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Measuring the mechanics of morphogenesis

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The past decades have seen a rapid increase in the understanding of plant morphogenesis at the molecular-genetic level. However, the control of growth and morphogenesis by molecular and signaling networks ultimately requires the coordinated regulation of mechanical properties in individual cells. There is also increasing evidence that mechanical stresses can feedback on hormone signaling and growth, and may have a central role in developmental patterning. Thus the development of techniques to investigate the mechanical properties of plant tissue at the cellular level is key to understanding growth and morphogenesis.

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Introduction

In most plant tissues, growth is largely symplastic, and cells do not slide with respect to each other. There is no cell movement as commonly occurs in animals. This puts limitations on the mechanisms available to the plant to generate its form and shape, with morphogenesis relying on carefully coordinated growth. Growth itself results from an interplay between biochemical and biophysical factors [1,2••,3,4•]. Plant cells are like small balloons, and are under considerable turgor pressure (typically 3–10 atm) resulting from the difference in osmotic potential inside and outside the cell. Containing this turgor pressure is the plant cell wall, a connected extracellular matrix made of cellulose, hemicellulose, pectins, other polysaccharides and some structural proteins [5,6]. Turgor pressure places the cell wall in a constant state of tension, and this gives the non-woody portions of the plant their shape and stiffness. A loss of pressure results in wilting, and collapse of the structure. In order for a plant cell to grow, it must be able to extend its cell wall, while simultaneously maintaining its structural integrity so that it does not weaken to the point of rupture. This is a remarkable accomplishment given that actively growing cells have relatively thin walls, and can increase in size by several orders of magnitude while withstanding tension in the range of 100–1000 atm [5,7].

The mechanism of cell wall extensibility is thought to occur through selective wall loosening, and remains relatively poorly understood [5,8]. Nonetheless many cell wall components and several enzymes that modify them have been identified [5,9–11]. In addition, many of the upstream molecular and signaling pathways driving morphogenesis have been uncovered [12–14]. In order to understand how these networks regulate cell expansion, there has been a recent trend towards integrating molecular level processes with mechanics. In the shoot apex differential mechanical properties have been suggested to have an instructive role in functional zonation [15••,16••]. It has also been suggested that mechanical signals can influence the transport of the growth hormone auxin through microtubule orientation [1,2••], cell pressure [3] or via specialized connections between the plasma membrane and the cell wall [17].

Elasticity, viscoelasticity, and plasticity

Plant cell growth occurs as the cell wall is deformed in response to loading caused by turgor pressure. Several types of deformation are possible when a material is subjected to load (see Box 1). It may deform purely *elastically*, such that when the load is removed it returns immediately to its original shape. Tensile tests on plant tissue often show considerable elastic deformation combined with a time-dependent or *viscoelastic* response [18–20]. In a viscoelastic response, the material eventually returns to its original size after releasing the stress, although this may take some time [21]. Finally a material may exhibit *plastic* behavior. In this case the material becomes permanently stretched, and does not return to its original shape after releasing the load. Growth is considered to be a plastic deformation, as the cell deforms permanently, and it is usually accompanied by the addition of new wall material, although in some cases it may involve substantial wall thinning [22]. The relationship between cell wall elasticity, viscoelasticity, turgor pressure and growth is still unclear; however, there is some evidence for a correlation between elasticity and growth [16••,23–25]. In single algae cells, step-wise increases in turgor pressure have been shown to enhance elongation rates [24,26], which likely reflect both growth and viscoelastic deformation. On the other hand, increased cell growth rates induced by low pH in maize roots do not involve differences in turgor pressure [27]. Nonetheless, most mathematical models for growth are in some way derived from the Lockhart model [7,28,29] and propose that the cell wall will irreversibly yield after reaching a certain threshold stress resulting from turgor. For a review of the mathematical models of growth see Goriely *et al.* [30]. It appears that plastic yielding is not a purely mechanical response, but

Box 1 Explanation of some useful mechanical terms

Mechanical behavior:

- *Elasticity* refers to a fully reversible deformation under load. The speed at which force is applied does not matter. If a load is removed from an elastic solid, it will come back instantly to its unloaded state. When the deformation (strain) is proportional to the force it obeys Hooke's law, and the behavior is called *linear elastic* or *hookean*. Biological materials often exhibit *non-linear elasticity*.
- *Viscosity* is a measure of the resistance of a liquid to flow. Unlike elasticity, it is a time-dependent response.
- *Viscoelastic* materials exhibit both viscous and elastic behavior when undergoing deformation. The effective stiffness depends on the rate of loading. The time-dependent, viscous response causes *force relaxation* under a constant deformation, or the sample to *creep* (deform with time) when a load is maintained. If a load is removed from a viscoelastic solid, it can slowly return to its original shape provided that no plastic deformation has occurred.
- *Plasticity* occurs when a solid does not recover its original shape after removing a load. The amount of plastic deformation depends on the force applied and time. Cell growth is due to a plastic deformation of the wall that *yields* under turgor pressure. Viscoelastic behavior can be misinterpreted as plastic deformation if the sample is not allowed enough time to come back to its original shape.

