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

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Abstract

From smooth to buckled, nature exhibits organs of various shapes and forms. How cellular growth patterns produce smooth organ shapes like those of leaves and sepals remains unclear. Here we show that unidirectional growth and comparable stiffness across both epidermal layers of Arabidopsis sepals are essential for smoothness. We identified a mutant with ectopic *ASYMMETRIC LEAVES 2* (*AS2*) expression that exhibits buckles on the outer epidermis due to conflicting growth directions and unequal epidermal stiffnesses. Aligning growth direction and increasing stiffness restores smoothness. Furthermore, buckling generates outgrowth by influencing auxin efflux transporter protein PIN-FORMED 1 polarity, indicating buckling can initiate organogenesis. 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.

One-Sentence Summary

The keys to unlocking sepal smoothness are unidirectional growth and even stiffness; misplace them, it's time to buckle up!

INTRODUCTION

During development, many organs such as the brain, intestine, and orchid flower petals form complex shapes with undulations, folds, and loops (*1-5*); conversely, other organs such as Arabidopsis leaves and fly wings develop smooth forms (*6-9*). These undulations, folds, and loops generally form through mechanical buckling (*10-12*), which is the bending of tissue when its growth patterns generate compressive forces (*13-16*). How cells within tissues regulate their growth and division to generate buckling in certain instances and maintain smoothness in others is not well understood.

Buckling plays a significant role in organ morphogenesis and development across organisms. In animals for instance, numerous studies demonstrate the necessity of buckling in morphogenesis (*2-4, 17-21*). Differential growth of connected tissue layers gives rise to buckling. Buckling creates complex organ shapes starting from simple geometries, which is important for functional specialization. These include gyrification of the cerebral cortex to increase neuron content (*2*), formation of gut villi and looping to increase contact with digested food and length of the gastrointestinal tract (*3, 4, 19, 20*), and initiation of new branches in lung airway epithelium to enhance gas exchange and oxygen transfer (*18*). Buckling also serves key roles in plant development. For instance, it generates helical twists in roots to enhance penetrative forces at the growing tip (*22-25*), triggers lobe deformation in Venus flytraps for rapid energy storage and release to capture prey (*26, 27*), and induces nanoscale cuticular wrinkles on petals to produce iridescence (*28-30*). Buckling has also been proposed to drive formation and positioning of primordia on the shoot apical meristem (*31, 32*).

Buckling, however, is not always beneficial. Inflorescence weight and external forces such as wind or rain can compress and buckle the stems of cereal crops, thereby causing damage and reduced yield (*33*). Similarly, slender tree stems are also vulnerable to buckling (*34, 35*). To counter this, plants need to allocate resources to increase stem thickness and stiffness (*36-38*). While buckling generates diverse leaf shapes (*5, 39*), majority of the leaves suppress buckling, generating smooth shapes that enhance light absorption and gas exchange for efficient photosynthesis (*40, 41*). Similarly, sepals, which are the outermost leaf-like floral organs, grow to smooth shapes to properly enclose and protect the developing flower bud (*42*). Therefore, plants need to modulate growth and tissue properties of leaves and sepals to determine whether they remain smooth or buckle during development. In this study, we used a gain-of-function mutant of the transcription factor *ASYMMETRIC LEAVES 2* (*AS2*), *as2-7D*, as a tool to determine how cell growth patterns are regulated to prevent buckling, and thus produce smooth sepals in the wild-type (WT).

RESULTS

Ectopic expression of *ASYMMETRIC LEAVES 2* (*AS2*) disrupts sepal smoothness

To determine how the sepals achieve smooth morphology, we performed a forward genetic screen (*43*), and identified an *Arabidopsis thaliana* mutant that disrupts sepal smoothness. This mutant exhibits an irregular morphology with folds and outgrowths (Fig. 1, A to F). The folds comprise ridges and invaginations, whereas the outgrowths, which are pointed epidermal projections, are typically located at the end of the folds (Fig. 1F and fig. S1, A and B). These folds and outgrowths are visible only on the outer (abaxial) epidermis, while the inner (adaxial) epidermis is smooth like the WT (Fig. 1, E to H, fig. S1, A and B and movie S1). Positional cloning identified a G-to-A point mutation 1,484 bps upstream of the translational start site of *ASYMMETRIC LEAVES 2* (*AS2*) (Fig. 1I). *AS2* encodes a LATERAL ORGAN BOUNDARIES (LOB) domain transcription factor that plays a critical role in leaf development, particularly in specifying the upper (adaxial) layer of the leaf (*44-49*). An identical mutation in the same locus was identified previously (*48*). However, the fold and outgrowth phenotype in the sepals was not reported (*48*), since the phenotype is very subtle in the Columbia (Col-0) background compared to what we observe in the Landsberg *erecta* (L*er*-0) background (hereafter referred to as WT) (Fig. 1, A to D and fig. S1, C to F). Previously, Wu et. al. (*48*) demonstrated that the mutation in the *AS2* promoter disrupts the binding site of the repressor KANADI, a transcription factor involved in specifying cell fate on the lower (abaxial) side of the leaf (*50-53*). They further showed that this mutation leads to ectopic *AS2* expression on the abaxial side, while normally in the WT, the *AS2* expression domain is restricted to the adaxial side (Fig.1I). To verify that this mutation also leads to ectopic *AS2* expression in sepals of the L*er*-0 background, we drove GFP expression under the WT and mutant *AS2* promoters (*pAS2::GUS-GFP*) in WT. While the *AS2* promoter from the WT drives GFP specifically on the inner sepal epidermal layer (Fig. 1, J and K), the mutant *AS2* promoter drives GFP on both the inner and outer epidermal layers (Fig. 1L). This confirms that the mutation causes a gain-of-function through ectopic *AS2* expression, consistent with the dominant nature of this allele (see Materials and methods). Therefore, following the convention of Wu et al. (*48, 54*), we named the mutant allele *as2-7D*.

To test whether ectopic *AS2* expression is sufficient to generate the folds and outgrowths, we ectopically expressed *AS2* on both inner and outer epidermis of the WT (*pML1::AS2*). All the transgenic plants form lumps and outgrowths on the sepal outer epidermis, similar to *as2-7D* (Fig. 1, M to P). Additionally, we observe folds and outgrowths form on the epidermis of the leaves and fruits (fig. S1, G to N), consistent with previous reports (*46*), indicating a potential stronger effect of constitutive epidermal *AS2* expression than in the *as2-7D* gain-of-function mutant. In summary, the mutation in *as2-7D* induces ectopic *AS2* expression on the outer epidermal layer, which leads to disruption of smooth sepal morphology by generating folds and outgrowths on the outer epidermal layer.

The *as2-7D* outer epidermis appears to buckle

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 (Fig. 2, A and B, fig. S2, A to D, and movie S2). Initially, both WT and *as2-7D* sepals are smooth (Fig. 2, A and B; 0-24h). While WT sepals remain smooth throughout, *as2-7D* starts to develop a fold in the middle of the sepal at 48 hours (Fig. 2, B and C). As the flower develops, folding intensifies further, forming several inward (invaginations) and outward (ridges) folds throughout the outer epidermis of *as2-7D* mutant (Fig. 2, B to D and movie S2). In addition to these folds, we also observe the development of phenotypically distinct pointed outgrowths. The outgrowths are not observed until the later stages when the folds intensify, suggesting they form downstream of folding (Fig. 2, B to D and fig. S2, C and D).

We considered two hypotheses for how the folds form: (1) due to localized growth underlying each ridge or (2) due to mechanical buckling caused by a surface-wide growth disruption. 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 every 48 hours over 96 hours (Fig. 2, E to H and fig. S2, E to N). We do not see any localized fast growth or cell division in the region where the first fold is formed in *as2-7D* at 48 hours (Fig. 2, C to F). In *as2-7D*, the cells grow at moderate rates throughout the epidermis (Fig. 2, E to F; 0-48 hrs). This pattern is also reflected in cell division rates, where we observe fast cell division throughout the sepal epidermis (Fig. 2, G and H; 0-48 hrs). This suggests that fold formation is a result of surface-wide disruption of cell growth patterns, and not because of localized cell growth and division. In the later time point (48-96 hrs) the growth is still relatively uniform along the places where the *as2-7D* buckles form; however, we do observe localized growth and division later at sites where outgrowths form (Fig. 2F and fig. S2, G and H; 48-96 hrs). We therefore considered the hypothesis that the folds on *as2-7D* outer epidermis form through mechanical buckling caused by surface-wide growth defects.

Analytical modeling predicts two criteria required for buckling

Based on our observations of *as2-7D* development, we built a simple two-dimensional (2D) analytical model to determine the conditions that can cause buckling (see Materials and methods). We consider a growing elastic system comprising three layers, corresponding to the inner epidermis, mesophyll, and outer epidermis of a sepal (Fig 2I). In the model, if the growth rate of the outer epidermis exceeds that of the inner epidermis, the tissue will curve (Fig. 2J). If the growth rate of the epidermal layers is sufficiently large compared to the mesophyll, then these two layers will be under compression, potentially causing buckling. If, in addition, the stiffness of the outer epidermis is less than the inner epidermis, the outer epidermis can, in theory, buckle, without any buckling of the inner epidermis (Fig. 2K). From the model and experimental observations, we predicted that (1) the growth of the outer epidermis of the *as2-7D*

mutant should exceed the inner epidermis and (2) the outer epidermis should be softer than the inner epidermis.

Synchrony of cell growth rate and directionality between epidermal layers is essential for the formation of smooth sepals

To test our model predictions, we live imaged WT and *as2-7D* sepals through pre-buckling stages (Fig. 3, A and B), and extracted the inner and outer epidermal layers of the same growing sepals by image processing (Fig. 3, C and D). We found that cells throughout the WT inner epidermis grow significantly faster than the outer epidermal cells (Fig. 3E and fig. S3, A, B, and E), overlapping with the cell division patterns (Fig. 3G and fig. S3, F, G, and J). This was surprising, as intuitively for flat sepal growth, we expected the cells on both epidermal layers to grow at similar rates. However, during the initial stages of sepal growth the sepal is highly curved in the longitudinal direction, due to the smaller surface area of the inner epidermis. This necessitates fast inner epidermal growth for achieving flatness (Fig. 3C and D, fig. S4F and movie S2). Likewise, in *as2-7D*, inner epidermal cells grow faster than outer epidermal cells; however, the inner epidermal cells of *as2-7D* do not grow as fast as the WT (Fig. 3, E and F and fig. S3, A to E). In concert with the growth rate, the cell division rate is also lower on the inner epidermis of *as2-7D* compared to the WT (Fig. 3, G and H and fig. S3, F to J). The fact that fast growth of the inner epidermis is disrupted in *as2-7D* suggests that this fast inner epidermal growth is an essential component of sepal smoothness.

Since buckling is a surface-wide phenomenon in our model, we tested the differences in whole inner versus outer epidermal growth rates. We found that the outer epidermal layers of the WT and *as2-7D* grow at the same rate, aligning with our cell growth rate data where the median epidermal cell growth rates show no significant difference (fig. S3E and fig. S4, A, B and E). However, the inner epidermis of *as2-7D* grows significantly slower than the WT (fig. S3E and fig. S4, C, D and E). Our findings, therefore, 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 (fig. S4F).

