry-RC12A	This study	pXH28
Oligonucleotides		
Oligonucleotides	This study	Table S1
Software and algorithms		
ImageJ2	Rueden et al. ¹²⁰	https://imagej.net/software/imagej2/
Fiji	Schindelin et al. ¹²¹	https://fiji.sc/
MorphoGraphX	Strauss et al. ⁶⁹	https://morphographx.org/
Analytical model	This paper	N/A
FEniCS	Alnæs et al. ¹²²	https://fenicsproject.org/
BVPy	Gacon et al. ¹²³	https://mosaic.gitlabpages.inria.fr/bvpy/
R codes	This study	Github: https://github.com/Avilash1993/Growth_directions_and_stiffness_across_cell_layers_determine_whether_tissues_stay_smooth_or_buckle
Numerical model implementation and the codes for exploration of the parameter space	This study	https://gitlab.inria.fr/mosaic/publications/growth_buckling

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Plant Materials

Arabidopsis thaliana genotypes used in this study are in the Landsberg *erecta* (Ler) background and are referred to as wild type unless otherwise mentioned. The mutants *rev-6*⁸⁶ and *as2-5D* mutant (in Col-0 background)⁵⁹ have been previously described, and were obtained from Arabidopsis Biological Resource Center (ABRC). The *kan1-2 kan2-1* mutant previously described⁴⁷ was a kind gift from John Alvarez (Monash University). The plants are homozygous for *kan1-2* while *kan2-1* is segregating; the phenotypes were assayed for plants that are homozygous for both *kan1-2* and *kan2-1*. The *as2-7D* mutant in the Ler-0 background was identified from ethyl methanesulfonate (EMS) based forward genetic screen. Briefly, M2 seeds (second generation) obtained from self-fertilized EMS mutagenized Ler plants were purchased from Lehle Seeds.¹²⁴ M2 plants were screened under a stereo microscope for sepal defects.^{77,125} The *as2-7D* mutant was isolated based on the lumpy sepal phenotype. A mapping population was generated by crossing the *as2-7D* mutant with a Columbia (Col-0) accession plant. Standard map-based cloning techniques were used to clone the underlying mutation.¹²⁶ The *as2-7D* mutation is a G to A change at base 1,484 upstream of the ATG translational start site of AT1G65620/AS2. To reduce background mutations, the *as2-7D* mutant was backcrossed twice to wild-type Ler plants before further analysis. In the backcrosses, we found that *as2-7D* mutant behaves as a dominant allele as the F1s exhibit the *as2-7D* phenotype, although it is not as strong. The *as2-7D* mutation can be PCR-genotyped by amplifying with primer pairs oLH108 and oLH324 at 55°C annealing temperature, followed by digesting the product with EcoRI to produce an 82-bp wild-type product or a 111-bp mutant product. Primers are listed in Table S1.

Plant Growth

Plants were grown in Percival (Percival-Scientific) growth chambers programmed for long day conditions (16-hour light/8-hour dark cycles). Growth temperature (22°C), relative humidity (65%) and fluorescence intensity (~100 μmol m⁻¹s⁻¹) under Philips Alto II F32T8/TL841 800 Series 32-W fluorescent tubes were maintained.

For soil-based experiments, plants were grown on soil premixed with Vermiculite (LM111). For stratification, the trays were placed in a 4°C cold room for three days prior to moving them to the growth chambers. For BASTA (glufosinate ammonium) selection, the

soil was autoclaved twice (Program 3– 15 min sterilization, 30 min dry, Primus ST-1 Autoclave (Model PSS5-B-MSSD)), and sterilized seeds were planted at a density of 50–100 seeds per pot. For live-imaging and adult-stage phenotyping experiments, one plant was grown per pot (6.5×8.5 inches) to facilitate healthy vegetative growth.

For plate-based experiments, seeds were washed twice with a sterilization solution containing 70% ethanol + 0.1% Triton X-100, replacing the solution after each wash. Two final rounds of washes were provided with 100% ethanol. Seeds were spread on filter paper (Whatman 1, diameter 90 mm) and allowed to dry inside a sterile safety cabinet (Class II, Type A2, Labgard). Dried seeds were evenly distributed on freshly prepared MS plates (1/2 MS basal salt mixture, 0.05% MES w/v, 1% sucrose w/v, pH ~ 5.8 (adjusted using KOH) containing 0.8% phytoagar. The plates were sealed with 3M micropore tape, wrapped in aluminum foil, and stratified for three days at 4 °C before being moved to the growth chamber.

METHOD DETAILS

Phenotyping whole inflorescences, flowers, and plants

Whole inflorescence and stage 14 flower images were taken either using Canon PowerShot A640 10MP Digital camera attached to Zeiss Stemi 2000-C stereo microscope or Accu-Scope Excelis 4K UHD camera attached to Zeiss Stemi 508 stereo microscope. For inflorescence images, and Stage 14 flowers, an optical zoom of 1.0 and 1.6 were used respectively. Whole plant images were captured using iPhone XR with a 26 mm focal length and aperture of f/1.8.

Schematic diagrams

The schematic diagrams were hand-drawn using the Notability application in Apple iPad (model A1701).

Genetic crosses

To generate *pML1::KRP1 as2-7D*, the *pML1::KRP1* in *Ler* background^{77,78} were crossed into *as2-7D* mutants. Segregation analysis and confocal microscopy was used to find *pML1::KRP1 as2-7D* plants. The *pPIN1::PIN1-GFP DR5::VENUS*⁹¹ plants and *pPIN1::PIN1-GFP pin1-4*⁹¹ plants were crossed into *as2-7D* mutants. The *pPIN1::PIN1-GFP pin1-4* plants (control) exhibit a mild phyllotaxy defect. Genetic segregation analysis and confocal microscopy was used to find *DR5::VENUS as2-7D* and *pPIN1::PIN1-GFP as2-7D* plants.