Quantities:

- *Stiffness (K)*: force divided by displacement. Measures the rigidity of an object, which depends on its shape. For example, the bending stiffness of a thin aluminum foil is much less than that of a spoon from the same metal. Unit: N/m.
- *Hardness (h)*: force over permanent (plastic) deformation. Values and unit depend on the experimental method used.
- *Strain* (ε): relative deformation. Unit-less.
- *Stress* (σ): force divided by area. It can be tensile or compressive. Unit: N/m^2, Pa, bar or atm.
- *Pressure (P)*: force divided by area. Unit: N/m^2, Pa, bar or atm.
- *Young's modulus (E)*: stress divided by strain. Intrinsic property of a linear elastic material, independent of the object shape or the measuring method. Same unit as stress.
- *Storage and loss modulus* (E', E''): measures of elastic (storage) and viscous (loss) component of the stress–strain response in viscoelastic solid. Same unit as stress.
- *Poisson's ratio* (υ): ratio of strain in one direction over strain in another. Measure of compressibility. Unit-less.

Models:

- *Analytical models* are purely mathematical models (equations) and can predict mechanical behavior if the system geometry is relatively simple (highly symmetrical). For example, the Hertz model was originally developed to describe the contact between two homogeneous elastic spheres. Variants of the Hertz model are used to analyze the contact between an indenter of known shape (cylindrical, pyramidal, conical) and an infinitely thick, flat piece of homogeneous linear elastic material.
- *Numerical simulations* are used when analytical models are too difficult to solve, often because of geometrical complexity. The *Finite Element Method (FEM)* is most often used for mechanical simulations because it can handle arbitrary geometries. The method can be used to predict local stresses and strains in cells and tissues. Thin objects such as the cell wall can be represented by a *membrane* (2D elements with no resistance to bending, no thickness), a *shell* (2D elements that possess bending stiffness) or solid elements (full 3D elements).

depends on both stress and enzymatic activity in the cell wall [21,31]. This behavior is enhanced in the presence of low extracellular pH, and has been called the *acid growth* theory [5,27,32]. Low pH is thought to enhance the activity of expansins, that cause cell wall remodeling and allow the wall to deform plastically, as well as modifying cell wall elasticity, making the walls less stiff [33]. Auxin is thought to trigger acid growth by enhancing ATPase activity and acidifying the cell wall. This appears to be independent of the TIR1 auxin signaling pathway [34,35•], triggering a direct short-term auxin growth response. However, extended growth induced by auxin likely depends on transcription and the downstream induction of the delivery of new wall material. The confluence of these processes and their interaction with turgor is what ultimately results in growth; however, a quantitative understanding of the mechanical changes that occur in the cell wall during growth is lacking [36]. Here we review recent developments in the measurement of the mechanical properties of plant cells and tissues, and the modeling techniques used to interpret the data.

Compression of single cells

A straightforward way to measure plant cell mechanical properties is to take single cells and compress them between two flat surfaces [33,37] (Figure 1). A force sensor records the load applied to the cell for a known displacement of one of the surfaces. The measured force will depend on several factors such as the size of the cell, wall thickness, cell wall elasticity and turgor pressure. In cases with simple cell geometries, it is possible to predict force–deformation curves from an analytical model of the cell. This has been done for the case of a single pressurized, liquid-filled spherical cell with a semi-permeable wall pressed between two plates [33]. Provided that initial parameters are known for the geometry of the cell, that water movement is neglected and that simple assumptions hold for the material model of the cell wall (linear, isotropic material, no bending stiffness, Poisson's ratio 0.4), we are left with two free parameters: turgor pressure and the Young's modulus. If either of these are known, the other can be calculated from the model fit of the force-displacement curve when large cell deformations are used [33,37]. Smith *et al.* [37,38] showed that a unique solution requires the measurement of water loss during the experiment, which for plant culture cells is likely to occur within seconds [39]. Thus without an accurate way to precisely measure the cell volume during compression, it is not possible to distinguish between purely elastic and viscoelastic or plastic behavior of the cell wall [37]. Similar