Since cell growth directions have been shown to be critical for determining organ shapes (*55-57*), we assessed the principal direction of maximal cell growth (PDG_{max}) in the WT and *as2-7D* mutant. We found that the WT cells on both epidermal layers show highly aligned growth along the sepal longitudinal axis (Fig. 3I). In *as2-7D*, although inner epidermal cells grow parallel to the sepal longitudinal axis, the outer epidermal cells exhibit highly misaligned growth relative to the longitudinal axis, with PDG_{max} tensors oriented transversely (Fig. 3J). We quantified the cell growth direction by the deviation angle of PDG_{max} tensors relative to the sepal's longitudinal axis (Fig. 3K). The heatmaps reveal minimal deviation in cell growth directions of the WT epidermal layers and the inner epidermal layer of *as2-7D* (Fig. 3, L and M and fig. S3, K to O). In contrast,

the outer layer of *as2-7D* displayed a high degree of deviation in growth directions (Fig. 3M and fig. S3, K to O). Since microtubules are known to align perpendicular to the maximal cell growth direction (*58*), we then tested the microtubule orientation pattern in comparable regions of the WT and *as2-7D* outer epidermis (Fig. 3, N and O). Microtubules show the typical transverse alignment pattern in WT, which is perpendicular to sepal longitudinal growth direction (Fig. 3N). Conversely, the microtubules are largely misoriented in the *as2-7D*, and in certain instances, mirror the transverse cell growth pattern (yellow arrowheads, Fig. 3O). Taken together, our data illustrates the complexity of biology beyond our simple 2D analytical model. Thus, we hypothesize that in the *as2-7D* mutant, buckling occurs on the outer epidermis because it overgrows in width while being constrained by the inner epidermis growing longitudinally. In theory, transverse growth in the outer epidermis favors longitudinal buckles (*59*), consistent with our observations. Overall, our data indicate that smooth sepal formation requires unidirectional growth on both epidermal layers, along with fast growth of the inner layer to achieve flatness.

Limiting transverse growth orientation and increasing stiffness of the outer epidermis rescues *as2-7D* phenotype, by preventing buckling

Having shown that *as2-7D* outer epidermal cells exhibit misaligned growth directions relative to the sepal longitudinal axis, we hypothesized that the transversal growth orientation of the outer epidermal cells causes buckling. Therefore, we tested whether prompting the cells to grow 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 trigger epidermal cells to skip division and undergo endoreduplication early during development, resulting in increased giant cell formation (*60, 61*). Giant cells, exclusive to the outer epidermis, exhibit the unique property of growing in a highly directional manner (*60*) along the longitudinal sepal axis (Fig. 4, A, C, E and G, fig. S5, A and C, and movie S3). Therefore, we wondered whether increasing giant cell formation can restore directional growth in *as2-7D* and consequently reduce buckling. Indeed, overexpressing *KRP1* in *as2-7D* (*pML1::KRP1 as2-7D*) results in a striking smoothing of the outer sepal epidermis (Fig. 4, A to H, fig. S5, A to D and movie S3). As expected, overexpressing KRP1 increases the number and size of the giant cells, which grow in a highly aligned manner along the longitudinal sepal direction (Fig. 4, E to P and movie S3). The PDG_{max} tensors of *pML1::KRP1 as2-7D* outer epidermal cells are similar to those of *pML1::KRP1*, wherein the transverse growth orientation exhibited by *as2-7D* is largely re-oriented longitudinally (Fig. 4, M to P and fig. S5, E to L). In addition to reduced buckling, the characteristic outgrowths of the *as2-7D* outer epidermis are also nearly absent in *pML1::KRP1 as2-7D* (Fig. 4, E to H), suggesting that outgrowths form as a result of buckling. Overall, consistent with our hypothesis, our findings show that misdirected growth of the outer epidermal cells is a key driver of buckling in *as2-7D*.

The second condition of our 2D analytical model states 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 (*62*) and compared the shrinkage of the WT and *as2-7D* epidermal layers. When exposed to a salt solution, cells undergo plasmolysis while the cell wall contracts (Fig. 4, Q and R). The extent of cell wall shrinkage gives a proxy measure for cell wall stiffness, with stiffness being inversely proportional to the wall shrinkage (*63*). We exposed early sepals to salt solution and measured the epidermal shrinkage as a proxy for epidermal stiffness (Fig. 4, S and T, fig. S5, M to P, and see Materials and methods). We found that the WT epidermal layers exhibit similar shrinkage, indicating comparable stiffness (Fig. 4, U and Y). In contrast, the outer epidermis of *as2-7D* shrank more than the inner epidermis, indicating that *as2-7D* outer epidermis is softer, which aligns with our model's prediction (Fig. 4, V and Y). We then wondered whether increasing giant cell formation affects the stiffness of the outer epidermis. To test this, we performed osmotic treatments on *ML1::KRP1* and *ML1::KRP1 as2-7D*. Indeed, we found increased stiffness of the outer epidermis in both *ML1::KRP1* and *ML1::KRP1 as2-7D* (Fig. 4, W to Y), suggesting that aligning growth along the longitudinal direction and increasing outer epidermal stiffness rescues the *as2-7D* phenotype. The osmotic treatment results are consistent with our initial hypothesis that reduced stiffness in the outer epidermis is another key factor in buckling. Taken together, our findings indicate that unidirectional growth and comparable stiffness across both epidermal layers are essential criteria for smooth sepal formation.

Buckling in early *as2-7D* sepal stages is primarily due to growth defects rather than abaxial-adaxial patterning disruption

It has long been established that the juxtaposition of inner (adaxial) and outer (abaxial) identity leads to the growth of the leaf blade (*50, 64, 65*). Ectopic boundary formation between these identities can result in abnormal growths on the leaf blade that disrupt the smooth leaf surface (*65-68*). An ectopic boundary can emerge when the inner identity-promoting transcription factors are mis-expressed on the outer side, creating a juxtaposition between inner and outer identity (*69*). Our evidence strongly supports our hypothesis that ectopic *AS2* expression disrupts growth patterns and tissue properties, leading to buckling. However, since *AS2* is known to promote inner epidermal identity (*44-46, 48, 70*), we tested the alternative hypothesis that *as2-7D* phenotype is caused by ectopic juxtaposition of inner and outer identity. We therefore looked at the expression pattern of *REVOLUTA* (*REV*) as an inner identity transcription factor (*53, 71, 72*), and *FILAMENTOUS FLOWER* (*FIL*) as an outer identity transcription factor (*73-75*). We observe no difference in *REV* and *FIL* expression patterns between WT and *as2-7D*, and particularly, no ectopic juxtaposition between inner and outer gene expression was formed in the *as2-7D* sepal surface (fig. S6, A to H). Since REV promotes adaxial identity in conjunction with AS2, we tested whether REV is necessary or sufficient for the *as2-7D* phenotype. Ectopic *AS2* expression in the *rev-6* mutant (*pML1::AS2* in *rev-6*) leads to folds and outgrowth formation

similar to *pML1::AS2* in WT (Fig. 1, M to P, fig. S1, G to N, S6, I to L and S7, A to I), indicating REV is not necessary for the phenotype. Conversely, ectopic *REV* expression in WT sepals is insufficient to generate buckles or outgrowths (fig. S7, J and K). Further, ectopic *REV* expression in *as2-7D* also does not enhance the phenotype (fig. S7, L and M). Overall, our investigations indicate no obvious inner-outer polarity disruption in *as2-7D*, which is consistent with our hypothesis that buckling in *as2-7D* is indeed attributable to growth defects. However, we cannot completely rule out the possibility that other polarity-associated genes are disrupted in *as2-7D*.

Buckling in *as2-7D* generates outgrowths via ectopic PIN convergence driving auxin maxima formation

In addition to the buckles, we observe the emergence of pointed outgrowths at the ends of the buckles in the later stages in *as2-7D* (Fig. 1F). While constitutive *AS2* overexpression has previously been reported to cause outgrowths (*46*), what local signal triggers the formation of these outgrowths at specific sites remains elusive. In previously characterized outgrowths, expression of auxin biosynthesis genes has been reported (*76*), suggesting auxin plays a role.

The polar localization of PIN-FORMED 1 (PIN1) protein, an auxin transporter, to the plasma membrane on a particular side of the cell determines the directionality of auxin flow. When PIN1 polarity from multiple neighboring cells converges to the same location, auxin accumulates at that site, creating an auxin maximum (*77*). In the shoot apical meristem (SAM), PIN1 converges to form auxin maxima causing the outgrowth of primordia (*77*). PIN1 polarity also responds to mechanical stress (*78*). Since buckling alters patterns of mechanical stress (*79, 80*), we hypothesized that bucking causes PIN1 convergence which subsequently leads to auxin accumulation and outgrowth formation in *as2-7D*. In the inflorescence and early-stage flowers, the *PIN1* expression pattern in *as2-7D* is similar to that of WT (Fig. 5, A and B). However, in stages where *as2-7D* exhibits both buckles and outgrowths, we observe PIN1 convergence specifically in the sites where the outgrowths are formed (Fig. 5, C and D, fig. S8, A and B). In contrast, no PIN convergence sites are observed in the middle of the WT sepals (Fig. 5, C and D, fig. S8, A and B). We also observe the expression of auxin-responsive promoter DR5 specifically where outgrowths are formed in *as2-7D* (fig. S8, C and D), which is consistent with the fact that *PIN1* convergence is essential for creating local auxin peaks (*81*).

We next tested whether PIN-mediated polar auxin transport is required for the formation of outgrowths. We treated growing *as2-7D* inflorescences with polar auxin transport inhibitor N-1-naphthylphthalamic acid (NPA) and compared the phenotype to mock-treated samples. While *as2-7D* live-imaged in the presence of NPA exhibited characteristic buckles, the formation of outgrowths was largely attenuated (Fig. 5, E and F, fig. S8, E to H, and movie S4). Buckling continues to occur since the characteristic growth patterns and misdirected sideways PDG_{max}

tensors leading to buckling are unaltered with NPA treatment (Fig. 5, G to J). However, the localized fast growth leading to outgrowth formation was largely reduced in the NPA-treated samples (Fig. 5, K and L). This indicates that polar auxin transport is critical for the formation of outgrowths, which are characterized by localized fast growth. Overall, our results are consistent with our hypothesis that mechanical buckling causes ectopic PIN1 convergence, generating local auxin maxima formation and fast growth, leading to outgrowth formation.

DISCUSSION

While buckling is critical for generating complex organ shapes required for specialized functional roles (*1-5, 10, 11*), buckling is not always advantageous, and needs to be circumvented to generate smooth organs like sepals (*82*). How cell growth patterns influence tissue growth, and thereby organ shape remains one of the major open questions in biology. Using Arabidopsis sepals as the model, our study demonstrates that unidirectional growth of both the outer and inner epidermis along the sepal longitudinal axis, and similar tissue stiffness between epidermal layers are key drivers of smooth sepal formation. Ectopic *AS2* expression on the outer epidermis of *as2-7D* creates an imbalance of growth and tissue stiffness across outer and inner epidermal layers, leading to outer epidermal buckling.

While our simple 2D analytical model directed us to the importance of analyzing growth and stiffness across epidermal layers, our experiments show that buckling of growing biological tissues is a complex three-dimensional phenomenon involving unequal growth directions and tissue stiffness. Interestingly, we find the growth rate and stiffness of the inner epidermis in *as2-7D* to be affected. Intuitively, one might expect that ectopic *AS2* expression on the outer epidermis would alter cell growth properties only on the outer epidermis of *as2-7D*. However, changes in inner epidermal growth properties in *as2-7D* could be attributed to the fact that the outer and inner epidermis are connected at the sepal margins. Altering growth patterns in the outer epidermis creates mechanical constraints on the inner epidermis, thereby affecting its growth indirectly. Overall, *as2-7D* buckles as the sideways growth of the outer epidermis is limited at the edges by the inner epidermis. This 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 epithelium (*83*)) relative to the tissue to which it is attached, drives organ shape.

AS2 has been studied primarily in the context of outer-inner polarity determination (*44-46, 48, 70, 84*). However, we demonstrate that the role of *AS2* extends beyond polarity specification. Given that ectopic *AS2* expression disrupts the coordination of growth patterns across epidermal layers, we propose that AS2 plays a central role as a growth coordinator. Growth coordination is a phenomenon that is barely understood across plants and animals (*83, 85*). We hereby define growth coordination as the exchange of positional, growth rate, and growth directional

information among cells across tissue layers for developing robust, functional organs. The precise mechanical and molecular mechanisms through which AS2 relays information across cell layers to coordinate growth across the organ, remain to be explored in future studies.

