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Mechanics, geometry and genetics of epidermal cell shape regulation: different pieces of the same puzzle

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Pavement cells in the leaf epidermis of many plant species have intricate shapes that fit together much like the pieces of a jigsaw puzzle. They provide an accessible system to understand the development of complex cell shape. Since a protrusion in one cell must fit into the indentation in its neighbor, puzzle cells are also a good system to study how cell shape is coordinated across a plant tissue. Although molecular mechanisms have been proposed for both the patterning and coordination of puzzle cells, evidence is accumulating that mechanical and/or geometric cues may play a more significant role than previously thought.

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Introduction

Cells with an elaborate, jigsaw puzzle-like shape appear in the epidermis of many plant species, including the model plant *Arabidopsis thaliana*. Progressing from simple polygon-shaped meristematic cells, they develop into large cells with many interlocking lobes (convex areas) and indentations (concave areas), that often resemble puzzle pieces (Figure 1). Because of this dramatic change in form during development, puzzle cells have become an attractive system for investigating cell-shape control.

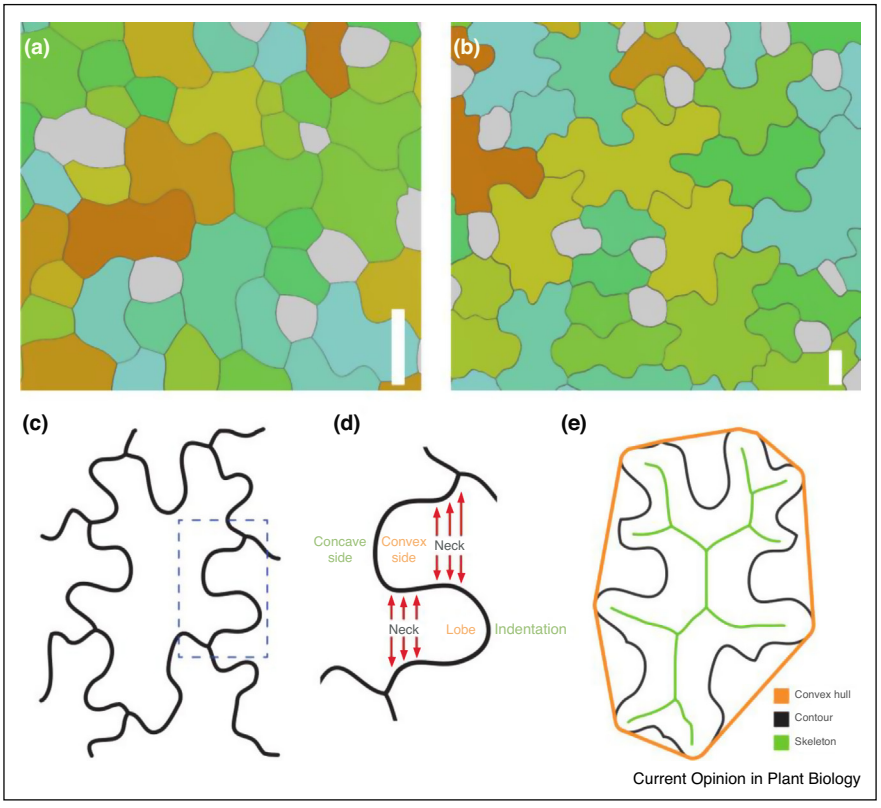
Understanding puzzle cell development has been challenging, as it appears to involve feedbacks and interactions at several scales. These feedbacks include various self-organizing components that act at the sub-cellular scale. Molecular interactions for cell-wall partitioning [1–7], sub-cellular cytoskeleton organization [8••,9,10] and differential cell wall mechanics [11,12••,13,14•] all interact to produce the lobes and indentations. Since a lobe in one puzzle cell must fit into the corresponding indentation in its neighbor, coordination of these processes must occur at the supra-cellular level. Possible candidates to provide this coordination are extra-cellular signaling molecules [15], mechanical or geometric cues [13,16••,17] or a combination of the two.