Cloning overexpression constructs and reporters

The pLH13 (*p35S::mCitrine-RCI2A*, hygromycin resistance) and pAR169 (*pML1::RCI2A-mCitrine*, basta resistance) plasma membrane markers have been described previously.^{62,77} The *pPDF1::mCitrine-MBD* microtubule marker⁷⁰ was a kind gift from Olivier Hamant (RDP institute, ENS de Lyon, France).

For cloning *pML1::AS2*, the AT1G65620/*AS2* coding sequence (600 bps) was amplified from Col-0 cDNA using primers oLH111 and oLH112 by Phusion PCR (NEB) and cloned into pENTR/D-TOPO (Invitrogen). The *AS2* coding sequence was cloned into the *pML1* containing gateway vector pAR176 (Basta resistance⁷⁷) by LR reaction.

For *pML1::REV*, the AT5G60690/*REV* full length coding sequence spanning 2,529 bp was amplified from Col-0 inflorescence cDNA using primer pairs oAS_005 and oAS_006. The resulting PCR product was subcloned into pCR8TOPO vector (Invitrogen) by TA cloning. LR reaction was used to clone the *REV* coding sequence into pAR176.

To clone *pAS2^{WT}::GFP-GUS*, the 4.1 kb upstream promoter region and part of the first exon of *AS2* was PCR amplified from Ler-0 genomic DNA using primer pairs oLH347 and oLH370 and cloned into pENTR/D-TOPO vector (Invitrogen). The resultant vector was LR recombined into the gateway vector pBGWFS7.¹²⁷ For cloning *pAS2^{as2-7D}::GFP-GUS*, the 4.1 kb upstream promoter region and part of the first exon of *AS2* was PCR amplified from *as2-7D* genomic DNA, and the subsequent cloning steps were similar to cloning *pAS2^{WT}::GFP-GUS*.

For *proREV::REV-GFP*, the 7.75 kb *proREV::REV* fragment (amplified from Ler-0) and 1.08 kb mGFP-T35S fragment amplified from *pML1::H2B-mGFP* (pAR180⁷⁷) were assembled into the PmeI site of pMOA33 (Kanamycin resistance).

For cloning *proFIL::mCherry-RCI2A*, a 3.92 kb *FIL* promoter fragment was amplified from Ler-0. This fragment was assembled with a 2.19 kb *omega-mCherry-RCI2A-T35S* fragment amplified from *p35S::mCherry-RCI2A* (pMZ11¹²⁸) into the SacI and SpeI site of pMOA34 destination vector (hygromycin plant resistance¹²⁹). All the intermediate and final constructs were verified for sequence and directionality by sequencing. Primers are listed in Table S1.

Plant transformation and selection

The binary vector constructs were transformed into *Agrobacterium tumefaciens* GV3101 strain (Rifampicin and Gentamycin resistant) by triparental mating method.¹³⁰ Briefly, two *E. coli* strains, one containing the binary vector and the other containing the helper (conjugative) plasmid, and *Agrobacterium* strain GV3101 were mated together. The GV3101 clones were streaked onto Rifampicin + Gentamycin + binary vector specific antibiotic plates for selection, and positive clones were isolated by colony PCR. The T-DNAs were transformed into plants by *Agrobacterium*-mediated floral dipping. Transformants were selected either on 1/2 MS media plates (for Hygromycin and Kanamycin selection) or on soil (for Basta selection). For selecting pLH13 in wild type and *as2-7D*, *proFIL::mCherry-RCI2A* in wild type and *as2-7D* T1s, a final concentration of 25–30 µg/mL Hygromycin was used. For selecting *proREV::REV-mGFP* in wild type and *as2-7D* T1s, a final concentration of 50 µg/mL Kanamycin was used. Healthy, non-chlorotic plants were

transplanted to the soil post two weeks of growth. For selecting *pML1::AS2* in wild type and in *rev-6*, *pML1::REV* in wild type and in *as2-7D*, *pAS2^{WT}::GFP-GUS* and *pAS2^{as2-7D}::GFP-GUS* in wild type, *pPDF1::mCitrine-MBD* in wild type and in *as2-7D* T1s, BASTA selection was performed on soil grown plants. Seedlings aged 1 to 2 weeks were selected with 100 μ g/mL BASTA solution by spraying, and non-chlorotic plants were transplanted to fresh pots after a week. At least twenty independent T1 plants were characterized for each construct. For fluorescent reporter lines, T1 plant was chosen for each line based on fluorescence. Confocal microscopy was used to assess the stability of fluorescence in every generation.

Flower staging

Floral staging was performed based on Smyth et al.⁶¹ Briefly, sepal primordia barely arise in Stage 3 and overlie the floral meristem in Stage 4. Sepals enclose the floral bud completely by late stage 6. Flowers beyond stage 6 were staged based on size and morphology.²⁹

Sepal selected for microscopy

For flower imaging across experiments (live imaging and single time point reporter imaging), we focused primarily on the outer sepal of the flower, unless specified. Briefly, flowers are enclosed within four sepals, wherein the foremost sepal, which faces away from the inflorescence meristem is denoted as the outer sepal, whereas the sepal that is diametrically opposite to the outer sepal and facing towards the inflorescence meristem is denoted as the inner sepal. The remaining two sepals positioned on either side are the lateral sepals. For a detailed description, please refer to Roeder²⁹

Scanning electron microscopy (SEM)

Scanning electron microscopy was performed as described previously.⁷⁷ Briefly, Stage 14 flowers were fixed in an FAA solution (50% ethanol, 5% acetic acid, and 3.7% formaldehyde) for 4 hours, dehydrated using an ethanol series, and critical point dried. Sepals were dissected, sputter-coated with platinum palladium and then imaged using a LEICA 440 scanning electron microscope.