Table 1

Overview of indentation experiments reported recently in the literature

Publication	Experiments, cell/ tissue type	Tip shape and size; cell diameter (D), wall thickness (t)	Indentation depth (I); max. force (F)	Model output	Data interpretation	Effect of reduction in turgor pressure on results
Milani 2011	Nano-indention, arabidopsis shoot apex	Pyramid (4 sides), $\alpha = 17.5^\circ$; D = 5–10 μm, t = 250 nm	I = 40–100 nm; F = 10 nN[a]	Cell wall modulus	Hertz model for pyramidal tip	No effect, same modulus on turgid and flaccid cells
Radotic 2012	Nano-indention, arabidopsis culture cells	Pyramid (3 sides), $\alpha = 20^\circ$; D = 15–80 μm, t = 1200 nm	I = 80 nm; F = 1.4 nN	Cell wall modulus at different depths	Hertz model for pyramidal tip	Only turgid cells used
Hayot 2012	Dynamic nano-indention, arabidopsis leaf	Pyramid (3 sides), $\alpha = 60^\circ$; D = 7 μm, t = 500 nm	I = 110 nm; F = 40 μN	Apparent stiffness, apparent modulus, cell wall modulus	Hertz model + FEM simulations of upper cell wall under pressure	Large effect, apparent modulus greatly reduced in flaccid cell
Fernandes 2012	Nano-indention, arabidopsis root	Pyramid (3 sides), α = ?; D = 10–20 μm[a], t = ?	$I \sim$ 1800 nm[a]; $F \sim$ 30 nN[a]	Ratio of hardness on apparent modulus, plasticity index	Hertz model for pyramidal tip	No significant effect on most cells, lower force in growing region of root
Routier-Kierzkowska 2012	Micro-indention, onion epidermis	Round, 2–3 μm; D = 40 μm, t = 2 μm	I = 1–2 μm; F = 6–10 μN	Apparent stiffness	FEM model, pressurized 3D cellular tissue with multiple wall layers	Large effects on both global stiffness and local apparent wall stiffness
Peaucelle 2011	Micro-indention, arabidopsis shoot apex	Round, 1 and 5 μm; D = 5–10 μm, t = 250 nm	I = 0.5 μm; F = 1–2 μN[a]	Apparent modulus at different depths	Hertz model for sphere	Only plasmolyzed tissue used
Forouzesh 2012	Micro-indention, arabidopsis leaf	Round, 5 μm; D = 15–30 μm, t = 500–1000 nm[a]	I = 1.8 μm[a]; F = 25 μN[a]	Apparent stiffness, cell wall thickness, cell wall modulus, turgor pressure	FEM simulations of u pper cell wall under pressure	Large effect, apparent stiffness greatly reduced in flaccid cell
Zerzour 2009	Micro-identation, lilly pollen tube	Flat, 4 μm; D = 20 μm[a], t = ?	I = few μm; F = ?	Apparent stiffness	No model used	Only turgid pollen tubes
Bolduc 2006 (data from Parre 2005)	Micro-indention, pollen tube	Flat, 11 μm; D = 6 μm, t = 200 nm	I = 0.5–1 μm[a]; F = 1.5 μN[a]	Apparent stiffness	FEM model, cylindrical cell, no internal pressure	Only turgid pollen tubes used
Wei 2001	Ball tonometry, onion epidermis	Ball, 300 μm; D = 120 μm, t = ?	I = 1–2 μm; F = 500–700 μN	Internal pressure	P = force/(contact area)	Measurements valid for cells under large pressure
Smith 2000	Compression, yeast cell	Plate, D = 5 μm, t = 92 nm	I = 3.5 μm; F = 100 μN (at bursting)	Cell wall modulus, hydraulic conductivity, strain at failure	FEM model, spherical cell, pressurized, permeable wall	Large effect. Rise in pressure during compression reflected in the force measured
Wang 2008	Compression, tomato culture cell, effect of pH and expansin	Plate, $D \sim$ 60 μm[a], t = 126 nm	I = 12 μm[a]; $F \sim$ 800 μN[a] (linear range)	Cell wall modulus, strain at failure	Numerical model, spherical cell, pressurized	Large effect. Fast compression used (<1 s) to avoid water loss