While how molecular cues influence tissue mechanics has been relatively well studied, how tissue mechanics inform molecular signals remain elusive. Our study shows that, not only does AS2 influence tissue mechanics generating buckles, but also that buckles feed back into molecular signals to influence organogenesis. We show that buckling influences the polarity of the PIN1-auxin efflux transporter. Our findings support the previous observations that mechanical perturbations affect PIN1 polarity (*78, 86, 87*). We further show that buckling is sufficient to converge PIN1 polarity causing the formation of an auxin maxima, and subsequent epidermal outgrowth. Thus, we find that buckling can initiate organogenesis. There is a long-standing debate as to whether primordial initiation in the shoot apical meristem (SAM) is influenced by buckling, a theory originally proposed by Paul Green (*31, 32, 88*). Contradicting evidence shows that PIN1 polarizes before primordia bumps are visible (*89*). While our evidence shows that buckling is sufficient to converge PIN1 and generate outgrowths, whether the same phenomenon occurs in the shoot apical meristem to generate primordia, requires further investigation.

ACKNOWLEDGEMENTS

We thank Isabella Burda, Frances Clark, Shuyao Kong, Byron Rusnak, Maura Zimmermann, Erich Schwarz, Phoebe Roeder, Scott D. Emr and Stéphane Verger for their valuable comments and suggestions on the manuscript. We thank Olivier Hamant for the *pPDF1::mCitrine-MBD* microtubule marker plasmid. We thank Soren Strauss for his guidance with image processing. We thank Mingyuan Zhu for his input on osmotic treatment experiments. We thank Trinh Duy Chi for his input on imaging adult sepals. Finally, we thank Arabidopsis Biological Resource Center for providing seed stocks for this work.

FUNDING

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number R01GM134037 to AHKR.
French National Research Agency (ANR, grant ANR-21-CE30-0039-01 GrowFlat, to AB).
National Natural Science Foundation of China grant no. 32270867 and Hundred-Talent Program of Zhejiang University to LH.
Cornell China Center and Zhejiang University for a joint seed fund grant to AHKR and LH.
IMB was supported by NSF REU Award (1850796).
The authors acknowledge the use of facilities and instrumentation supported by NSF through the Cornell University Materials Research Science and Engineering Center DMR-1719875. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or other funders.

AUTHOR CONTRIBUTIONS

Conceptualization: AHKR, ASY, LH. Experimentation: ASY, LH, PMK, XH, MH. Imaging: ASY, LH, MH, PMK. Image processing: ASY, LH, PMK, IMB, AMG, RSS. Data analysis: ASY, LH, AK. Analytical modeling: AK, AB. Funding acquisition: AHKR, AB, LH. Project administration: AHKR, AB. Supervision: AHKR, AB, LH. Writing – original draft: ASY, LH, AHKR. Writing – review & editing: ASY, LH, PMK, AK, XH, IMB, MH, AMG, RSS, AB and AHKR.

COMPETING INTERESTS

Authors declare that they have no competing interests.

DATA AND MATERIALS AVAILABILITY

The raw datasets used for making the graphs are provided in Supplementary Data S1. Primers used for this study are available in Table S1 in Supplementary materials. The meshes and images will be uploaded in Zenodo. The R codes used for generating the plots can be accessed from GitHub: https://github.com/Avilash1993/R-codes-for-the-paper-Yadav-et.-al.-2023-as2-7D

FIGURE TITLES AND LEGENDS

Fig. 1. Ectopic expression of *ASYMMETRIC LEAVES 2* (*AS2*) on the outer epidermal layer disrupts sepal smoothness in *as2-7D* mutant.

(**A** and **B**) Whole inflorescences of (**A**) WT (L*er*-0) and **B**) *as2-7D* mutant (in L*er*-0 background). Yellow arrowheads indicate outgrowths. Scalebar, 1 cm; representative images of *n* = 3 inflorescences for both genotypes.

(**C** and **D**) Mature flowers (stage 14 (*90*)) of (**C**) WT and (**D**) *as2-7D* mutant. 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 cm; 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 µm; 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-RCI2A*), a yellow fluorescent protein-based cell membrane marker (*91*). Scale bar, 100 µm; 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 1484 bps upstream of ATG in *as2-7D* (Top half). The *AS2* expression (red dots) domain in the WT and *as2-7D* sepals is 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**) Longitudinal section of stage 6 flowers of WT. Cell walls are stained with propidium-iodide (PI, greyscale). Images show the GFP expression pattern (green) driven by (**K**) WT *AS2* promoter ($pAS2^{WT}$) and (**L**) *AS2* promoter from *as2-7D* ($pAS2^{as2\text{-}7D}$). Arrows indicate the outer and inner epidermal layers of the outer sepal. Scale bar = 20 µm; *n* = 3.

(**M**) Whole inflorescence of a representative transgenic (T1) harboring *pML1::AS2* (*AS2* expression driven by *ATML1* epidermal specific 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** to **P**) Confocal images of PI-stained (greyscale) 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 µm; *n* = 3 inflorescences per genotype.

Fig. 2. The *as2-7D* sepal outer epidermal morphology transitions from smooth to buckles and subsequently outgrowths.

(**A** and **B**) Representative live-imaging series of (**A**) a WT flower and (**B**) an *as2-7D* mutant flower starting from developmental stage 4 over a course of 96 hours, imaged at 24-hour intervals (n = 3). Images capture morphological changes of the outer sepal. Both genotypes harbor the pLH13 (*35S::mCitrine-RCI2A*) cell membrane marker (greyscale). Trichomes were removed from the outer sepal epidermis by image processing to better visualization the sepal morphology. Scale bar, 50 µm.

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

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

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

(**G** and **H**) Cell division rate over a 48-hour period for the (**G**) WT and (**H**) *as2-7D*. Cell division rate is calculated based on the number of daughter cells per lineage, represented as a heatmap projected onto the later time point. Red to blue gradient shows more to less division. The cell lineages were tracked using MorphoGraphX software (*92*). 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 µm.

(**I** to **K**) Visual representation of the analytical model (see Materials and methods) used to determine buckling criteria. (**I**) A growing elastic system composed of three layers, corresponding to the outer epidermis (denoted o), mesophyll (m), and inner epidermis (i) of a sepal. Letters E, g and h represent Elastic modulus, growth rate, and thickness respectively. Diagrams represent growth conditions causing (**J**) the tissue to curve and (**K**) buckling of the outer epidermis.

Fig. 3. Unidirectional growth on both epidermal layers is essential for smooth sepal development.

(**A** and **B**) Representative confocal images of the (**A**) WT and (**B**) *as2-7D* mutant live-imaged with a high laser intensity (~16-20%) every 24 hours. Both the genotypes harbor pLH13 (*35S::mCitrine-RCI2A*) cell membrane marker (green). Scale bar, 20 µm. One of the three representative replicates (n = 3) is shown.

(**C** and **D**) Segmented cells in grey superimposed onto the plasma membrane signal (also in grey) projected on mesh surfaces generated using MorphoGraphX (*92*). Mesh surfaces for 0 and 48-hour 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-hour time point. Scale bar, 20 µm; n = 3.

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

(**G** and **H**) Cell division rates over a 48-hour 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. Red to blue gradient shows more to less/no cell division respectively. Scale bar, 20 µm; 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 (**K**) WT and (**L**) *as2-7D*. Segmented cells in grey are superimposed on the plasma membrane signal (also in grey). 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 µm; 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 90º (highly deviated, red) to 0º (no deviation, blue). Scale bar, 20 µm; n = 3.

(**N** and **O**) Cortical microtubule orientation (greyscale) from comparable regions of stage 6 flower outer epidermis for the (**I**) WT and (**J**) *as2-7D* mutant is shown. These plants harbor *pPDF1::mCitrine-MBD*, a YFP based microtubule marker (*93*). Images show MorphoGraphX generated meshes from PI-stained images, onto which the microtubule signal has been projected. Segmented cells are outlined in cyan. White arrows point towards the longitudinal axis of the sepals. Microtubules in the WT are oriented perpendicular to the sepal longitudinal axis, whereas, in *as2-7D*, the microtubules are misoriented. Yellow arrowheads point towards the misoriented microtubules. Scale bar, 10 µm; n = 3.

Fig. 4. Aligning growth along the longitudinal direction and increasing outer epidermal stiffness is sufficient to rescue buckling in *as2-7D*.

(**A** to **D**) Representative stage 14 flowers of (**A**) WT, (**B**) *as2-7D* mutant, (**C**) *AtML1::KRP1*, and (**D**) *AtML1::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 *AtML1::KRP1 as2-7D* compared to *as2-7D*. Scale bar, 1 cm; n = 3 flowers for all genotypes.

(**E** to **H**) Confocal images of the outer epidermis of (**E**) WT, (**F**) *as2-7D* mutant, (**G**) *AtML1::KRP1*, and **(H)** *AtML1::KRP1 as2-7D* stage 14 sepals. The sepals were stained with PI (grey, cell walls). Note the increased number of highly elongated giant cells in *AtML1::KRP1* and *AtML1::KRP1 as2-7D* sepal, and the smooth outer epidermis of *AtML1::KRP1 as2-7D* sepal compared to the highly rugose morphology of *as2-7D*. Scale bar, 200 µm; n = 3.

(**I** to **L**) Magnified insets of the (**I**) WT, (**J**) *as2-7D* mutant, (**K**) *AtML1::KRP1*, and (**L**) *AtML1::KRP1 as2-7D* sepal for the regions marked by white boxes in panels E-H respectively. Asterisks denote a typical giant cell. Scale bar, 200 µm; n = 3.

(**M** to **P**) The principal direction of growth (PDG_{max}) for the outer epidermal cells of the **(M)** WT, **(N)** *as2-7D*, (**O**) *AtML1::KRP1*, and (**P**) *AtML1::KRP1 as2-7D* sepals. Yellow lines mark the PDG_{max} tensors calculated over a 48-hour 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 grey are superimposed onto the plasma membrane signal (also in grey). Scale bar, 20 µm; n = 3.

(**Q** and **R**) Schematic diagrams demonstrating the concept of cell wall stiffness assessment by osmotic treatment assays (*62*). (**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 using 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** to **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**) *AtML1::KRP1* and (**X**) *AtML1::KRP1 as2-7D*, post osmotic treatment in 0.35M 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 µm. 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), *AtML1::KRP1* (grey dots), and *AtML1::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.

Fig. 5. Mechanical buckling drives ectopic PIN convergence creating auxin maxima, causing fast local growth and subsequent outgrowth formation.

(**A** to **D**) Confocal stack images showing the expression pattern of auxin efflux carrier protein PIN-FORMED 1 (PIN1) in the WT and *as2-7D*. (**A** and **B**) Expression pattern of PIN1 protein reporter (*pPIN1::PIN1-GFP* (*94*)) in the inflorescence meristem of (**A**) the WT, (**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 towards the insipient primordia and growing sepal edges, as described previously (*94*). Scalebar, 50 µm. (**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 panels A to D, the images are representative of three *pPIN1::PIN1-GFP* and *pPIN1::PIN1-GFP as2-7D* plants (n = 3). PI is shown in greyscale and GFP in green. Trichomes were removed from the outer sepal epidermis by image processing. Scale bar, 50 µm.

(**E** to **F**) Representative live-imaging series of (**E**) Mock treated *as2-7D* and (**F**) 10µM NPA treated *as2-7D* developing flowers over 96 hours, imaged at every 24-hour interval (representative of n = 3 flowers). Note the development of outgrowths from 72-hour time-point onwards (yellow arrowheads) in the mock-treated *as2-7D*, which are absent in NPA treated *as2-7D* flowers. Plants harbor pLH13 (*35S::mCitrine-RCI2A*) cell membrane marker (greyscale). Scale bar, 50 µm.

(**G** to **H**) The PDG_{max} tensors (yellow) of the cells on the outer epidermis of (**G**) mock-treated and (**H**) 10µM NPA treated *as2-7D* over the first 48-hour growth period for the representative samples shown in panels E to 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 µm.