Sepal Contour analysis

Analysis of sepal shape was performed as described previously.⁵⁸

Confocal Microscopy

Live imaging

For live imaging sepal development, primary inflorescences from six- to eight-week-old plants containing the *p35S::mCitrine-RCI2A* membrane marker (*pLH13* in wild type, *as2-7D*, *pML1::KRP1*, and *pML1::KRP1as2-7D* T2s) were excised. Dissection was performed as described previously,¹²⁸ with modifications. Briefly, siliques were removed using surgical scissors (Artman Instruments). Flowers older than stage 7 were dissected using Dumont tweezers (Electron Microscopy Sciences, Style 5) under a stereo microscope (ZEISS Stemi 2000). Dissected inflorescences were inserted upright into small petri-plates containing live imaging media (1/2 MS media supplemented with 1X Gamborg Vitamin mix and 1000-fold diluted plant preservative mixture) with 1.2% w/v phytoagar. The plates were filled with autoclaved water and allowed to stand for two-three hours for the dissected inflorescence to rehydrate. Residual buds were dissected down to Stage 3-4 in water using a needle (BD Micro-Fine IV Insulin Syringes). Inflorescences were moved to fresh media plates and transferred to the growth chamber for at least 24 hours for recovery from dissection, prior to imaging. Stage 4 flowers were selected as the 0-hour time point across all live imaging series.

Post recovery, dissected tissues for live imaging were positioned at an angle, to ensure the outer sepal of the chosen stage 4 flower directly faces the objective of the confocal microscope.⁶⁸ The flowers were submerged in MillQ water containing 0.01% v/v SILWET L-77 surfactant and imaged using Zeiss 710 upright confocal microscope using 20X water dipping objective (Plan-Apochromat 20x/1.0 DIC D=0.17 M27 75mm). The following settings were used: mCitrine (yellow fluorescent protein), excitation laser 514 nm (intensity- 2.4-6.0%), emission collection range 519-596 nm and a z-step of 0.415-0.420 μ m. For high laser intensity based live imaging of *pLH13* in wild type and in *as2-7D* to visualize the adaxial sepal epidermis, imaging settings for mCitrine used: excitation laser 514 nm (intensity- 16-18%), emission collection range 519-596 nm and a z-step of 0.244 μ m. Post imaging, the plates were returned to the growth chamber, and the process was repeated for each time-point. Inflorescence tissues were transferred to fresh media every 48 hours to prevent fungal contamination.

NPA treatment assay

Primary inflorescences of six- to eight-week-old *pLH13* in *as2-7D* T2s were dissected down to stage 3 and were divided into two groups of 5-6 samples. One group of samples were placed in 1/2 MS live imaging media plates containing 10 μ M NPA. The other group, serving as the control, was placed in media plates containing an equivalent amount of DMSO (20 μ L DMSO per 100 ml media). The tissues were dissected 48 hours prior to imaging. Post dissection, the tissues were treated every 12 hours with a 100 μ L liquid solution, applied directly above the inflorescence tissue by pipetting. For the 10 μ M NPA treatment samples, the liquid solution comprised of 1 μ L of 1M NPA+19 μ L DMSO+10 μ L Silwet in 100 ml water, whereas the control (mock) samples were treated with 20 μ L DMSO+10 μ L Silwet in 100 ml water. The aim of administering liquid solution was to enhance the effectiveness of the treatment, which was performed every 12 hours throughout the experimental duration. Stage 4 flowers, 48 hours post dissection were selected as the 0-hour time point for imaging mock and 10 μ M NPA treated samples. Live imaging was performed as described above, with the samples being moved to fresh mock or 10 μ M NPA plates every 48 hours. Imaging settings used: mCitrine (yellow fluorescent protein), excitation laser 514 nm (intensity -2.6-2.8%), emission collection range 519-596 nm, and a z-step of 0.415 μ m.

Propidium Iodide (PI) cell wall staining and imaging

A 5 mg/ml stock solution was prepared for PI staining, with 5 mgs of PI solubilized in 1 mL of MilliQ water. The stock solution was stored in a foil-covered 1.7 ml microcentrifuge tube and refrigerated at 4°C. For staining, stock solution was five-fold diluted with water containing 0.01% v/v Silwet surfactant (PI working solution). Whole inflorescences and flowers below Stage 10 were stained for 6–8 minutes, stage 14 sepals were stained for 12 minutes, and leaves were stained for 25 minutes. Care was taken to ensure that the tissue was fully submerged in the staining solution, and no air bubbles surrounded the tissue. Following staining, the tissue was washed in a fresh solution containing just 0.01% v/v Silwet, prior to imaging. Fresh PI working solution was prepared before every experiment. For imaging PI-stained inflorescences of wild type, *as2-7D* and the transgenics (T1s) of *pML1::AS2* in wild type, *pML1::REV* in wild type, *pML1::REV* in *as2-7D* and the inflorescences, flowers of *kan1-2 kan 2-1* mutants, the following settings were used: excitation 514 nm (laser intensity -18–22%), emission 566–650 nm. For imaging sections of PI-stained *kan1-2 kan 2-1* mutant leaves, samples were excited with 514 nm (laser intensity - 70%) and emission was collected between 566–643 nm range.

Imaging reporters

For single time point imaging of the reporters, inflorescences were dissected down to stage 3–4. The recovery period was extended to 48 hours to allow ample time for stabilization of the reporter signal. The inflorescences were placed upright in the live imaging media plates with the meristem facing the objective. Prior to taking images of late-stage flowers, the inflorescences were allowed to grow for a few days. For the reporters imaged together with PI, fresh inflorescences were dissected for imaging late-stage flowers. Images were taken with Zeiss 710 upright confocal microscope using 20X water dipping objective.