When striking or unusual cell shapes are observed, it is natural to wonder about their function, as form often follows function in biology (for a recent review see [18]). Several hypotheses have been proposed to explain the interlocking puzzle shape of these cells. It has been proposed that puzzle shapes may be important for the correct spacing of other epidermal cell types such as stomata and trichomes [19] or to help the leaf to remain flat and thereby optimize light capture [20]. Another hypothesis is that the interlocking shapes may increase adhesive strength between cells, increasing the stability of the epidermis [21,22] that is often under considerable tension from internal cells [23]. A related idea is that puzzle cells might help the tissue to undergo large reversible deformations, such as when the tissue is stretched or bent [14•].

Sapala *et al.* (2018) have recently proposed a different function for the puzzle cell shape, that is related to the mechanical stresses the cell walls encounter due to their turgor pressure [16••]. Green plant tissue relies on turgor pressure for its shape. It behaves like a pressurized cellular structure, not unlike an inflatable mattress. When turgor pressure is reduced, the structure collapses and the plant wilts. Sapala *et al.* (2018) propose that the puzzle shape allows the formation of large pressurized cells in the epidermis of plant organs that grow isotropically, as seen in many leaves. If the cells had simple shapes, large cells would bulge out excessively under pressure and burst (imagine an air mattress without the vertical seams) [16••]. Of course, long, thin cells would also work, as in the air mattress, however these would only be possible in plant organs that grow mostly anisotropically, such as roots or stems.

Studies on pavement cell development have been impeded by difficulties in reliably quantifying the growth, shape and mechanics of these cells, and in understanding the nature of patterning within cells and its coordination between adjacent cells. Here we review recent work addressing these shortfalls and highlight how they begin to substantially change our understanding of pavement cell function and development, which may be directly related.

Figure 1



Pavement cell shape. (a,b) Cell shape in the epidermis of an *Arabidopsis thaliana* cotyledon. **(a)** Small cells (2 days after germination) have relatively isodiametric shapes, while **(b)** larger cells (6 days after germination,) display very complex, jigsaw puzzle-like shapes. The emergence of the puzzle shape occurs early in organ development. Scale bars, 20 μm. Cells belonging to stomatal lineage (grey) do not become puzzle-shaped. **(c)** Cells with a jigsaw-puzzle like shape that interlock with neighboring cells are called puzzle cells. The inset (dashed box), is shown in **(d)** and demonstrates the basic terminology used to describe puzzle cell morphology. **(e)** Measures of puzzle cell shape are typically computed from the cell contour (black), its convex hull (orange), or a skeleton approximating the overall form of the cell (green).

Quantifying puzzle cell shape

Puzzle-cells have complex, recognizable shapes that nevertheless are highly variable. This has made it challenging to reliably quantify cell shape changes during development or identify cell-shape differences between various mutants. Shape measures provide a means to determine specific geometric aspects of cell shape. The simplest one is circularity, which indicates how close a cell shape is to a circle (see Box 1A for definitions). The perimeter or area of a cell can be compared to its convex-hull (Figure 1e) to give a measure of the convexity of a cell representing the amount of indentations or concave regions the cell has. Conversely, one can take the ratio of the largest empty circle (LEC) that fits inside a cell and compare it to the cell area, giving another simple measure of how the cell deviates from a circular shape. These simple to compute measures are useful in coarsely evaluating differences in cell-shape, focusing on the general degree of lobeyness. Although most are not directly related to the mechanism of pavement cell formation, the LEC by itself (without the area ratio) provides a proxy for stress in the cell [16••]. It follows that measures related to the mechanism controlling pavement-cell morphogenesis may be especially useful in characterizing the phenotypes of various puzzle cell shape mutants.

For more advanced quantification, measures characterizing the number and geometry of lobes and indentations are required. These measures are often directly relevant to proposed mechanisms, for example the number of lobes at a given cell size could indicate the periodicity of an intra-cellular partitioning mechanism. Manual measurement of these features indicate they are indeed biologically relevant [2,7,24], however it does not offer a reliable means of objective quantification. To address this problem several methods for puzzle cell quantification have been proposed. They can be roughly divided into two categories: those that focus on the cell contour and those that use skeletons to approximate the overall form of the cell (Box 1B, Figure 1e).