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

MATERIALS AND METHODS

Plant Materials

Arabidopsis thaliana genotypes used in this study are in Landsberg *erecta* (L*er*) background, which is referred to as the wild type (WT), unless otherwise mentioned. The mutants *rev-6* (*71*) and *as2-5D* mutant (in Col-0 background) (*48*) have been previously described, and were obtained from Arabidopsis Biological Resource Center (ABRC). The *as2-7D* mutant in the L*er*-0 background was identified from ethyl methanesulfonate (EMS) based forward genetic screen. Briefly, M2 seeds (second generation) obtained from self-fertilized EMS mutagenized L*er* plants were purchased from Lehle Seeds (*95*). M2 plants were screened under a stereo microscope for sepal defects (*60, 96*). 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 (*97*). 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 L*er* plants twice 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 WT product or a 111-bp mutant product. Primers are listed in Supplementary 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 (22ºC), relative humidity (65%) and fluorescence intensity (~100 µmol $m^{-1}s^{-1}$) were maintained.

For soil-based experiments, plants were grown on soil premixed with Vermaculite (LM111). For stratification, the trays were placed in a 4 ºC 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 ºC before being moved to the growth chamber.

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 shown in Fig. 1I and Fig. 4 Q to T were hand-drawn using the Notability application in Apple iPad (model A1701).

Genetic crosses

Crosses were performed as described previously (*98*). The *pATML1::KRP1* in L*er* background (*60, 61*) were crossed into *as2-7D* mutants. Segregation analysis and confocal microscopy was used to find *pATML1::KRP1 as2-7D* plants. The *pPIN1::PIN1-GFP DR5::VENUS* (*94*) plants and *pPIN1::PIN1-GFP pin1-4* (*94*) 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 (*pATML1::RCI2A-mCtrine*, basta resistance) plasma membrane markers have been described previously (*60, 99*). The *pPDF1::mCitrine-MBD* microtubule marker (*93*) 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 *pATML1* containing gateway vector pAR176 (Basta resistance (*60*)) 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 pCR™8TOPO™ vector (Invitrogen™) by TA cloning (*100*). 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 L*er*-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 (*101*). For cloning *pAS2*$^{as2\text{-}7D}$*::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::GFP-GUS.*

For *proREV::REV-GFP*, the 7.75 kb *proREV::REV* fragment (amplified from L*er*-0) and 1.08 kb mGFP-T35S fragment amplified from *pML1::H2B-mGFP* (pAR180 (*60*)) 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 L*er*-0. This fragment was assembled with a 2.19 kb *omega-mCherry-RCI2A-T35S* fragment amplified from *p35S::mCherry-RCI2A* (pMZ11 (*102*)) into the SacI and SpeI site of pMOA34 destination vector (hygromycin plant resistance, (*103*)). All the intermediate and final constructs were verified for sequence and directionality by sequencing. Primers are listed in Supplementary 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 (*104*). 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 (*105*). Transformants were selected either on ½ MS media plates (for Hygromycin and Kanamycin selection) or on soil (for Basta selection). For selecting pLH13 in WT and *as2-7D*, *proFIL::mCherry-RCI2A* in WT and *as2-7D* T1s, a final concentration of 25 -30 µg/mL Hygromycin was used. For selecting *proREV::REV-mGFP* in WT 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 WT and in *rev-6*, *pML1::REV* in WT and in *as2-7D*, *pAS2*^{WT}*::GFP-GUS* and *pAS2*$^{as2\text{-}7D}$*::GFP-GUS* in WT, *pPDF1::mCitrine-MBD* in WT and in *as2-7D* T1s, BASTA selection was performed on soil grown plants. Seedlings aged 1 to 2 weeks were selected with 100 µg/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 (*90*). 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 (*42*).

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 meristem is denoted as the outer sepal, whereas the sepal that is diametrically opposite to the outer sepal and facing towards the 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 Roeder et. al (*42*).

Scanning electron microscopy (SEM)

Scanning electron microscopy was performed as described previously (*60*). 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.

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 WT, *as2-7D*, *pML1::KRP1*, and *pML1::KRP1as2-7D* T2s) were excised. Dissection was performed as described previously (*102*, *106*), 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^{TM} 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. 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 µm. For high laser intensity based live imaging of pLH13 in WT 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 µm. 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 ½ MS live imaging media plates containing 10 µM NPA. The other group, serving as the control, was placed in media plates containing an equivalent amount of DMSO (20 µL 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 µL liquid solution, applied directly above the inflorescence tissue by pipetting. For the 10 µM NPA treatment samples, the liquid solution comprised of 1 µL of 1M NPA+19 µL DMSO+10 µL Silwet in 100 ml water, whereas the control (mock) samples were treated with 20 µL DMSO+10 µL 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 µM NPA treated samples. Live imaging was performed as described above, with the samples being moved to fresh mock or 10 µM 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 µm.

Propidium Iodide (PI) cell wall staining and imaging

A 5 mg/ml stock solution was prepared for PI staining, with 5 mgs of PI (Sigma) solubilized in 1 mL of MilliQ water. The stock solution was stored in a foil-covered 1.7 ml microcentrifuge tube and refrigerated at 4ºC. 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, and stage 14 sepals were stained for 12 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 WT, *as2-7D* and the transgenics (T1s) of *pML1::AS2* in WT, *pML1::REV* in WT and *pML1::REV* in *as2-7D*, the following settings were used: excitation 514 nm (laser intensity -18-22%), emission 566-650 nm.

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* $^{as2\text{-}7D}$*::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 µm. For *proREV::REV-GFP*, Chlorophyll and GFP were excited with 488 nm laser (intensity- 40%), emission range was collected between 647-722 nm for Cholorophyll and 493-550 nm for GFP. Z-step, 0.240 µm (inflorescences), 0.4 µm (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 µm. For *pPIN1::PIN1-GFP*, 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 µm. For *pDR5::VENUS*, samples were excited with an argon laser (514 nm), and data were collected in the YFP (520–565 nm) channelFor *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 µm.

Imaging adult sepals

For imaging fully mature sepals, all flowers were dissected off the inflorescence except for a single stage 14 flowers. All the internal floral organs from the stage 14 flower were carefully dissected off, leaving just the outer sepal (*42*) 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 WT 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 WT, *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 (*pATML1::RCI2A-mCtrine*) 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.

Osmotic treatments for measuring epidermal stiffness

Osmotic treatments were performed as described previously (*43, 62*), with modifications. In brief, primarily inflorescences from pLH13 in WT, 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 in 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 (*107*). For multichannel images, images were split based on channels, which were saved individually. The MorphoGraphX (MGX) (*92*) 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. Colormaps 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. In addition, 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 Zhu et. al. and Hong et. al. (*43, 108*), with modifications. The following process was used to generate the surfaces for analyzing cell growth parameters on the outer epidermis of WT, *as2-7D*, *pML1::KRP1*, *pML1::KRP1 as2-7D*, *as2-7D* mock and 10 µM 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 Sets (*109*) 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 µm, followed by smoothing once (Smooth Mesh, passes = 10) and subdividing twice. The membrane signal was projected onto the 2.5D surface with a min. and max. distance range of 1 to 8 µm from the image.

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

To obtain the inner epidermal surface, clipping plane 1 (View Tab/Clip 1), set to a width of 10 to 15 µm, 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 (µm) = 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 µm, followed by smoothing once (passes = 10) and subdividing twice. Plasma membrane signal from the inner surface was projected onto the mesh surface with a min. and max. distance range of 3 to 8 µm.

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 (µm) = 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 µm distance range from the image.

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 (*110*). 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 assumed 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.

Analytical model for buckling

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) rates and have different elastic moduli. To simplify the formulae obtained here, we assume that the epidermal layers have similar thicknesses. We assume that the Poisson ratios of all layers are equal (value ν). Finally, we neglect the bending modulus of each layer in view of their small thickness. These three assumptions do not affect the qualitative behavior of the system.

Formulation. Let E_i, E_m, and E_o be the values of elastic modulus, h,h_m, and h the values of thickness. g_i,g_m, and g_o are the values of strain associated with target 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

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

Results. 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

$$c = \frac{2A}{h+h_m}\left(2E_i E_o h(g_o - g_i) + E_m E_o h_m(g_o - g_m) - E_m E_i h_m(g_i - g_m)\right).$$

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:

$$\varepsilon_o = A E_m E_i h_m (2g_m - g_i - g_o).$$

ε_o can become compressive (<0) only if $2g_m - g_i - g_o < 0$. When it falls beyond a threshold (*111*), this leads to buckling of the abaxial side, with patterns as described (*59*), depending on the anisotropy of the residual strain.

Interestingly, the elastic strain of the adaxial epidermis,

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

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

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

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

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.

SUPPLEMENTARY FIGURE TITLES AND LEGENDS

Fig. S1. *AS2* overexpression causes characteristic ridges, invaginations, and outgrowths on the outer epidermis, associated with Main Fig. 1.

(**A** and **B**) Representative optical cross-sections of Stage 11 flowers of (**A**) the WT and (**B**) *as2-7D* mutant. Images are representative of T2s harboring pAR169 (*pATML1::RCI2A-mCitrine*), an epidermal layer specific plasma membrane marker. Green and grey represent epidermal cells and chlorophyll containing mesophyll cells respectively. WT sepals (A) have a smooth shape, while the *as2-7D* sepal (B) outer epidermis exhibit outward protrusions (ridges), inward indents (invaginations) and pointed epidermal extensions (outgrowths). Note that the outgrowths lack mesophyll cells (grey) underneath whereas mesophylls occupy the space beneath ridges and invaginations.

(**C** and **D**) Whole inflorescence images of (**C**) Col-0 (control) and (**D**) *as2-5D* (in Col-0, isolated by Wu et. al (*48*)). Images are representative of $n = 3$ inflorescences. Scalebar, 1 cm.

(**E** and **F**) Stage 14 flowers of (**E**) Col-0 and (**F**) *as2-5D* mutant, representative of $n = 3$ flowers. Yellow arrowhead points toward an outgrowth. Note the mild nature of outgrowth formation in *as2-5D* mutant as compared to *as2-7D* (Fig. 1). Scalebar, 1 cm.

(**G** to **J**) Representative six-week-old adult plants of (**G**) WT, (**H**) *as2-7D* and (**I-J**) *pML1::AS2* in WT T1s. The transgenics show strong (17/28 plants, panel I) to extreme (9/28 plants, panel J) developmental defects, categorized qualitatively by the extent of leaf deformity. The transgenics exhibit outgrowths on the lower (abaxial) surface of the leaves as well, in addition to severely stunted growth. Scalebar, 1 cm.

(**K** to **N**) Fully formed siliques of (**K**) WT, (**L**) *as2-7D*, (**M-N**) *pML1::AS2* in WT T1s. Note the outgrowths on the silique epidermis. In panels M and N, siliques are representative of strong and extreme categories, described for panels I and J respectively. Three siliques on each panel correspond to three independent plants. Total number of independent transgenics (T1s) assessed $n = 28$.

Fig. S2. The outer epidermis of *as2-7D* exhibits loss of stereotypical growth patterns, associated with Main Fig. 2.

(**A** to **D**) Confocal stack images of (**A**) WT replicate 2, (**B**) WT replicate 3, (**C**) *as2-7D* replicate 2 and (**D**) *as2-7D* replicate 3 developing flowers, transformed with the pLH13 (*35S::mCitrine-RCI2A*) membrane marker. Images correspond to the 48-hour (left) and 96-hour (right) time points from WT and *as2-7D* live imaging experiment (n = 3). Yellow arrowheads show outgrowths. Trichomes were removed from the outer sepal epidermis by image processing. Scalebar, 50 µm.

(**E** to **H**) Cell area growth rates of (**E**) WT replicate 2, (**F**) WT replicate 3, (**G**) *as2-7D* replicate 2 and (**H**) *as2-7D* replicate 3. The heatmap is projected onto the later time point. Red to blue gradient shows fast to slow growth. Yellow arrowheads point towards the outgrowth which exhibit localized fast growth. Scalebar, 50 µm.