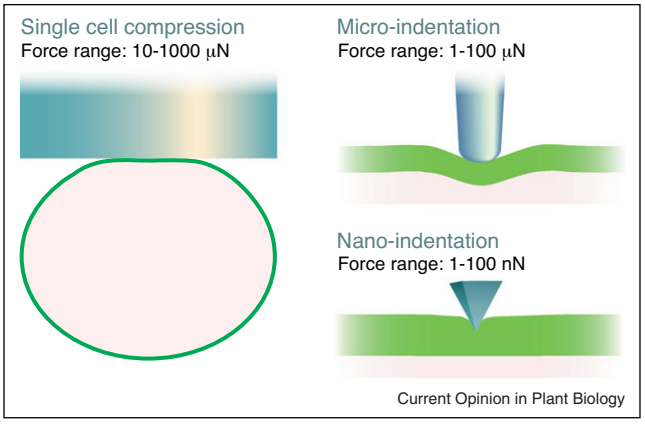
Diameters are given for flat and rounded tips. For pyramidal tips, α = tip semi-opening angle (°).
[a] Values estimated from figures.

model assumptions using small displacements with flat surfaces or large spherical indenters can be used to calculate pressure directly if the contact area can be accurately measured [40]. This is the principle behind ball tonometry [41,42].

Micro-indentation

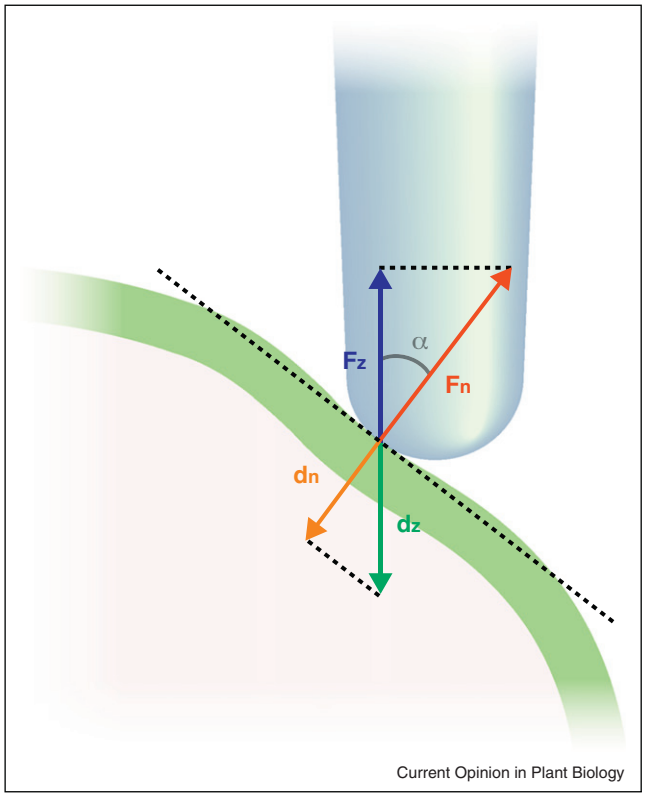
Micro-indentation can be used to measure and apply forces to both single cells and cells in intact plant tissues. Similar to compression experiments, micro-indentation involves monitoring the force needed to indent a small probe into the cell wall. The indenter is usually flat or rounded, a few micrometers in diameter (1–5 μm), and is typically used with indentation depths comparable to or larger than the cell wall thickness (see Table 1 and Figure 1). The force–displacement data from micro-indentation experiments are affected by a variety of factors. Cell and indenter geometry, turgor pressure, contact angle (Figure 2) and cell wall elasticity all play a role in the resulting measurements [43,44••,45]. As with compression experiments, analytical models can be used to extract values of elasticity and turgor pressure, but only for quite restrictive assumptions and very simple cell and indenter geometries. This is possible, for example, in the case of a spherical cell with a point load [46•]. More complicated geometries and a much wider range of assumptions can be accommodated with numerical models, such as those simulated with the finite element method (FEM) [37,43,44••,45]. This allows the analysis of how various geometric factors such as cell and indenter size and shape, contact angle, indentation depth and so on affect the measurement. If the indenter diameter is much larger than cell wall thickness, local compression of the cell wall can be neglected, and the wall can be modeled as a membrane (no bending stiffness) or shell [43,45,46•]. If the indenter diameter is similar to, or smaller than the cell wall thickness, the measurement is influenced by both local cell wall compression and a more a global deformation. To accurately model this situation, full 3D solid elements can be used in place of (or in conjunction with) shell or membrane elements (see Box 1). Routier-Kierzkowska *et al.* [44••] used this approach to model onion