Box 1 Glossary

(A) Basic terminology used in pavement cell studies

- Lobe — a convex (protruding) portion of the cell-contour in a puzzle-shaped epidermal cell.
- Indentation — a concave (indented) portion of the cell-contour in a puzzle-shaped epidermal cell. Indentations facing each other across the cell form a 'neck'. A lobe in one cell is matched with an indentation in a neighboring cell.
- Puzzle cell — a cell with lobes and indentations along its contour which interdigitate with those of neighboring cells.
- Circularity — a shape measure of how closely an object resembles a perfect circle (4π Area/Perimeter^2).
- Largest Empty Circle (LEC) — the largest empty circle that can fit into a cell; provides a proxy for mechanical stress [16••].
- Convex hull — the smallest convex shape that contains an object. The convex-hull of the cell contour is used in the simple shape measures solidity (convex-hull area/cell-area) and convexity (cell-perimeter/convex-hull perimeter) [16••,29•,30] and as the basis for detailed analysis of lobe geometry [29•].
- Lobeyness — a scalar cell shape measure indicating how lobed or puzzle shaped a cell is. Several measures have been proposed for this purpose, and capture different aspects of cell geometry, including: convexity, solidity, lobe number or scalars computed from the application of variants of Fourier-analysis of the cell contour (e.g. cumulative difference in [31•]).

(B) Puzzle cell quantification software developed in recent years

- LobeFinder [29•] — Contour-based. A MatLab application focused on extraction of lobes based on the convex hull (lobe number, distance to convex hull). Extracts lobes and can track their development.
- PaCeQuant [30] — An ImageJ tool that provides a suite of measures based on both the cell contour and skeleton. The tool calculates many simple measures, but also pavement cell specific measures such as lobe number.
- LOCO-EFA [31•] — Contour-based. Extends traditional Elliptical-Fourier-Analysis (decomposes the contour into waves of different frequencies) to provide rotation-invariance. These coefficients can then be used to analyze cell shape.
- MorphoGraphX [16••] — Plugins to calculate largest empty circle fitting into the cell (LEC), circularity and both perimeter-based and area-based convex hull measures. Quantification can be performed on both flat images and curved surfaces.

Interestingly, there is a great deal of similarity between these techniques and those used to quantify leaf-shape. Indeed, systems quantifying leaf shape based on skeletonization [25] or contour-based lobe-extraction [26] have been recently proposed. There seems to be ample opportunity for the cross-pollination of ideas, and the application of techniques based on machine-learning [27] or persistent homology [28] to pavement cells is particularly appealing. Initial works applying these approaches to leaves has demonstrated they can be used to broadly compare leaves with diverse shapes for the purpose of automatic classification of species or family (e.g. [27,28]) and to identify differences that are not easily captured by simple shape measures (e.g. circularity or solidarity [28]).

Quantifying growth

Although the analysis of static images is useful in classifying and discriminating between different puzzle shape phenotypes, time lapse data can provide insight into pavement cell formation. At the sub-cellular level, puzzle cells have complicated patterns of growth that, nonetheless, occurs within the context of the broadly coordinated tissue growth shaping organs [10,29•,32]. To track sub-cellular growth, recent studies in *A. thaliana* have used microbead labeling to randomly mark the outer wall of cells in the epidermis with fluorescent beads and monitored the positions of beads over time [11,33]. Both of these studies confirm that individual puzzle cells grow heterogeneously in a pattern related to lobe outgrowth, but suggest different patterns of sub-cellular growth. Armour *et al.* (2015) report that growth in puzzle cells is isotropic, but lobe creation is enhanced by higher growth rates in the convex sides of the cell wall [11]. Whereas, Elsner *et al.*'s (2018) observations suggest lobe outgrowth is not limited to the lobe tip, but involves anisotropic extension of the entire lobe (i.e. diffuse anisotropic growth) [33]. These inconsistencies may stem from the sparse covering of fluorescent beads, or differences in the computational techniques used to infer growth. Cellular growth tracking in whole organs [16••] shows that sub-cellular and tissue level growth are related, with increased lobeyness of cells in isotropically growing regions and decreased lobeyness in areas where growth is more anisotropic.

The correlation between organ growth isotropy and puzzle-cell development makes it tempting to speculate that organ shape and the lobeyness of pavement cells are strongly correlated. Although this is sometimes the case, organ level growth analysis of leaves in several eudicot species with different leaf forms (*A. thaliana* [34], petunia, tobacco [35,36], and bay laurel [37]) indicate that growth is nonetheless isotropic at later developmental stages when we expect puzzle cells to form. Consequently, an important direction for further work is to jointly quantify both pavement cell shape and growth isotropy during organ expansion in diverse species and backgrounds, as the latter cannot always be inferred from organ shape alone.