(**I** to **L**) The cell division rates of (**I**) WT replicate 2, (**J**) WT replicate 3, (**K**) *as2-7D* replicate 2 and (**L**) *as2-7D* replicate 3. Red to blue gradient shows more to less division. Cell division over 0-48 hours is shown on the left and over 48-96 hours on the right. Scalebar, 50 µm.

(**M** and **N**) Graphical representation of (**M**) cell growth rates and (**N**) cell division rates across all three independent biological replicates of the WT (black dots) and *as2-7D* mutant (red dots). Dot represents the growth rate (M) and division rate (N) of a given cell lineage. Dot plots are superimposed with box plots, where the central line indicates the median. Left and right panels display data for 0-48 and 48-96 hours, respectively. Note the reduced spatial variability in the growth rates of *as2-7D* (panel M). Lines above data points indicate statistical comparisons. The *p-values* for Bonferroni corrected pairwise t-test (two-tailed) are shown. *p-values*: ***<0.0001, **<0.001, *<0.05, ns = non-significant. Exact *p-values* for growth rates: WT vs *as2-7D* for 0-48 hours, 0.9884; WT vs *as2-7D* for 48-96 hours, 1.565172×10^{-93}. Exact *p-values* for cell division rates: WT vs *as2-7D* for 0-48 hours, 7.800489×10^{-05}; WT vs *as2-7D* for 48 - 96 hours, 9.47882×10^{-07}.

Fig. S3. Slower growth on the inner epidermis together with misdirected outer epidermal growth trigger buckling in *as2-7D*, associated with Main Fig. 3.

(**A** to **D**) Growth rate heatmaps for (**A**) WT replicate 2, (**B**) WT replicate 3, (**C**) *as2-7D* replicate 2 and (**D**) *as2-7D* replicate 3 developing sepals. Red to blue heatmap gradient shows fast to slow growth. Outer (left) and inner (right) epidermal growth rates over 48 hours is projected as a heatmap onto the later time point. Scalebar, 20 µm.

(**E**) Graph shows cell growth rates over a 48-hour period for the WT (black) and *as2-7D* (red) mutant (n = 3 biological replicates). Each dot represents the growth rate for a given cell lineage. Dot plots are overlayed with boxplots, where the central line represents the median. The *p-values* for Bonferroni corrected pairwise t-test (two-tailed) are shown. *p-values*: ***<0.0001, **<0.001, *<0.05, ns = non-significant. Exact *p*-values for epidermal cell growth rate: WT outer vs inner, 5.751359×10^{-35}; *as2-7D* outer vs inner, 3.039502×10^{-25}; WT vs *as2-7D* outer, 0.1011; WT vs *as2-7D* inner, 8.496122×10^{-16}.

(**F** to **I**) Cell division heatmaps for (**F**) WT replicate 2, (**G**) WT replicate 3, (**H**) *as2-7D* replicate 2 and **(I)** *as2-7D* replicate 3. Red to blue color gradient shows more to less cell division. The data is presented in the same format as described for panels A-D. Scalebar, 20 µm.

(**J**) Graph shows cell division rates for the WT and *as2-7D* mutant (n = 3 biological replicates) Dots represent the number of cell divisions for each cell lineage. Data presentation and statistical comparisons are same as described above for panel E. Exact *p*-values for epidermal cell division rates: WT outer vs inner, 4.08502×10^{-28}; *as2-7D* outer vs inner, 0.004; WT vs *as2-7D* outer, 3.677026×10^{-23}; WT vs *as2-7D* inner, 1.865456×10^{-06}.

(**K** to **N**) Heatmap representing the angle of deviation of PDG_{max} tensors from the sepal longitudinal axis for (**K**) WT replicate 2, (**L**) WT replicate 3, (**M**) *as2-7D* replicate 2 and (**N**) *as2-7D* replicate 3. Scalebar represents a deviation of 90º in red and 0º (no deviation) in blue. Scalebar, 20 µm.

(**O**) Graph showing the angle of deviation for the WT and *as2-7D* mutant (n = 3 biological replicates). Each dot represents the angle of deviation for a given cell lineage. Data representation and statistical comparisons are shown in the same format as described for panels E and J. Exact *p-values* for epidermal angle of deviation: WT outer vs inner, 3.793354×10^{-05}; *as2-7D* outer vs inner, 1.572509×10^{-20}; WT vs *as2-7D* outer, 8.052118×10^{-10}; WT vs *as2-7D* inner, 0.349.

Fig. S4. Slower growth on the inner epidermis leads to overgrowth of the outer epidermis in *as2-7D*, associated with Main Fig. 3.

(**A** to **D**) Heatmaps represent growth rates of (**A**) WT outer, (**B**) *as2-7D* outer, (**C**) WT inner, and (**D**) *as2-7D* inner epidermal surface at every 24-hour interval, over a 48-hour live imaging period. Growth rate is projected onto the later time point. Growth rate for the region demarcated in cyan at 0-hour time point was tracked over time. Heatmap: red, fast growth, and blue, slow growth. Representative heatmaps for $n = 3$ biological replicates are shown. Scalebar, 20 µm.

(**E**) Graph shows epidermal surface growth rates every 24 hours for the WT (black) and *as2-7D* (red) is shown ($n = 3$). Lowercase letters on top of the data points represent significant differences based on one-way ANOVA with Tukey's HSD post-hoc test ($p < 0.05$, similar letters represent no significant difference).

(**F**) Graph shows the ratio of the outer vs inner epidermal surface area of WT (black) and *as2-7D* (red) for each time point ($n = 3$). Note that the outer vs inner ratio of the WT decreases sharply over time compared to *as2-7D*. Error bars indicate standard error.

Fig. S5. Analysis of cell growth direction and stiffness due to *KRP1* overexpression in WT and *as2-7D*, associated with Main Fig. 4.

(**A** to **D**) Confocal images of the inner epidermis of (**A**) WT, (**B**) *as2-7D* mutant, (**C**) *AtML1::KRP1* and (**D**) *AtML1::KRP1 as2-7D* stage 14 sepals. The sepals were stained with PI. Note the smooth morphology of the inner epidermis across all genotypes. Scalebar, 200 µm, images representative of $n = 3$ adult sepals.

(**E** to **L**) Principal direction of growth (PDG_{max}) for the outer epidermal cells of (**E**) WT Replicate 2, (**F**) *as2-7D* mutant Replicate 2, (**G**) *AtML1::KRP1* Replicate 2, (**H**) *AtML1::KRP1 as2-7D* Replicate 2, (**I**) WT Replicate 3, (**J**) *as2-7D* mutant Replicate 3, (**K**) *AtML1::KRP1* Replicate 3 and (**L**) *AtML1::KRP1 as2-7D* Replicate 3 sepals. PDG_{max} tensors (yellow) calculated over a 48-hour live imaging period starting from stage 4, projected on a later time point surface. Segmented cells in grey are superimposed onto the plasma membrane signal (also in grey). Scalebar, 20 µm, $n = 3$.

(**M** and **N**) Representative confocal images of WT ($n = 5$) early stage 6 flower (**M**) before and (**N**) after osmotic treatment in 0.35M NaCl. The PI (cell wall stain) and membrane marker (pLH13, *35S::mCitrine-RCI2A*) signals have been merged together and shown. The highlighted region of the outer sepal (white rectangle) indicates the comparable longitudinal section extracted for analyzing epidermal shrinkage.

(**O** and **P**) MorphoGraphX generated surface of the longitudinal section (**O**) before treatment and (**P**) after osmotic treatment, extracted from flower images shown in panels M and N respectively. Comparable regions of the outer and inner sepal epidermis were segmented (outlined in cyan) to calculate the percentage areal shrinkage.

Fig. S6. Buckling in *as2-7D* is not caused due to disruption of outer (abaxial) – inner (adaxial) polarity.

(**A** to **D**) REVOLUTA (REV) protein reporter (*proREV::REV-mGFP*) shows nuclear localization of REV, restricted exclusively to the inner epidermis of WT and *as2-7D* sepals. (**A** and **B**) Confocal images of inflorescence meristems show adaxial specific REV expression (yellow arrowheads) in the young sepals of (**A**) WT and (**B**) *as2-7D* mutant. (**C** and **D**) Representative middle stage (Stage 8-9) flowers of (**C**) WT and (**D**) *as2-7D* show no detectable REV expression 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 greyscale. Scalebar, 50 µm.

(**E** to **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 greyscale. (**G** and **H**) Representative middle stage (Stage 7-8) 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). Scalebar, 50 µm.

(**I** to **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 (Fig. 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.

Fig. S7. Ectopic *AS2* expression mediated buckles and subsequent outgrowth formation occur independent of *REVOLUTA*.

(**A**) Representation of the previously characterized meristem initiation defect phenotype of *rev-6* mutant. Images show an excised lateral inflorescence meristem of the WT (left) and *rev-6* mutant (right). Majority of the cauline leaves axils (white arrows) in the *rev-6* mutant are bare, as they fail to produce axillary meristem, as described by Otsuga et. al. (*71*). Scalebar, 1cm.

(**B** to **D**) Fully developed siliques of (**B**) *rev-6* and (**C** and **D**) *pML1::AS2* in rev-6 T1s. No of independent T1s assessed n = 19. The three siliques correspond to three independent plants. The numbers represent the total number of plants showing strong (12/19 plants, panel C) to extreme (7/19 plants, panel D) phenotypes, categorized based on the extent of leaf deformity in the adult vegetative stage.

(**E** to **I**) Representative six-week-old adult plants of (**E**) WT, (**F**) *rev-6*, (**G**) *as2-7D* and (**H** and **I**) *pML1::AS2* in *rev-6* T1s. Total number of T1s assessed n = 19. The transgenics show strong (12/19 plants, panel H) to extreme (7/19 plants, panel I) defects in leaf development, characterized by outgrowth formation on the abaxial epidermis and stunted growth. Scalebar, 1 cm.

(**J** to **M**) Confocal images of PI-stained (greyscale) inflorescences of (**J**) WT, (**K**) representative *pML1::REV* in WT T1, (**L**) *as2-7D* mutant, and (**M**) representative *pML1::REV* in *as2-7D* T1. Note that the transgenics ectopically expressing *REV* looks like their respective backgrounds in either the WT (panels J and K) or *as2-7D* (panels L and M). Yellow arrowheads point to the outgrowths. For each *pML1::REV* transgenics in WT and in *as2-7D* backgrounds, number of independent T1s assessed, n = 28. Scalebar, 50 µm.

Fig. S8. Outgrowths on *as2-7D* outer epidermis are formed due to buckling mediated PIN1 convergence and subsequent auxin maxima formation, associated with Main Fig. 5.

(**A** and **B**) Confocal images show the expression pattern of PIN1 protein reporter (*pPIN1::PIN1-GFP*) in **(A)** WT and (**B**) *as2-7D* inflorescence meristems. Note the expression of *PIN1* directed towards the emerging outgrowths on the outer epidermis of *as2-7D* older flowers. Scalebar, 50 µm, $n = 3$.

(**C** and **D**) Expression pattern of auxin response reporter (*pDR5::VENUS*) in stage 11 flower of *as2-7D* mutant. Shown is (**C**) VENUS signal by itself (green) and (**D**) VENUS signal merged with PI (grey). Note the presence of DR5 signal specifically in the outgrowths (yellow arrowheads). Scalebar, 100 µm, $n = 3$.

(**E** to **H**) Representative live-imaging series showing the second replicate of (**E**) Mock treated, (**F**) 10µM NPA treated developing flowers, and third replicate of (**G**) Mock treated, (**H**) 10µM NPA treated developing flowers of *as2-7D* mutant. Images taken at every 24-hour interval over 96-hour period. Note the development of outgrowths from 72-hour time-point onwards (yellow arrowheads) in the mock treated *as2-7D* samples (panels E and G), which are absent in NPA treated *as2-7D* flowers (panels F and H). The *as2-7D* flowers harbor pLH13 (*35S::mCitrine-RCI2A*) cell membrane marker (greyscale). Trichomes were removed from the outer sepal epidermis by image processing. Scalebar, 50 µm.