Figure 1



Different indentation methods at various scales. In all cases an indenter deforms the cell while both position and the force applied are recorded. The size of the indenter tip and indentation depth relative to cell dimensions (size and wall thickness) determine what material properties are reflected in force-indentation data. Large cell deformations resulting from single cell compression are used to measure both turgor pressure and how stretchable (elastic) the cell wall is laterally [33,37]. Micro-indentation results are sensitive to turgor pressure, cell wall elasticity, cell and tip geometry as well as indentation angle [43,44••,45,56]. Nano-indentation is used to deform the cell wall locally [15••,50,52] and determine its elastic modulus mostly in the direction normal to the surface [15••]. Under some conditions, nano-indentation data can also be sensitive to turgor pressure [51••].

Figure 2



Effect of the indentation angle on force measurements. Plant tissues have a curved surface, and during micro-indentation or nano-indentation experiments the probe axis will not always be perpendicular to the cell surface. The probe tip indents the cell at angle α, which influences force-displacement data used to extract the sample elasticity. Only the component of the force in the axis of the probe F_z will be measured, and will be less than the actual force F_n for $\alpha > 0$. In addition, the vertical displacement d_z will be larger than the actual indentation depth d_n, again resulting in a lower stiffness measurement. In the case of frictionless contact illustrated here, the reaction force is normal to the surface. Friction between the tip and the sample would result in a higher force measured in the z direction and a higher stiffness measured on the slope. A lateral force is acting on the probe tip during indentation. If the indenter is long and flexible, it will bend by a small angle, resulting in a smaller indentation depth d_n. Hence, the tip bending stiffness, which depends on its shape and stiffness modulus, has to be taken into account in the measurements if long, thin probes are used.

epidermal cells. The mechanical effects of the presence of neighbor cells beside the cell being indented were considered in the model. It was shown that the level of turgor pressure influences both the global apparent stiffness and how local the measurement is for a given force. For higher pressure, cell wall deformation is restricted to a small region, mostly resulting in wall compression, whereas lower pressures result in a deeper indentation and a more global deformation the cell.

Nano-indentation

Nano-indentation is commonly used in material sciences to determine elasticity (Young's modulus) and plasticity (hardness) of solid materials (see Box 1 for terminology). A common device that can be used for nano-indentation is the Atomic Force Microscope (AFM). The AFM uses very precise piezoelectric actuators to impose a deformation (in the nanometer range) that is negligible compared to the specimen thickness. The force obtained during the deformation is determined by optically monitoring the deflection of a cantilever upon which a pyramidal tip is mounted. The sample elasticity is then determined is then determined by fitting the force–displacement curve to a mathematical model of the contact between tip and sample. Data from nano-indentation experiments are most commonly interpreted by using the Hertz model and its extensions [47,48]. The sample is approximated as an isotropic, linear elastic solid occupying an infinitely extending half space. It is assumed that the indenter is not deformable and that contact is frictionless. If these conditions are met the sample Young's modulus (also called elastic modulus, E) can be calculated provided that the indenter shape and Poisson's ratio are known.

Nano-indentation methods have been recently applied to living plant cells to measure elasticity and viscoelasticity of the primary cell wall [15••,49•,50,51••,52] (Table 1). Using AFM with a sharp indenter tip and small displacements (<100 nm), the cell wall modulus was directly extracted from experimental data [15••,52]. The idea is to deform the cell wall so locally that the effects of turgor pressure and the support by the underlying tissue would be negligible (Figure 1). Young's modulus was computed from force-deformation curves using a Hertz model for a pyramidal indenter shape. In order to apply the Hertz model, it is assumed that the experiment is equivalent to indenting a flat, homogeneous elastic material of infinite thickness. This assumption can be valid for very shallow indentations [15••,51••] that do not result in cell wall bending, or displacement of the inside surface of the outermost cell wall. To overcome this issue, Hayot and co-workers combined the classical Hertz-based interpretation with a finite element mechanical simulation that includes turgor pressure from inside the cell [51••]. Using a sharp pyramidal tip on a nano-indentation machine, they indented to a depth of 110 nm in order to avoid surface effects, such as the presence of a soft cuticle layer, adhesion and surface irregularities. Stiffness values were extracted from the slope of unloading curves close to maximal indentation and analyzed using an extended Hertz model [48]. The *apparent elastic modulus* values obtained reflect both cell wall modulus and the influence of other factors such as cell wall thickness and turgor pressure. An FEM model of the upper cell wall under pressure was then used to simulate indentation experiments. Values of Young's modulus for the cell wall were fit so that the simulation produced the same apparent modulus as in the experiments. The work of Hayot *et al.* [51••] showed that the apparent modulus extracted directly from experimental data using Hertz-based models could be as much as eight times lower than the elastic modulus of the cell wall estimated using FEM simulations.