Local patterns of cell wall properties

The intricate shape of puzzle cells implies that their growth must be regulated in a non-trivial way. As cell mechanics ultimately determines cell growth, the waves or lobes in the periclinal cell wall of epidermal cells likely arise from subcellular differences in cell-wall composition. Panteris *et al.* (1993) have shown that the cell walls at the bottoms of the indentations are thicker in the developing puzzle cells of *Vigna sinensis* [38]. Thickening of the cell-walls in these locations is likely to restrict growth, and counteract the increased stress at indentations predicted by mechanical models [13,16••]. More recently, Majda *et al.* (2017) showed that even minor alterations in cell-wall composition can alter puzzle cell shape, by changing lobe number [12••]. They also demonstrated mechanical heterogeneity in the

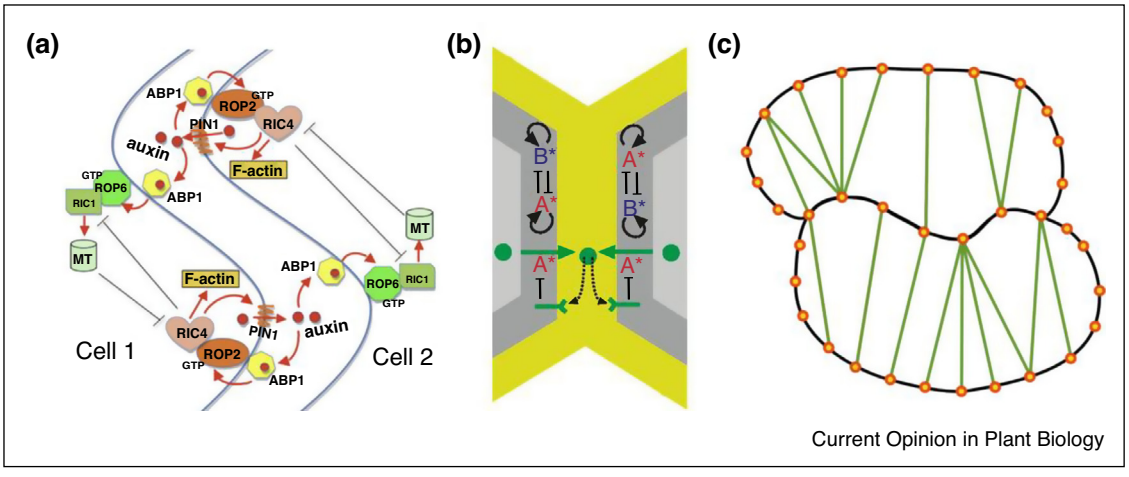
anticlinal walls of puzzle-shaped epidermal cells, which is asymmetric across the wall (stiffer in indentations and softer in lobes). Using a finite-element model of anticlinal cell walls they show that this heterogeneity (interchangeable regions of softer and stiffer wall creating subcellular variations of growth rates) can create waves. Similarly, Sotiriou *et al.* (2017) show de-methylesterified homogalacturonans can be found in the curved areas of the anticlinal cell-wall [14•]. When stretching the epidermis parallel to the direction of lobe outgrowth these curved areas straighten, but return to their original shape when released. This implies a correlation between differences in mechanical properties and the local biochemical composition of the cell wall.

These studies help understand how sub-cellular wall composition may contribute to the establishment and growth of lobes, but leave open the question of how the molecular pathways positioning lobes are coordinated with mechanics. Some attention has been paid to tissue-level effects of mechanical forces, but in the context of signal transduction during stomata development [39] rather than cell shape formation.