The following settings, specific for each of the reporters, were used. For *pAS2^{WT}::GFP-GUS* and *pAS2^{as2-7D}::GFP-GUS* images, PI - excitation laser 594 nm (intensity- 2%), emission range 599–650 nm and GFP - excitation laser 488 nm (intensity-20%), emission range 493–550 nm, with z-step of 0.240 μ m. For *proREV::REV-GFP*, Chlorophyll and GFP were excited with 488 nm laser (intensity-40%), emission range was collected between 647–722 nm for Chlorophyll and 493–550 nm for GFP. Z-step, 0.240 μ m (inflorescences), 0.4 μ m (flowers). For *proFIL::mCherry-RCI2A*, Chlorophyll and mCherry were excited with 561 nm laser (intensity- 12–15%), emission range was collected between 655–722 nm for Chlorophyll and 578–650 nm for mCherry. Z-step, 0.240 μ m. For *pPIN1::PIN1-GFP* single time-point images, PI - excitation laser 594 nm (intensity- 2%), emission collection range 599–650 nm and GFP - excitation laser 488 nm (intensity- 25–30%), emission collection range 493–550 nm, with z-steps of 0.240–0.5 μ m. For *pPIN1::PIN1-GFP as2-7D* live-imaging, GFP - excitation laser 488 nm (intensity - 20–25%), emission collection range 493–538 nm and Chlorophyll - excitation laser 488 nm (intensity - 20–25%), emission collection range 647–722 nm, with z-steps of 0.5 μ m. For *pDR5::VENUS*, samples were excited with an argon laser (514 nm), and data were collected in the YFP (520–565 nm) channel. For *pPDF1::mCitrine-MBD*, PI and mCitrine were excited with 514 nm (intensity- 30–32%), and emission range was collected between 603–643 nm for PI and 519–567 nm for mCitrine. Z-step, 0.2 μ m.

Imaging adult sepals

For imaging fully mature sepals, all flowers were dissected off the inflorescence except for a single stage 14 flower. All the internal floral organs from the stage 14 flower were carefully dissected off, leaving just the outer sepal²⁹ attached to the pedicel. The sepal was wetted in 0.01% v/v Silwet water and mounted between two coverslips filled with 0.01% v/v Silwet water. To prevent the sepal from drying out during imaging, the coverslips were sealed with vacuum grease. Sepals were imaged using 10X objective (C-Apochromat 10X/0.45 W M27). Three horizontally tiled images were taken with an overlap of 5% and were auto stitched with a threshold of 0.70 in the ZEISS ZEN software. For imaging fully mature sepals of pLH13 in wild type and in *as2-7D*, mCitrine was excited with 514 nm laser (intensity- 20–22%) and emission was collected at 519–621 nm. For imaging PI-stained sepals of wild types, *as2-7D*, *pML1::KRP1* and *pML1::KRP1 as2-7D*, PI was excited at 514 nm (intensity- 27–30%) and emission was collected at 566–648 nm. For epidermal structure observation, mature flowers harboring pAR169 (*pML1::RCI2A-mCitrine*) were transverse sectioned using a razor blade. Both mCitrine and chlorophyll were excited using 514 nm laser, and emission was collected at 520–565 nm for mCitrine and 651–700 nm for chlorophyll.

Laser ablations

For laser ablations, outer sepals of floral developmental stage 6 were chosen for both *35S::mCitrine-RCI2A* WT and *35S::mCitrine-RCI2A as2-7D*. The laser ablations were performed on an Olympus FVMPE-RS Twin Laser upright Multiphoton Microscope, controlled by the Fluoview software. For ablations, cells were brought into focus, and a line-shaped Region of Interest (ROI) was positioned over the target cells. The ROI was irradiated twice (50 msec per irradiation) with an 800 nm laser line (provided by a Spectra Physics MaiTai Ti:Sapphire laser) set to 60% power (which corresponds to ~130 mW), using the main scanner set at 10 μ s/pixel. Images of the samples before and after ablation were taken using a 25X objective (XL/PLAN N/1.05 W MP). For imaging, mCitrine was excited with a 920 nm laser line (provided by a SpectraPhysics Insight OP) and the emission was collected between 498–542 nm.

Osmotic treatments for measuring epidermal stiffness

Osmotic treatments were performed as described previously,^{58,79} with modifications. In brief, primarily inflorescences from pLH13 in wild type, pLH13 in *as2-7D*, pLH13 in *pML1::KRP1* and pLH13 in *pML1::KRP1 as2-7D* six- to eight-week-old plants were dissected down to stage 3 to 4 and placed in ½ MS live imaging media plate. Dissected tissues were allowed to recover for 24 hours in the growth chamber. Early stage 6 buds were precisely chosen for osmotic treatment, since this is the stage right before *as2-7D* outer epidermis starts buckling.

Each bud (corresponding to one replicate) was subjected to osmotic treatment individually and was imaged pre- and post-treatment. Pre-treatment imaging involved staining the bud with a PI working solution for precisely 6 minutes. After washing off the PI stain, the tissue was transferred to a fresh media plate and was oriented to focus on the outer sepal. The tissue was submerged

in 0.01% v/v Silwet water, and the pre-treatment image was captured. Imaging settings used for mCitrine – excitation 514 nm (intensity- 20-25%), emission 519-567 nm and PI - excitation 514 nm (intensity- 20-25%), emission 603-643 nm.