Peaucelle *et al.* [49•] performed indentation experiments on the shoot apex using AFM. Their work can be classified as micro-indentation because they used indenter sizes of 1 μm and 5 μm. Experiments were done on plasmolized tissues at an indentation depth of 0.5 μm, and analyzed with a Hertz model. The apparent modulus extracted using the 1 μm and 5 μm indenters was interpreted by the authors as reflecting tissue elasticity at different depths, which does not satisfy the homogeneity constraint of the Hertz model. Nonetheless, if indentation parameters are kept consistent the method can be used to give a qualitative view of the relative stiffness of different areas in a sample.

Manipulation of turgor pressure

The manipulation of turgor pressure can also be used to measure cell wall stiffness by monitoring the change in cell size in relation to pressure changes. Unlike nano-indentation methods, the method is mostly sensitive to in-plane cell wall stiffness, and therefore can be used to test the influence of the cellulose microfibrils on cell expansion. For very large cells, turgor pressure can be manipulated directly with the pressure probe (reviewed by Tomos and Leigh [53]). By injecting or removing a known volume of liquid, the resulting pressure change can be used to determine the average cell wall stiffness [24,54,55]. In addition to direct measurements of turgor pressure, the pressure probe can be used to obtain internal osmotic potential [27] by withdrawing a sample of the cell contents and determining its freezing point. By following pressure changes over time, water permeability of the cell can be determined [55].

It is also possible to manipulate turgor by submersing plant tissue into solutions with different osmotic potentials [31]. This can be used with any cell size, and is less invasive than the pressure probe. Osmotic treatments require a means to precisely quantify the deformation of the tissue in response to change in osmolarity since, unlike turgor manipulation with the pressure probe, the

change in volume is generally not known. This can be a challenge for smaller cells, especially when the deformations are very small. Kierzkowski *et al.* [16••] have developed a method to precisely track surface expansion by combining osmotic treatments with confocal laser scanning microscopy. Using this method different zones of elastic behavior in the shoot apex were shown. Fast growing areas in the peripheral zone were much easier to expand with hypo-osmotic treatment than the slower growing cells in the central zone.

Conclusions

Indentation experiments on the nano-scale and micro-scale are being increasingly used to determine turgor pressure and cell wall elastic and viscoelastic properties, and in some cases these have been combined with genetic approaches [4•,49•,51••]. Depending on indentation depth and tip geometry, experimental results will be more or less sensitive to turgor pressure and cell wall elasticity [44••,46•,51••]. To extract these quantities, a model is required to fit the force-indentation curves. For indentations with very small tips at depths that are significantly smaller than the cell wall thickness, the measurement is mostly sensitive to wall elasticity in the direction normal to the surface [15••,52]. In this case the Hertz model or one of its derivatives can be used to interpret the experiments [15••,50,51••,52]. Larger indentations can be used to measure both turgor pressure and in-plane elasticity if the deformation is large enough to significantly stretch the cell wall [37,44••,51••]. However, except for very simple cell and indenter geometries, the interpretation of results is much more complex. To address this issue, engineering-style mechanical models of cells and tissues are used, with FEM simulation being the most common approach. Even if all of the geometric parameters in the model are known, the simulations typically still have too many free parameters to get a direct measurement of elasticity and pressure. To address this, some authors combine indentation experiments with other approaches, such as osmotic treatments or pressure-probe measurements [37,40,45]. In addition to pressure and elasticity of the cell wall, viscous and plastic behavior of the cell might also be observed, depending on the timescale of the experiments. This can come from several sources, such as water movement in and out of the cell and viscous or plastic behavior of the cell wall itself.

In the past few years several exciting new techniques have been developed to examine plant tissue mechanics in the context of morphogenesis. All of these techniques rely on mathematical and simulation models to interpret experimental results. Currently, quantitative models exist only for very simple geometries involving single cells. A future challenge is to extend these models to multi-cellular structures, such as the shoot and root apex, so that they can be used to interpret indentation experiments in plant organs. This, combined with genetic approaches, will be a crucial step towards understanding how genes and signaling networks regulate the mechanical properties of cells controlling growth and development.

Acknowledgments

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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