Supplementary Table S1: List of primers used in this study.

Primer Name	Sequence	Purpose
oLH108	5'-TTAATGCGAATTACTAGTAGAGAGT-3'	F_Primer for PCR genotyping *as2-6D* mutation
oLH324	5'-TGAAGAATTTTGCTCTTTTTATTCGAATT-3'	R_Primer for PCR genotyping *as2-6D* mutation
oLH347	5'-CACCTGGTAGCTAGCGTTGTTGACA-3'	F_Primer for primer for cloning *AS2* (At1G65620) promoter from WT and *as2-7D* into pENTR
oLH370	5'-GAACGTTTGCGAATTTTTGTGG-3'	R_primer for cloning *AS2* (At1G65620) promoter from WT and *as2-7D* into pENTR
oLH111	5'-CACCATGGCATCTTCTTCAACAAACTCAC-3'	F_Primer for Cloning *AS2* CDS into pENTR
oLH112	5'-TCAAGACGGATCAACAGTACG-3'	R_Primer for Cloning *AS2* CDS into pENTR
oXH172	5'-ctctagacacggggtggtttGTGTGAGCCAAAAACGAG-3'	F_Primer for amplifying *proREV::REV* fragment from Ler-0
oXH176	5'-cggcagcagccgcagcagaaacCACAAAAGACCAGTTTACAAAG-3'	R_Primer for amplifying *proREV::REV* fragment from Ler-0
oXH177	5'-aaactggtctttgtggtttctgctgcggctgctgccg-3'	F_Primer for amplifying mGFP for cloning *proREV::REV-GFP*
oXH173	5'-gtggtaccgcggccgcgcgtttAGGTCACTGGATTTTGGTTTTAG-3'	R_Primer for amplifying mGFP for cloning *proREV::REV-GFP*
oXH174	5'-atatatcctgtcaaagagctTCTTTGTTCTGCGCAACAG -3'	F_Primer for amplifying promoter of *FIL*
oXH178	5'-ggtaattgttgtaaaaatacgcccAGTGAGAAGGGAAAGGGG-3'	R_Primer for amplifying promoter of *FIL*
oXH179	5'-CCCCTTTCCCTTCTCACTgggcgtattttacaacaattacc-3'	F_Primer for amplifying mCherry for cloning *proFIL::mcherry-RCI2A*
oXH175	5'-taaacgcggccgcggtaccaAGGTCACTGGATTTTGGTTTTAG -3'	R_Primer for amplifying mCherry for cloning *proFIL::mcherry-RCI2A*
oAS_005	5'-aaaATGGAGATGGCGGTGGC-3'	F_Primer amplying full length *REVOLUTA* CDS
oAS_006	5'-TCACACAAAAGACCAGTTTACAAAGG-3'	R_Primer amplying full length *REVOLUTA* CDS
oAS_007	5'-TCCGTGAATGTTCCATTTTG-3'	F_Primer *REVOLUTA* qRT-PCR, product size - 129 bp
oAS_008	5'-CAGAGAGCTTCCGGTTTACG-3'	R_Primer *REVOLUTA* qRT-PCR, product size - 129 bp
oAS_017	5'-GTAAAACGACGGCCAG-3'	M13 forward primer
oAS_031	5'-CCACATCCTCTTCCTCACCT-3'	F_Primer on *ATML1* for colony PCR & sequencing

Supplementary Data S1: Raw data for making the plots

Supplementary Movie S1: Confocal images of the outer and inner epidermis of adult sepals of the WT (left) and *as2-7D* mutant (right). Genotypes harbor pLH13 (*p35S::mCitrine-RCI2A*) plasma membrane marker. Scalebar, 200 µm.

Supplementary Movie S2: Confocal images of developing WT (left) and *as2-7D* mutant (right) flowers imaged every 24 hours, over 96-hour time period. Genotypes harbor pLH13 (*p35S::mCitrine-RCI2A*) plasma membrane marker. Trichomes were removed from the outer sepal epidermis by image processing for better visualization of sepal morphology. Scalebar, 50 µm.

Supplementary Movie S3: Confocal images show the outer epidermis of adult sepals of WT (top left), *as2-7D* (top right), *pML1::KRP1* (bottom left) and *pML1::KRP1 as2-7D* (bottom right). Note the largely rescued sepal smoothness in *pML1::KRP1 as2-7D* compared to *as2-7D*, due to an increase in the longitudinally aligned giant cells. Sepals were stained with Propidium-iodide, which stains the cell walls. Scalebar, 200 µm.

Supplementary Movie S4: Confocal images of developing *as2-7D* mutant flowers grown in the presence of mock (left) or 10µM NPA (right). Images were captured every 24 hours over the 96 hour time period. The *as2-7D* mutant harbors pLH13 (*p35S::mCitrine-RCI2A*) plasma membrane marker. Trichomes were removed from the outer sepal epidermis by image processing. Note the reduction in outgrowth formation in 10µM NPA-treated samples despite buckling. Scalebar, 50 µm.

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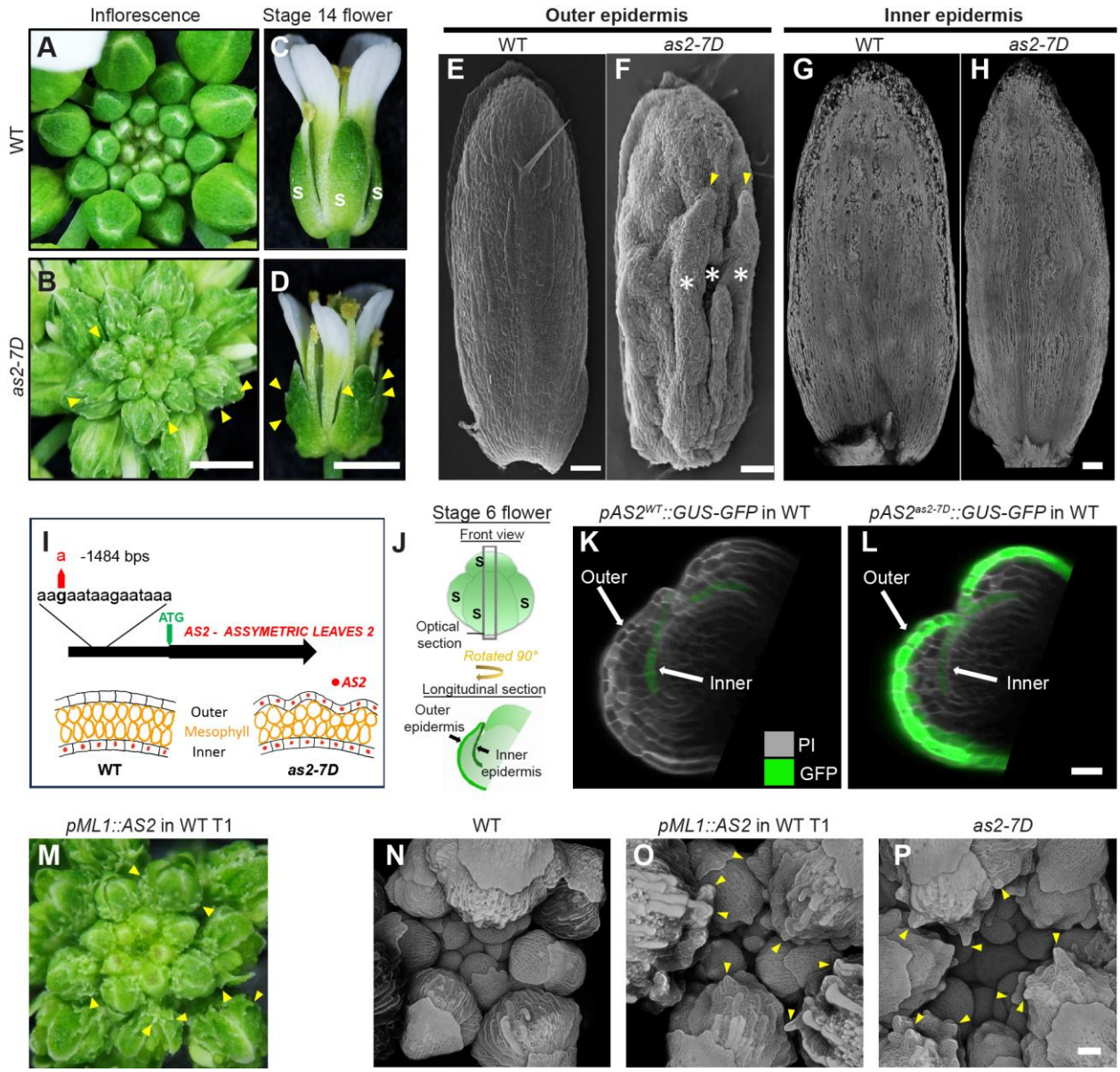


Fig. 1. Ectopic expression of *ASYMMETRIC LEAVES 2* (*AS2*) on the outer epidermal layer disrupts sepal smoothness in *as2-7D* mutant.

(**A** and **B**) Whole inflorescences of (**A**) WT (L*er*-0) and **B**) *as2-7D* mutant (in L*er*-0 background). Yellow arrowheads indicate outgrowths. Scalebar, 1 cm; representative images of *n* = 3 inflorescences for both genotypes.

(**C** and **D**) Mature flowers (stage 14 (*1*)) of (**C**) WT and (**D**) *as2-7D* mutant. 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 cm; 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 µm; 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-RCI2A*), a yellow fluorescent protein-based cell membrane marker (*2*). Scale bar, 100 µm; 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 1484 bps upstream of ATG in *as2-7D* (Top half). The *AS2* expression (red dots) domain in the WT and *as2-7D* sepals is 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**) Longitudinal section of stage 6 flowers of WT. Cell walls are stained with propidium-iodide (PI, greyscale). Images show the GFP expression pattern (green) driven by (**K**) WT *AS2* promoter ($pAS2^{WT}$) and (**L**) *AS2* promoter from *as2-7D* ($pAS2^{as2\text{-}7D}$). Arrows indicate the outer and inner epidermal layers of the outer sepal. Scale bar = 20 µm; n = 3.

(**M**) Whole inflorescence of a representative transgenic (T1) harboring *pML1::AS2* (*AS2* expression driven by *ATML1* epidermal specific 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** to **P**) Confocal images of PI-stained (greyscale) 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 µm; n = 3 inflorescences per genotype.