For osmotic treatment, the bud was immersed in 0.35M NaCl solution containing 0.01% v/v Silwet for exactly 20 minutes. Post treatment, the bud was re-stained in a PI working solution prepared in 0.35M NaCl (1 part 5 mg/mL PI (solubilized in 0.35M NaCl) and 4 parts 0.35M NaCl solution containing 0.01% v/v Silwet) for 10 minutes, leading to a cumulative osmotic treatment duration of 30 minutes. This timeframe was found to be optimum for inducing plasmolysis in epidermal cells of early- stage 6 flowers, wherein the plasma membrane gets visibly detached from the cell wall at the corners. Re-staining the buds with PI is necessary since NaCl solution erodes off the PI stain. The bud was finally re-positioned on a fresh media plate and submerged in 0.35M NaCl solution. Before image acquisition, the tissue was allowed to stabilize for a few minutes due to its increased fragility post osmotic treatment. The post-treatment image acquisition setting was as follows: mCitrine – excitation 514 nm (intensity- 25-35%), emission 519-567 nm and PI - excitation 514 nm (intensity- 25-35%), emission 603-643 nm. This procedure was iterated for all samples individually.

Image processing

Converting raw images to tiffs, taking screenshots and videos

The raw images, generated either in lsm or czi formats from ZEN imaging software, were converted to tiff files in ImageJ distribution Fiji.¹²¹ For multichannel images, images were split based on channels, which were saved individually. The MorphoGraphX (MGX)⁶⁹ software was used for image processing and taking the screenshots using the built-in screenshot option in MGX. For taking screenshots of the reporter lines, the reporter image was loaded onto Stack 1, and either chlorophyll or PI was loaded onto Stack 2. Color-maps for Stack 1 and Stack 2 were set to green and gray respectively. Flower images and meristems were rotated in the desired orientation. The voxels not associated with the flower or meristem of interest, were removed using the Voxel Edit tool in MGX. Additionally, trichomes from the outer sepal epidermis were removed in a similar manner, for better visualization of the sepal surface. Following this, screenshots were captured. From flower images, trichomes on the outer sepal were removed by image processing for better visualization of the sepal epidermis. The brightness and contrast were uniformly adjusted across samples for any given experiment. Screenshots of MGX generated heatmaps were taken using the default brightness and contrast settings, without any adjustments.

Videos were captured as a series of two-dimensional tiffs files using the built-in “Record movie” option in MGX. The 3D stack images were rotated in the MGX window for the desired duration and time points. For capturing videos involving two genotypes/treatments, the images were loaded onto Stack 1 and Stack 2 and rotated independently. The stack of tiffs generated was loaded onto Fiji and saved as a.avi file without compression. The.avi files were assembled and labeled in Adobe Premiere Pro 2023.

Generating surfaces for analyzing outer epidermal growth

Making the surfaces and analysis of growth data was performed in MGX as previously described in Hong et al.,⁵⁸ and Zhu and Roeder¹³¹ with modifications. The following process was used to generate the surfaces for analyzing cell growth parameters on the outer epidermis of wild type, *as2-7D*, *pML1::KRP1*, *pML1::KRP1 as2-7D*, *as2-7D* mock and 10 μ M NPA treatment samples. Briefly, the tiffs were imported onto Stack 1 Main Store in the software. Trichomes on the outer epidermis were removed using the voxel edit tool to get a better surface. Since the outer epidermal morphology of *as2-7D* becomes increasingly irregular in later stages, Level Sets¹³² was used to extract the structure representing the general flower shape. For this, Level Sets was initialized with an up/down threshold value of 3 to 4. The structure was allowed to evolve (Level Set Evolve) with default parameters, except for “view steps”, which was set to 1 for visualization at more frequent intervals. For performing Edge Detect Angle, the mask was oriented such that the outer sepal directly faces the viewer. The Edge Detect Angle process was then run with default parameters to get a finer structure that closely resembles the image. The holes generated in the structure (which corresponds to places in the image with low membrane marker signal) were closed using Stack/Morphology/Closing - X/Y/Z radius of 3 to 8. A 2.5D surface fitted with a triangular mesh was created from the structure using Marching Cube Surface with a cube size of 3 to 5 μ m, followed by smoothing once (Smooth Mesh, passes = 10) and subdividing twice. The membrane signal was projected onto the 2.5D surface with a minimum and maximum distance range of 1 to 8 μ m from the image.

Generating surfaces for analyzing outer vs inner epidermal growth in wild type and *as2-7D*

To obtain the inner epidermal surface, clipping plane 1 (View Tab/Clip 1), set to a width of 10 to 15 μ m, was used to clip the flower image along the longitudinal direction. The voxel editing tool was used to carefully remove the voxels below the inner epidermis of the sepal within the clipping plane region. The clipping plane was then moved to the adjacent region, and the same process was repeated, while tilting the plane to ensure voxels from the inner epidermis itself are not removed. Once the voxels below the inner epidermis were removed throughout, the edited tiff was copied back to the main stack and saved. To generate the surface for the inner epidermis, the Z axis of the stack was reversed (Stack/Canvas/Reverse Axes). The stack was blurred using Gaussian blur (X/Y/Z sigma (μ m) = 2 to 2.5), and structure was extracted using Edge Detect with a threshold of 2000 to 3000. A triangular mesh surface was created using a cube size of 3 μ m, followed by smoothing once (passes = 10) and subdividing twice. Plasma membrane signal from the inner surface was projected onto the mesh surface with a minimum and maximum distance range of 3 to 8 μ m.

To obtain the outer epidermal surface, the processed tiff, prior to reversing the axes, was used. To process the stack, Gaussian blur (X/Y/Z sigma (μ m) = 1 to 1.5) followed by Edge detect (threshold of 8000 to 10,000) were used sequentially. Mesh surface was created as described above, and the membrane signal was projected within a 2 to 4 μ m distance range from the image.