Molecular network for puzzle cell patterning

Much work has been put into deciphering the molecular components driving puzzle cell formation. Current thinking is that Rho of Plants (ROP) proteins interact with RIC proteins to direct the cytoskeleton and thereby locally regulate cell growth. Specifically, it appears that ROP6 accumulates in indentations and recruits cortical microtubules through RIC1, inhibiting growth in that region [3], while ROP2 recruits actin filaments through RIC4 in lobes to promote further outgrowth [2]. ROP6 and ROP2 are believed to mutually inhibit each other, thereby creating an alternating pattern along the anticlinal cell wall (Figure 2a). Simulation models of a ROP2–ROP6 style co-repression network can partition cells into sub-cellular domains, and intra-cellular coordination of these domains can be accounted for by an extracellular signal [42] (Figure 2b). Several authors have proposed that auxin could be such a signal, acting in concert with ABP1 and the PIN-FORMED (PIN) auxin transporters [2,7,43]. However, recent work of Gao *et al.* (2015) have undermined the function of ABP1 as a key component in auxin signaling [40]. In addition, Belteton *et al.* (2017) showed that PIN proteins, which are expressed during pavement cell development, have no apparent influence on lobe patterning [41••]. Consequently, the hypothesis that auxin controls the ROP2/ROP6 patterning of lobes and indentations via PIN and ABP1 [7] has been put in question. Nonetheless, the idea of an extra-cellular signal that coordinates a partitioning mechanism within the cell seems likely. But does this signal have to be molecular? Another possibility is that it could be transmuted mechanically, or through the geometry of the cells themselves [16••] (Figure 2c).

Figure 2



Models of puzzle cell formation. **(a)** Proposed ROP2–ROP6 molecular network for cell partitioning into domains that correspond to lobes and indentations. ROP2 accumulates in lobes promoting growth via RIC4 and actin, and ROP6 accumulates in indentations restricting growth via RIC1 and cortical microtubules (reproduced from Ref. [7]). The lobes in one cell are coordinated with the indentations in its neighbors via a mobile signal. Auxin has been proposed as the mobile signal and ABP1 as the receptor, however recent work casts doubt on this interpretation [40,41••]. **(b)** Cartoon of a simulation model of a signaling network similar to (a) that is able to partition cells into distinct domains (reproduced from Ref. [42]). A and B represent the inactive (non-membrane bound) forms of ROP2 and ROP6, with A* and B* the active forms. A* and B* self-activate, and co-repress each other. An extracellular signal (green circles) coordinates polarity between cells. **(c)** A growing 2D mechanical model of lobe formation (adapted from Ref. [16••]). Growth restrictions are placed across the cell (green lines) and represent the action of mictrotubules that direct downstream cellulose deposition. These restrictions act when their length exceeds a threshold, a proxy for when the stress becomes too high. Small indentations attract springs and protrusions lose them, causing the lobes and indentations to be enhanced. The coordination of the lobe in one cell with the indentation in its neighbor results from the geometry (curvature) of the cell wall, without requiring a mobile coordinating signal.

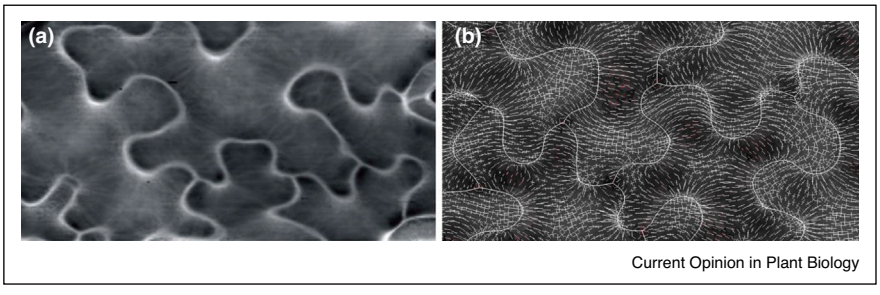
A role for mechanical or geometric cues

Cortical microtubules are believed to organize themselves along the principle direction of stress [44,45]. Since microtubules guide cellulose synthases (CESA) [46], they can trigger anisotropic reinforcement of the cell wall along the direction of stress, limiting growth in this direction [47–49]. This can alter the shape of the cell, which in a pressurized structure is a primary determinant of stress ([16••,50,51]). This suggests a feedback where stress patterns orient microtubules (and thereby cellulose, see Figure 3a), causing changes in growth and the shape of the cell, which in turn affects stress. Such a feedback has been proposed to be the primary driver of puzzle cell formation [13].