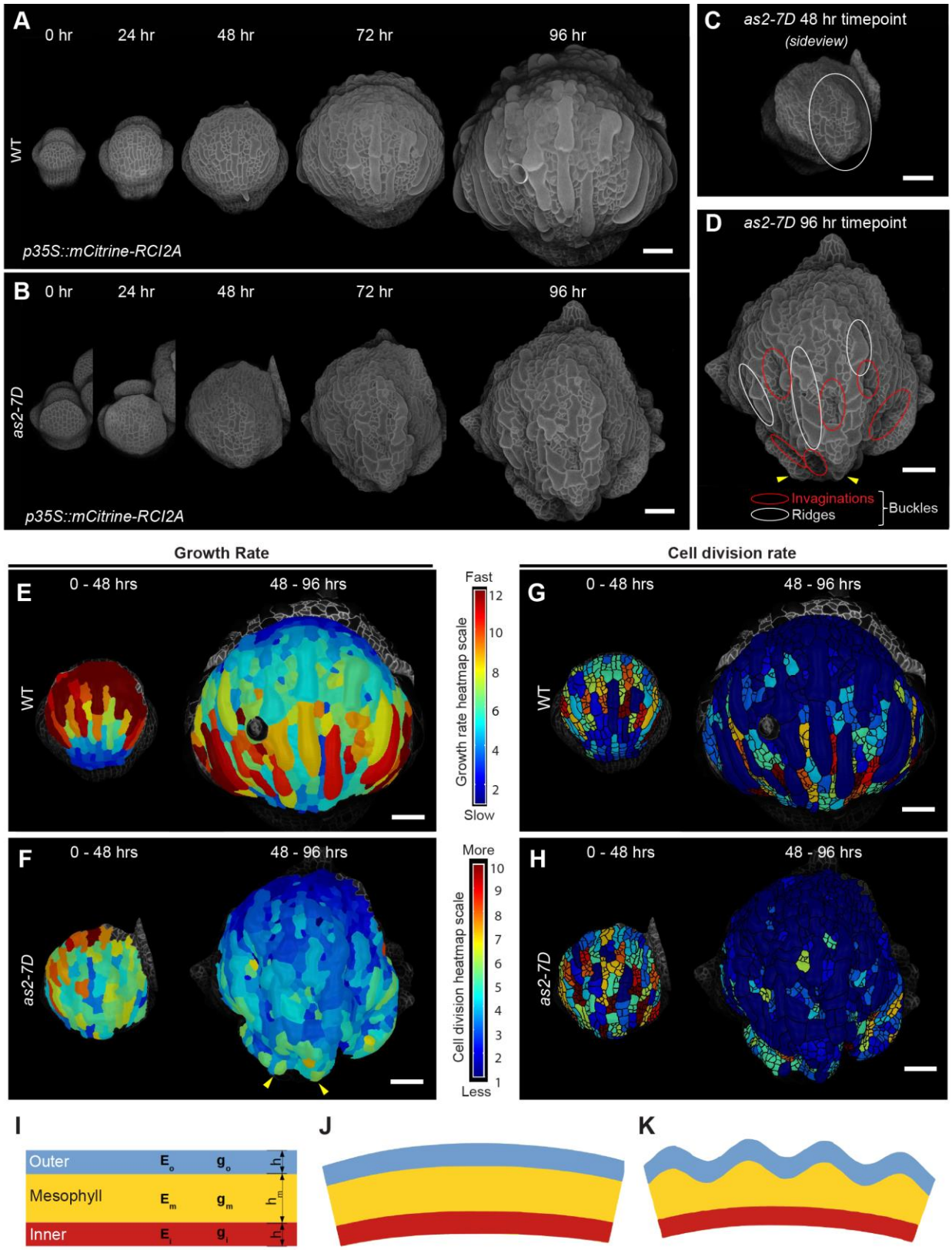

A
0 hr
24 hr
48 hr
72 hr
96 hr
WT
p35S::mCitrine-RCI2A
B
0 hr
24 hr
48 hr
72 hr
96 hr
as2-7D
p35S::mCitrine-RCI2A
C
as2-7D 48 hr timepoint
(sideview)
D
as2-7D 96 hr timepoint
Invaginations
Ridges
Buckles
Growth Rate
Cell division rate
E
0 - 48 hrs
48 - 96 hrs
WT
F
0 - 48 hrs
48 - 96 hrs
as2-7D
Fast
Growth rate heatmap scale
Slow
12
10
8
6
4
2
More
Cell division heatmap scale
Less
10
9
8
7
6
5
4
3
2
1
G
0 - 48 hrs
48 - 96 hrs
WT
H
0 - 48 hrs
48 - 96 hrs
as2-7D
I
Outer
Mesophyll
Inner
J
K

Fig. 2. The *as2-7D* sepal outer epidermal morphology transitions from smooth to buckles and subsequently outgrowths.

(**A** and **B**) Representative live-imaging series of (**A**) a WT flower and (**B**) an *as2-7D* mutant flower starting from developmental stage 4 over a course of 96 hours, imaged at 24-hour intervals (n = 3). Images capture morphological changes of the outer sepal. Both genotypes harbor the pLH13 (*35S::mCitrine-RCI2A*) cell membrane marker (greyscale). Trichomes were removed from the outer sepal epidermis by image processing to better visualization the sepal morphology. Scale bar, 50 µm.

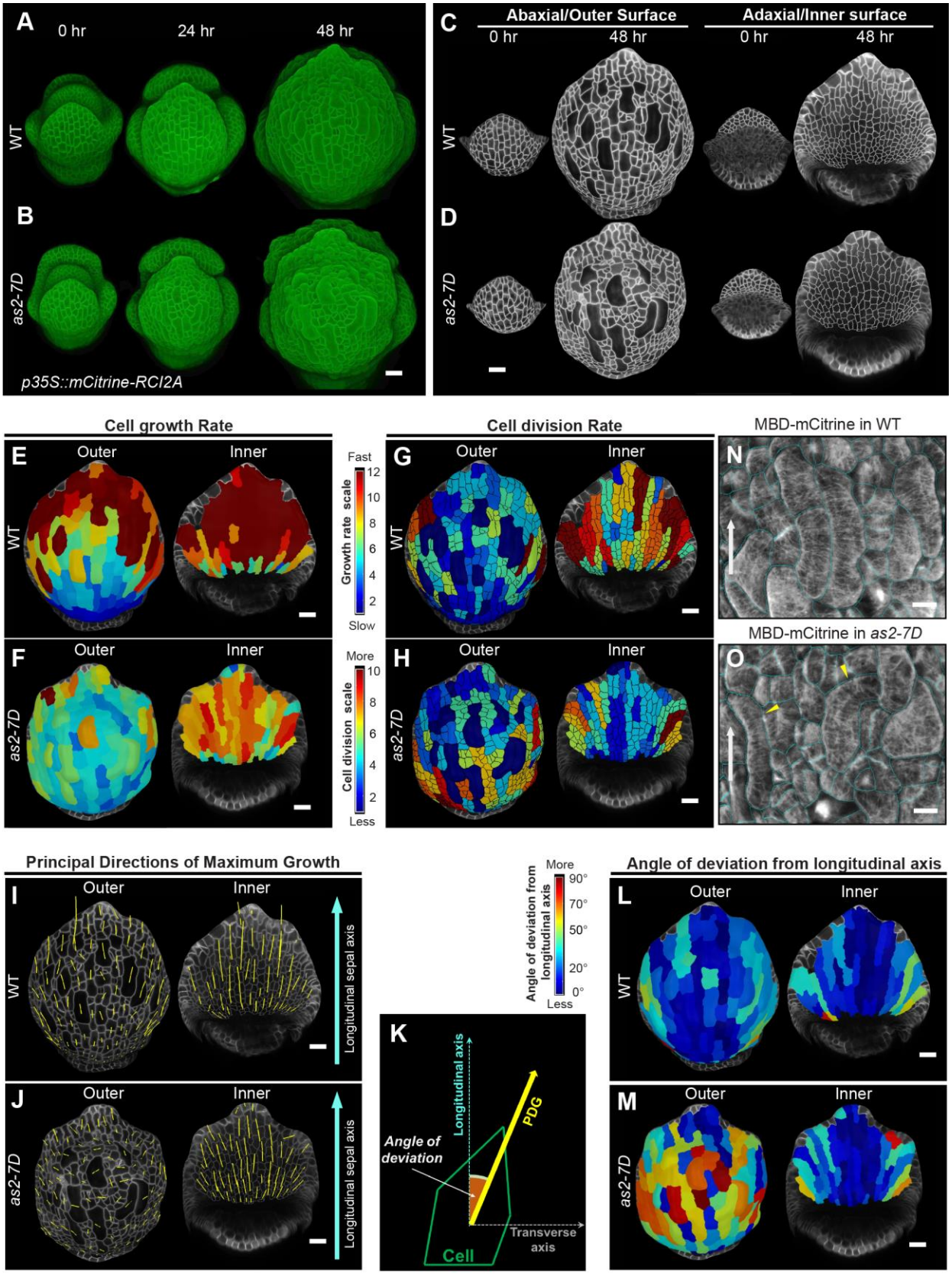
(**C**) Tilted view of *as2-7D* mutant flower at the 48-hour time point. Note the appearance of the out-of-plane protrusion (ridge) in the middle of the sepal, circled in white. Scale bar, 50 µm.

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

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

(**G** and **H**) Cell division rate over a 48-hour period for the (**G**) WT and (**H**) *as2-7D*. Cell division rate is calculated based on the number of daughter cells per lineage, represented as a heatmap projected onto the later time point. Red to blue gradient shows more to less division. The cell lineages were tracked using MorphoGraphX software (*3*). 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 µm.

(**I** to **K**) Visual representation of the analytical model (see Materials and methods) used to determine buckling criteria. (**I**) A growing elastic system composed of three layers, corresponding to the outer epidermis (denoted o), mesophyll (m), and inner epidermis (i) of a sepal. Letters E, g and h represent Elastic modulus, growth rate, and thickness respectively. Diagrams represent growth conditions causing (**J**) the tissue to curve and (**K**) buckling of the outer epidermis.


A
0 hr
24 hr
48 hr
WT
B
as2-7D
p35S::mCitrine-RCI2A
C
Abaxial/Outer Surface
Adaxial/Inner surface
0 hr
48 hr
0 hr
48 hr
WT
D
as2-7D
Cell growth Rate
E
Outer
Inner
WT
F
Outer
Inner
as2-7D
Fast
Growth rate scale
12
10
8
6
4
2
Slow
More
Cell division scale
10
8
6
4
2
Less
Cell division Rate
G
Outer
Inner
WT
H
Outer
Inner
as2-7D
MBD-mCitrine in WT
N
MBD-mCitrine in as2-7D
O
Principal Directions of Maximum Growth
I
Outer
Inner
WT
Longitudinal sepal axis
J
Outer
Inner
as2-7D
Longitudinal sepal axis
More
Angle of deviation from longitudinal axis
90°
70°
50°
20°
0°
Less
K
Longitudinal axis
PDG
Angle of deviation
Transverse axis
Cell
Angle of deviation from longitudinal axis
L
Outer
Inner
WT
M
Outer
Inner
as2-7D

70

71

Fig. 3. Unidirectional growth on both epidermal layers is essential for smooth sepal development.

(**A** and **B**) Representative confocal images of the (**A**) WT and (**B**) *as2-7D* mutant live-imaged with a high laser intensity (~16-20%) every 24 hours. Both the genotypes harbor pLH13 (*35S::mCitrine-RCI2A*) cell membrane marker (green). Scale bar, 20 µm. One of the three representative replicates (n = 3) is shown.

(**C** and **D**) Segmented cells in grey superimposed onto the plasma membrane signal (also in grey) projected on mesh surfaces generated using MorphoGraphX (*3*). Mesh surfaces for 0 and 48-hour 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-hour time point. Scale bar, 20 µm; n = 3.

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

(**G** and **H**) Cell division rates over a 48-hour 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. Red to blue gradient shows more to less/no cell division respectively. Scale bar, 20 µm; 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 (**K**) WT and (**L**) *as2-7D*. Segmented cells in grey are superimposed on the plasma membrane signal (also in grey). 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 µm; 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 90º (highly deviated, red) to 0º (no deviation, blue). Scale bar, 20 µm; n = 3.

(**N** and **O**) Cortical microtubule orientation (greyscale) from comparable regions of stage 6 flower outer epidermis for the (**I**) WT and (**J**) *as2-7D* mutant is shown. These plants harbor *pPDF1::mCitrine-MBD*, a YFP based microtubule marker (*4*). Images show MorphoGraphX generated meshes from PI-stained images, onto which the microtubule signal has been projected. Segmented cells are outlined in cyan. White arrows point towards the longitudinal axis of the sepals. Microtubules in the WT are oriented perpendicular to the sepal longitudinal axis, whereas, in *as2-7D*, the microtubules are misoriented. Yellow arrowheads point towards the misoriented microtubules. Scale bar, 10 µm; n = 3.