Generating surfaces for analyzing growth of outermost mesophyll layer in wild type and *as2-7D*

To enhance the membrane marker signal for the mesophyll cells, we utilized a deep learning approach based on a Convolutional Neural Network (CNN). We used the inbuilt CNN Unet 3D prediction algorithm `VijayanUnet.pt`¹³³ in MorphoGraphX to precisely predict the cell outlines. The algorithm then uses these predictions for enhancing and de-noising the membrane marker signal and generates a new image based on the predictions.

For both wild type and *as2-7D* mutants harboring pLH13 membrane marker, the CNN algorithm was run on the pre-processed confocal images of the outer sepal, where the parts of the flower not associated with the outer sepal were already removed by voxel editing (see ‘generating surfaces for analyzing outer vs inner epidermal growth in wild type and *as2-7D*’ section in STAR Methods). Using the pre-processed sepal images instead of whole flowers for running the CNN algorithm reduced computational processing times. Briefly, for wild type and *as2-7D* mutants, CNN-generated images of the sepals for each time point were obtained by running Stack/CNN/UNet3D Prediction/VijayanUnet.pt using default parameters. The CNN Unet3D prediction process usually also generates voxels unrelated to the sepals, which were removed using the ‘Voxel edit’ tool. These edited CNN-generated images were then used to run Stack/Mesh Interaction/Annihilate function, to remove the voxels associated with the outer epidermis and the inner layers and obtain the outermost mesophyll layer (layer closest to the outer epidermis). For sepals at 0-hour and 24-hour time points, the outermost mesophyll layer was extracted by setting the minimum distance values for Annihilate function to 5/6 and the maximum distance values to 15. For the 48-hour time points, the minimum and maximum distance value for the Annihilate function was set to 6 and 20 respectively. The resulting images generated after running Annihilate function were used to generate the 2.5D surface for the outermost mesophyll layer. Briefly, for each time-point and genotype, the annihilated image stack was loaded onto Stack 1 and blurred using Gaussian Blur, with X/Y/Z sigma (μm) value of 3. The surface was extracted using Edge Detect threshold of 2000 to 3000. The mesh surface was created using a cube size of 3 μm , followed by smoothing once (passes = 10) and subdividing twice. The membrane signal was projected onto the mesh surface with a minimum and maximum distance range of 7 to 8 μm .

Lineage tracking

For meshes across all time points, individual cells were manually seeded. Cells were segmented using the watershed algorithm, and unlabeled vertices were fixed by running Cell Mesh/Fix Corner Triangles. Cell lineages were parent tracked to the earlier time-point to analyze cell growth. Parent (lineage) tracking involves transferring the parent labels from cells in the earlier time point onto the daughter cells at the later time point. For this, the meshes corresponding to the earlier (Mesh 1) and later (Mesh 2) time points were opened side by side on the same MGX window. A few daughter cells in Mesh 2 were manually parent labeled, and using these as landmarks, the remaining daughter cells were auto parent labeled (by running Deformation/Morphing/Set Correct Parents followed by Deformation/Mesh 2D/Auto Parent Labelling 2D). Errors in segmentation or parent tracking were manually corrected. Parent tracking was checked for accuracy using Cell Axis/PDG/Check Correspondence, which checks whether the cell junctions are identical across time points. The final parent labels were saved as.csv files. A custom R script (combinelineages.r) was run to automatically correlate the lineage information through multiple time points, followed by manual correction.

Analyzing cell growth parameters

Meshes corresponding to the earlier (Mesh 1) and later time points (Mesh 2) were loaded side by side in the same MGX window, with parent labels loaded onto Mesh 2. Cell growth rate was calculated based on the total area of the daughter cells in the second time point divided by the area of the parent cell in the earlier time point. Growth rate heatmaps were generated using Heat Map/Heat Map Classic/Heat Map with the Type set to Area. The cell division rate was calculated based on the total number of daughters formed in the second time point for each cell in the earlier time point. Cell division heatmaps were generated using Lineage Tracking/Heat Map Proliferation. The values for growth rate and cell division rate were exported to.csv files.

The Principal Direction of Growth (PDG) uses information of corresponding junctions between the earlier and later time points, to compute the direction of maximum and minimum growth of each individual cell in the earlier time point.⁶⁹ In the figures, the PDGs represent the maximum growth direction (PDG_{max}) tensor (yellow lines), the length of which is directly proportional to the magnitude along the direction of maximum growth. The PDGs were calculated for the cells in the earlier time point (using the information of parent labels from the later time point), the PDG_{max} tensors were projected onto the later time point for visualization. For displaying PDG_{max} , the values for line width and line scale were set to 5 and 3 respectively.

The Angle of Deviation of the PDG_{max} tensor was calculated relative to the longitudinal axis of the sepal. The sepal longitudinal axis was set using a Bezier line on the earlier time point, since PDGs and deviation angles are calculated on the earlier time point. Briefly, a new Bezier grid (Misc/Measure/New Bezier) was created, which was then collapsed into a Bezier line (Misc/Bezier/Collapse Bezier Points). The supporting points on the Bezier line were placed on the mesh in a manner such that the Bezier line is parallel to the sepal longitudinal direction, and the line shape fits the curvature of the sepal. The custom axis for each cell was set using this Bezier line (Cell Axis/Custom/Create Bezier Line Directions) followed by smoothing (Smooth Custom Directions). The PDGs were loaded onto the mesh, and the angle of deviation of the PDG_{max} from custom X direction (which represents the longitudinal axis) was calculated using Cell Axis/Custom/Custom Direction Angle. The values of the angle of deviation heatmap were exported as.csv files.