It has also been proposed that cell geometry itself may account for microtubule orientations [8••,52]. Based on simple rules derived from observation of microtubule behavior, Chakrabortty *et al*. (2018) simulate the interaction of microtubules with each other and the curvature of the cell wall. They are able to reproduce patterns resembling those observed *in planta* [8••]. Similar simulations by Mirabet *et al*. (2018) indicate that highly curved cell shapes (i.e. with sharp edges) have more anisotropic microtubule distribution than those with smooth edges [9], which may lead to more focused cell wall reinforcement by CESA. The alignment of microtubules perpendicular to sharp-edged corners can be overcome by CLASP (cytoplasmic linker-associated proteins) which accumulate in corners and create microtubule bundles [53]. The tendency for microtubules to bundle when they interact may provide an additional mechanism for the accumulation of microtubules in indentations. This could work in concert with the self-enhancing behavior of the membrane bound form of ROP6 proposed in molecular models of pavement cell patterning [3,42] with ROP2 in the lobes promoting enhanced growth rates [11]. At present, however, it is unclear if the ROP2–ROP6 co-repression network prepatterns lobes and indentations prior to their emergence, or if this network acts in concert with changes in cell geometry, making cell shape itself an integral part of the feedback producing puzzle shaped cells.

Sapala *et al*. (2018) propose that jigsaw puzzle cell shapes are an outcome of self-enhancing growth restriction in the lobes that helps the cells mitigate excessive stress from large, isodiametric shapes. In their model, when cells (and stresses) become too large, microtubules orient to direct growth restrictions. Small indentations attract microtubules and are enhanced, whereas lobes become enhanced by the loss of microtubules (Figure 2c). The coordination of a lobe in one cell with the indentation in its neighbor is transmitted through cell geometry. The model is able to reproduce a wide variety of pavement cell patterns similar to those observed in different plant species [16••]. Nonetheless, there are alternative mechanisms cells could employ to limit cell wall stress. Another possibility to prevent the cell wall from bursting is to increase its thickness. As mechanical stress is defined as the ratio of force acting on a material to the area of its cross section, both increasing cell wall thickness and decreasing LEC radius would reduce stress locally. However, thickening the cell wall likely requires more resources than adjusting cell shape. As epidermal cells are relatively thin compared to their surface area [54,55] even a large increase in cell perimeter (by adding lobes and indentations) only leads to a modest increase in cell wall material (surface area will not increase dramatically as the addition and loss of area due to lobes and indentations would, on average, cancel out). Additional strategies to manage cell wall stress include cell division, and modification of the cell wall composition in order to make it stiffer.

Figure 3



Cell wall reinforcements follow stress directions in the periclinal cell walls. **(a)** Surface of pavement cells in *Arabidopsis thaliana*. Image obtained with an Atomic Force Microscope, darker regions are soft and lighter regions are stiffer. Note the orientated pattern, thought to reflect that of cellulose fibrils which are the stiffest component of the cell wall. Their deposition is guided by cortical microtubules. Adapted from Ref. [13]. **(b)** A finite element method (FEM) simulation of pressurized 3D puzzle cells (adapted from Ref. [16••]) with stress directions (the directions of maximum and minimum stress) visualized as white lines. Stress orientations are similar to the cellulose fibrils in (a), radiating out from the high stress indentations, and oriented across the lobes.

Could cells sense stress through geometry?

Since plants are pressurized cellular structures, there is a close correlation between cell shape and stress [50,51], where curvature and turgor pressure are the primary determinants of cell wall stress. The idea of curvature sensing controlling shape through gene expression has been proposed in rod-like elongated bacterial cells [56•], animal intestinal stem cell niches [57], and other systems (for a recent review, see [58]). Self-organization of microtubules may be central to curvature sensing [58] as indicated by simulation studies in plants [8••,9], as well as experiments in *Drosophilla melanogaster* embryos where cell shape aligns microtubules [52]. In the case of plant puzzle cells, creating intricate forms via a mechanism of shape or curvature sensing may be the outcome of optimizing mechanical stress in cell walls (Figure 3b). Although there is increasing evidence that stress is a central factor in morphogenesis and signal transduction, it has remained elusive how, or even if, it is possible for the cell to measure stress in the wall. Most stress measurement methods ultimately rely on measuring strains of some kind, yet strain-based feedbacks on growth do not seem to be sufficient [59]. The pressurized nature of plant cells offers an exception. Plant cells could be using geometry (i.e. curvature) sensing as a proxy for stress.

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