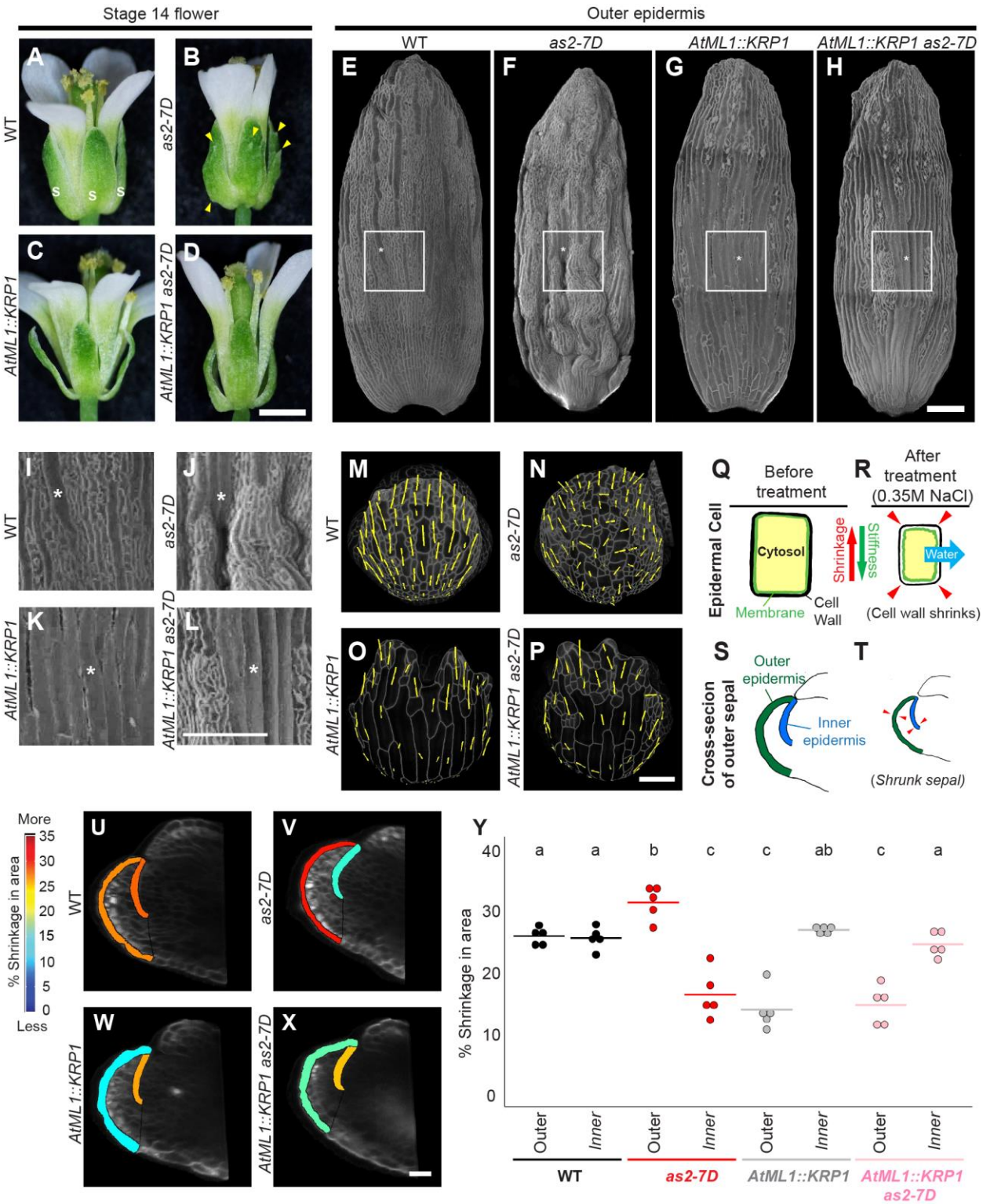


Fig. 4. Aligning growth along the longitudinal direction and increasing outer epidermal stiffness is sufficient to rescue buckling in *as2-7D*.

(**A** to **D**) Representative stage 14 flowers of (**A**) WT, (**B**) *as2-7D* mutant, (**C**) *AtML1::KRP1*, and (**D**) *AtML1::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 *AtML1::KRP1 as2-7D* compared to *as2-7D*. Scale bar, 1 cm; n = 3 flowers for all genotypes.

(**E** to **H**) Confocal images of the outer epidermis of (**E**) WT, (**F**) *as2-7D* mutant, (**G**) *AtML1::KRP1*, and **(H)** *AtML1::KRP1 as2-7D* stage 14 sepals. The sepals were stained with PI (grey, cell walls). Note the increased number of highly elongated giant cells in *AtML1::KRP1* and *AtML1::KRP1 as2-7D* sepal, and the smooth outer epidermis of *AtML1::KRP1 as2-7D* sepal compared to the highly rugose morphology of *as2-7D*. Scale bar, 200 µm; n = 3.

(**I** to **L**) Magnified insets of the (**I**) WT, (**J**) *as2-7D* mutant, (**K**) *AtML1::KRP1*, and (**L**) *AtML1::KRP1 as2-7D* sepal for the regions marked by white boxes in panels E-H respectively. Asterisks denote a typical giant cell. Scale bar, 200 µm; n = 3.

(**M** to **P**) The principal direction of growth (PDG_{max}) for the outer epidermal cells of the **(M)** WT, **(N)** *as2-7D*, (**O**) *AtML1::KRP1*, and (**P**) *AtML1::KRP1 as2-7D* sepals. Yellow lines mark the PDG_{max} tensors calculated over a 48-hour 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 grey are superimposed onto the plasma membrane signal (also in grey). Scale bar, 20 µm; n = 3.

(**Q** and **R**) Schematic diagrams demonstrating the concept of cell wall stiffness assessment by osmotic treatment assays (*5*). (**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 using 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** to **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**) *AtML1::KRP1* and (**X**) *AtML1::KRP1 as2-7D*, post osmotic treatment in 0.35M 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 µm. 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), *AtML1::KRP1* (grey dots), and *AtML1::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.

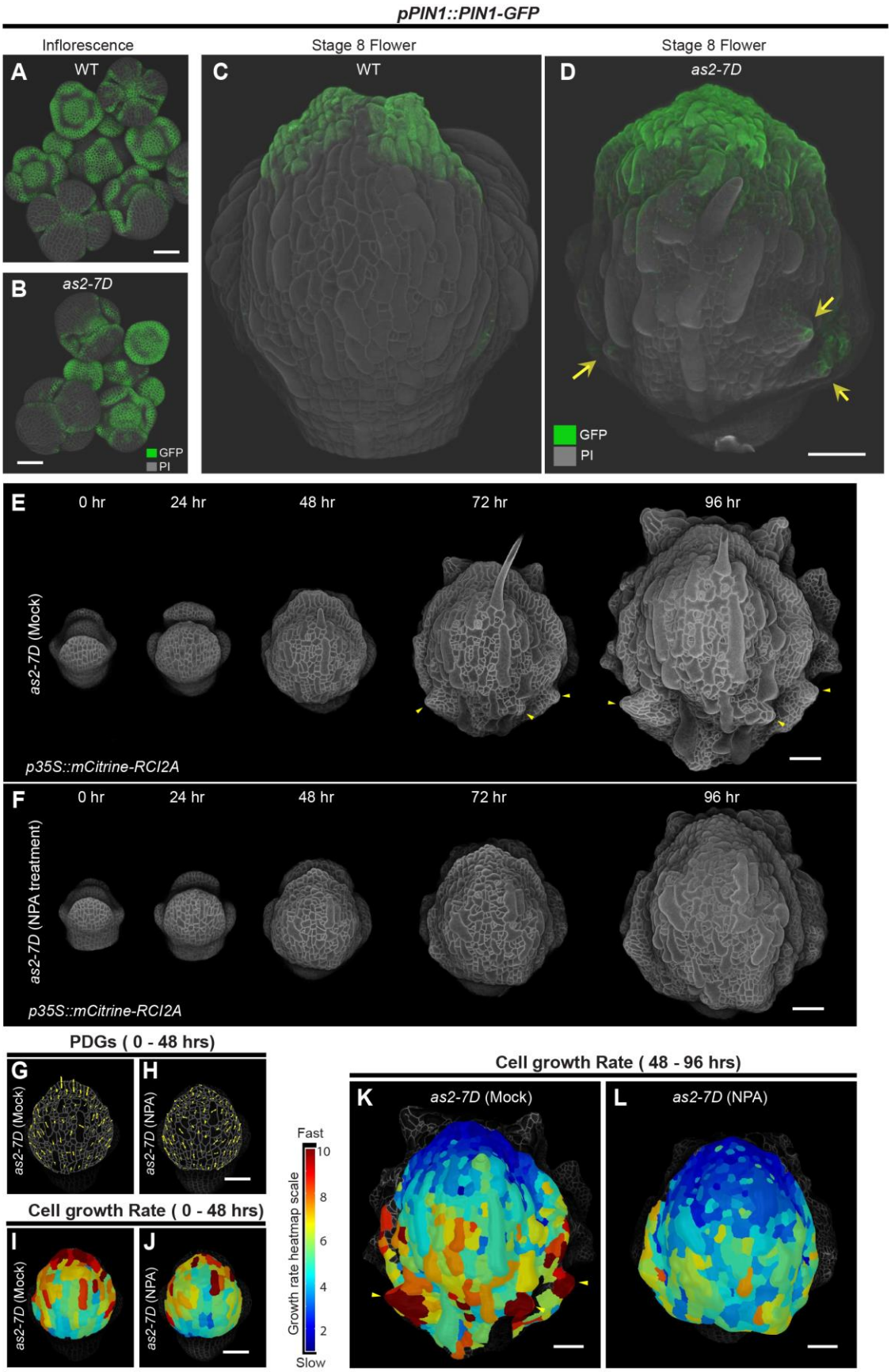

pPIN1::PIN1-GFP
Inflorescence
Stage 8 Flower
Stage 8 Flower
A
WT
B
as2-7D
GFP
PI
C
WT
D
as2-7D
GFP
PI
E
0 hr
24 hr
48 hr
72 hr
96 hr
as2-7D (Mock)
p35S::mCitrine-RCI2A
F
0 hr
24 hr
48 hr
72 hr
96 hr
as2-7D (NPA treatment)
p35S::mCitrine-RCI2A
PDGs (0 - 48 hrs)
G
as2-7D (Mock)
H
as2-7D (NPA)
Cell growth Rate (0 - 48 hrs)
I
as2-7D (Mock)
J
as2-7D (NPA)
Fast
10
8
6
4
2
Slow
Growth rate heatmap scale
Cell growth Rate (48 - 96 hrs)
K
as2-7D (Mock)
L
as2-7D (NPA)

Fig. 5. Mechanical buckling drives ectopic PIN convergence creating auxin maxima, causing fast local growth and subsequent outgrowth formation.

(**A** to **D**) Confocal stack images showing the expression pattern of auxin efflux carrier protein PIN-FORMED 1 (PIN1) in the WT and *as2-7D*. (**A** and **B**) Expression pattern of PIN1 protein reporter (*pPIN1::PIN1-GFP* (*6*)) in the inflorescence meristem of (**A**) the WT, (**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 towards the insipient primordia and growing sepal edges, as described previously (*6*). Scalebar, 50 µm. (**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 panels A to D, the images are representative of three *pPIN1::PIN1-GFP* and *pPIN1::PIN1-GFP as2-7D* plants (n = 3). PI is shown in greyscale and GFP in green. Trichomes were removed from the outer sepal epidermis by image processing. Scale bar, 50 µm.

(**E** to **F**) Representative live-imaging series of (**E**) Mock treated *as2-7D* and (**F**) 10µM NPA treated *as2-7D* developing flowers over 96 hours, imaged at every 24-hour interval (representative of n = 3 flowers). Note the development of outgrowths from 72-hour time-point onwards (yellow arrowheads) in the mock-treated *as2-7D*, which are absent in NPA treated *as2-7D* flowers. Plants harbor pLH13 (*35S::mCitrine-RCI2A*) cell membrane marker (greyscale). Scale bar, 50 µm.

(**G** to **H**) The PDG_{max} tensors (yellow) of the cells on the outer epidermis of (**G**) mock-treated and (**H**) 10µM NPA treated *as2-7D* over the first 48-hour growth period for the representative samples shown in panels E to 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 µm.

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

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