Analyzing osmotic treatment induced epidermal shrinkage

Our osmotic treatment conditions were optimized such that the cell walls shrank, but the cell membrane barely plasmolyzed. Post osmotic treatment, the membrane just visibly pulled away from the cell wall corners, thereby rendering the wall and membrane area almost comparable. Since the PI signal (corresponding to the cell wall) is very weak post-treatment, especially for the inner layers, the PI and mCitrine (membrane marker) images were merged as a single image MGX (Stack/Multistack/Merge Stacks).

For analyzing epidermal stiffness, we assume that the surface tension across genotypes is comparable. The shrinkage in area of the epidermal layers along a comparable longitudinal section was analyzed to infer tissue stiffness. For this, the pre- and post-treatment merged stacks for any given sample were opened side by side in the same MGX window. Using clipping plane 1 (Clip 1), longitudinal cross-section corresponding to the same regions were extracted from the flower images. The tiffs were processed using Levels Sets to extract the global shape. Mesh surfaces were made with a cube size of 3 μm , which was smoothed once (10 passes) and subdivided twice. Signal was projected with a 1.5 to 3 μm distance range from the image. From the cross-section surface, the outer epidermis and inner epidermis were seeded and segmented as single “cells”. Care was taken to ensure that the epidermal region being seeded was comparable between the pre- and post-treatment surfaces. For measuring shrinkage, parent labels from the pre-treatment mesh were transferred onto the post-treatment mesh, following which the shrinkage ratios were quantified (Mesh/Heatmap/Heatmap Classic). The areal shrinkage values were stored as a.csv file, which was converted to percentages for generating heatmaps shown in the figures.

Computing minimum curvature

In MorphographX, minimal curvature analysis quantifies the least degree of curvature at any given location on the surface. The output is displayed in the form of a heatmap where warm colors represent regions of higher curvature or outward bulging, whereas cool colors represent regions of lower curvature, such as inward deformations (saddles). For calculating the minimal curvature for the sepals from the live imaging series of *pPIN1::PIN1-GFP as2-7D* flowers, we used the images obtained from the chlorophyll autofluorescence channel to construct the mesh surfaces. Briefly, the chlorophyll image corresponding to a given flower (for which the minimal curvature is to be analyzed) was loaded onto Stack 1. The structure representing the general shape of the flower was extracted using Level Sets (Stack/Lyon/Init Level Set), with an up and down threshold values set to 4. The structure was allowed to evolve (Stack/Lyon/Level Set Evolve) with default parameters. The triangular 2.5D mesh was created using Mesh/Creation/Marching Cube Surface with 5 μm cube size, followed by one round of smoothing (Mesh/Structure/Smooth Mesh, passes = 5) and subdividing (Mesh/Structure/subdivide). Minimal curvature was then calculated using Mesh/Signal/Project Mesh Curvature, selecting Type = Minimal and setting the neighborhood radius value to 50 μm . For visualizing the resulting heatmap, the transfer function was set to “Jet”.

Analytical model for a growing trilayer

System and assumptions. We consider a growing elastic system made of three layers, corresponding to the inner epidermis (denoted *i*), mesophyll (*m*), and outer epidermis (*o*) of a lateral organ. We consider that the three layers may grow at different (specified or target) rates and have different elastic moduli. To simplify the formulae, we assume that the epidermal layers have similar thicknesses. We also assume that the Poisson ratios of all layers are equal (value ν). Finally, we neglect the bending modulus of each layer considering their small thickness. Note, these three assumptions do not affect the qualitative behavior of the system.

We assumed E_i, E_m , and E_o be the values of elastic modulus, h_i, h_m , and h the values of thickness, and g_i, g_m , and g_o as the values of strain associated with target (specified) growth rates for the inner epidermis, the mesophyll, and the outer epidermis, respectively.

The differences in growth rates between the layers may lead to changes in system geometry. As the three layers are bound to each other, they have approximately the same curvature c (corresponding to the radius of curvature measured at the middle of the mesophyll), while the strain in the inner and outer layers can be deduced from the strain in the middle layer ε using curvatures and assuming that the reference state is flat. From kinematics of the system, the elastic (residual) strains in the three layers are given by

$$\varepsilon_i = \varepsilon - \frac{1}{2}(h_m + h)c - g_i$$

$$\varepsilon_m = \varepsilon - g_m$$

$$\varepsilon_o = \varepsilon + \frac{1}{2}(h_m + h)c - g_o$$

The elastic energy of the system takes the form

$$E = \frac{1}{2(1 - \nu^2)} [E_i h \varepsilon_i^2 + E_m h_m \varepsilon_m^2 + E_o h \varepsilon_o^2]$$

Minimizing the elastic energy with respect to strain and curvature yields their values at equilibrium. In particular, using the notation

$$A = \frac{1}{(4E_i E_o h + E_m (E_i + E_o) h_m)} > 0$$

we obtain the equilibrium values for the strain

$$\varepsilon = A(2E_i E_o h(g_i + g_o) + E_m (E_i + E_o) h_m g_m)$$

and the curvature

$$c = \frac{2A}{h+h_m}(2E_iE_o h(g_o - g_i) + E_mE_o h_m(g_o - g_m) - E_mE_i h_m(g_i - g_m)).$$

This curvature is in general non-zero, the system curves with the abaxial side on the convex side as soon as the abaxial growth rate is sufficiently larger than the adaxial growth rate.

To determine whether buckling of the abaxial epidermis may occur, we compute the elastic (residual) strain in this layer at equilibrium:

$$\varepsilon_o = AE_mE_i h_m(2g_m - g_i - g_o).$$

ε_o can become compressive (<0) only if $2g_m - g_i - g_o < 0$. When it falls beyond a threshold,¹³⁴ this leads to buckling of the abaxial side, with patterns as described,¹³⁵ depending on the anisotropy of the residual strain.

Interestingly, the elastic strain of the adaxial epidermis,

$$\varepsilon_i = AE_mE_o h_m(2g_m - g_i - g_o)$$

always has the same sign as that of the abaxial epidermis. This means that either the two epidermal layers are in compression, or the two epidermal layers are in tension. A situation where one epidermis would be in tension and the other would be in compression does not correspond to mechanical equilibrium. Nevertheless, the amount of compression may differ between the two epidermal layers: the ratio of the elastic deformations is related to the ratio of Young's moduli

$$\frac{\varepsilon_o}{\varepsilon_i} = \frac{E_i}{E_o}.$$

Accordingly, it is possible that only one of the two epidermal layers reaches the buckling threshold. For instance, a much smaller Young's modulus for the outer epidermis compared to the inner epidermis would lead to buckling of the outer epidermis exclusively.

Finally, we mention that the elastic strain in the mesophyll has an opposite sign

$$\varepsilon_m = -2AE_iE_o h(2g_m - g_i - g_o).$$

This means that as soon as the epidermis is under compression, the mesophyll is under tension and vice versa.

Numerical model for a growing layered sepal

We again considered a growing elastic system comprising of three layers (inner epidermis (i), mesophyll (m), and outer epidermis (o)). To better represent the geometry of the transversal section of a sepal, we also considered two lateral semi-circles (the edges) to connect the inner and outer epidermis (Figures 2I–2K). These semi-circles are denoted “end”, while the region enclosed by the epidermal layers and the edges denote the mesophyll of the trilayer. This geometry is characterized by three parameters: the epidermal layer thickness h , the mesophyll thickness h_m and the width of the trilayer part L .

We adopt the framework of morpho-elasticity,¹³⁶ assuming that the residual stresses are solely created by a local growth deformation tensor F_g , and the observed deformation gradient can be decomposed multiplicatively on the elastic and the growth deformation tensors. In this framework we consider a quadratic energy density in the Green-Lagrange strain tensor E_e ,

$$W = \frac{1}{2}E_e : H : E_e,$$

where H , the elasticity tensor of rank four, represents the mechanical properties of the material. The proposed model assumes all regions of geometry to be isotropic materials. Therefore, the elasticity tensor field H , is characterized by independent parameters: the Young's moduli of the four regions (E_i , E_m , E_o and E_{end}), and the common Poisson ratio ν . An equation for the time evolution of the growth tensor F_g completes the model,

$$\begin{aligned} \frac{\partial F_g}{\partial t} &= GF_g, & t > 0 \\ F_g &= 1, & t = 0, \end{aligned}$$

with G the growth rate tensor. We consider here that the growth rate has both an imposed target (or specified) component G_T , as well as a component that stands for an influence of the elastic strain on growth:

$$G = G_T + CE_e,$$

with C being the extensibility (assumed to be scalar). We assume that the target growth is unidirectional along the trilayer, so that the target growth rates γ_i , γ_m , γ_o and γ_{end} for the inner epidermis, the mesophyll, the outer epidermis, and the edge respectively are the only independent parameters encoded in the target growth rate tensor G_T . As for the extensibility C , we have four additional parameters C_i , C_m , C_o and C_{end} corresponding to the four regions.

In comparison with the analytical model presented above, all geometrical and mechanical parameters are the same, while the growth parameters g_i, g_m , and g_o can be seen as the relative growth cumulated until time t , which can be approximated as when $C = 0$.

The model of the growing layered sepal was solved using the Finite Element Method and implemented with the FEniCS computing platform¹²² with its Python interface, with a special use of the BVPy Python library.¹²³

We simulated the deformation of the defined geometry when the outer epidermis is growing ($\gamma_o \neq 0$), while the other regions do not ($\gamma_i = \gamma_m = \gamma_{end} = 0$). This is one of the simplest situations, when the mean growth rate of the two epidermal layers is higher than the growth rate of the mesophyll, and according to the predictions of the analytical model the epidermal layers are under compression. This first condition for buckling being satisfied, we studied whether buckling indeed occurs depending on the moduli of the mesophyll and epidermal layers (Figure S3A). To validate the approach, we also compared the equilibrium curvature predicted by the analytical model to the mean midline curvature of the deformed sepal in the numerical model (Figures S3B and S3C).

For all our simulations we considered fixed values for the length $L = 10$, and thickness $2h + h_m = 5$ of the sepal, the target growth rate of the outer epidermis $\gamma_o = 0.1$, and the Poisson ratio $\nu = 0.49$. The value of the extensibility is chosen low for all regions but relatively higher for the mesophyll region, $C_o = C_i = C_{end} = 0.01$ and $C_m = 0.1$. The Young's modulus of the mesophyll is chosen as the lowest among all regions, $E_m = 1$, while the Young's modulus of the edge epidermis is a high value, $E_{end} = 10^6$. As for the Young moduli of the outer and inner epidermis, we performed an exploration of the parameter space defined by these two extreme values, $(E_o, E_i) \in [1, 10^6] \times [1, 10^6]$. We present the results of this analysis for different values of the epidermis thickness, $h \in \{0.1, 0.2, 0.3, 0.4\}$ (Figure S3).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed in R (version 4.2.2 (2022-10-31 ucrt)). The exact code used to perform the statistical analyses is embedded in the R scripts used to generate each plot. The R scripts are available at Github. (https://github.com/Avilash1993/Growth_directions_and_stiffness_across_cell_layers_determine_whether_tissues_stay_smooth_or_buckle) under GNU General Public License v3.0. Descriptions of the statistical analyses for each plot are provided in the corresponding figure legends.

Graph plotting

Graphs were generated in R (version 4.2.2 (2022-10-31 ucrt)) using ggplot, raw data for which is provided in Data S1.