The stomatal flexoskeleton: How the biomechanics of guard cell walls animate an elastic pressure vessel

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Highlight

This article reviews progress on measuring and modeling the biomechanics of stomatal complexes and highlights knowledge gaps and promising future directions in the field.

Abstract

In plants, stomatal guard cells are one of the most dynamic cell types, rapidly changing their shape and size in response to environmental and intrinsic signals to control gas exchange at the plant surface. Quantitative and systematic knowledge of the biomechanical underpinnings of stomatal dynamics will enable strategies to optimize stomatal responsiveness and improve plant productivity by enhancing the efficiency of photosynthesis and water use. Recent developments in microscopy, mechanical measurements, and computational modeling have revealed new insights into the biomechanics of stomatal regulation and the genetic, biochemical, and structural origins of how plants achieve rapid and reliable stomatal function by tuning the mechanical properties of their guard cell walls. This review compares historical and recent experimental and modeling studies of the biomechanics of stomatal complexes, highlighting commonalities and contrasts between older and newer studies. Key gaps in our understanding of stomatal functionality are also presented, along with assessments of potential methods that could bridge those gaps.

Keywords: Stomatal Complex; Guard Cell Wall; Stomatal Geometry; Guard Cell Mechanics; Turgor Pressure; Finite Element Modeling

Introduction

Plants control gas exchange with their environment through stomatal complexes that consist of paired guard cells surrounding a stomatal pore of tunable size. The mechanical origin of stomatal opening and closure has been hypothesized to be a harmonious combination of controlled change in turgor and mechanical responses to this turgor change in the walls of the guard cells. Because the mechanics of stomatal complexes are the culmination of biochemical synthesis, cellular morphology, and biophysical processes, we refer to these as being biomechanical. Morphologically, the guard cell wall behaves as an elastic material because a guard cell pair can return to its original shape despite any potential non-linear or time-dependent responses, and it is a pressure vessel because it bears altered internal pressure (turgor) during stomatal function. Putting these characteristics together, the guard cell wall can be defined as a "flexoskeleton" (flexible exoskeleton), and its biomechanics contribute to the repeated and controlled dynamics of the stomatal complex to allow for efficient photosynthesis and water transport in plants.

There has been longstanding interest in understanding how the biomechanics of guard cell walls make it possible to animate guard cell pairs and regulate stomatal opening and closure. To that end, several mechanics-based biomechanical models of stomatal guard cells have been proposed (DeMichele and Sharpe, 1973; Aylor et al., 1973; Shoemaker and Srivastava, 1973; Cooke et al., 1976; Meckel et al., 2007) to explain how stomatal opening is regulated by the mechanical responses of guard cells in response to turgor changes. However, a complete picture has yet to be drawn that explains the causal mechanisms of stomatal opening from the standpoint of the guard cell wall. This incomplete mechanistic understanding is partly due to the complexity of biomechanical elements in guard cells and guard cell walls, including their diverse molecular structures, architectural configurations, and physiological control mechanisms.

The stomatal complex is a turgor-bearing structural system that is contained by guard cell walls, and both the geometric arrangement and the constitution of the guard cell wall contribute to stomatal function. To elucidate the biomechanical origins of stomatal dynamics, it is useful to dissect the geometry of the stomatal complex from the mechanical properties of the guard cell wall. At the same time, it is important to keep a holistic perspective when investigating the dynamics of stomatal complexes. *In silico* experimentation based on computational models is a

promising approach to investigate the holistic behavior of stomatal complexes that manifests the contributions of several different biomechanical elements.

As pointed out by Yi *et al.* (2018) and Marom *et al.* (2017), understanding the geometric configuration of a stomatal complex and its interaction with neighboring cells is important for explaining how the specific deformation patterns of stomatal guard cells facilitate the opening and closure of the stomatal pore. Acknowledging that stomatal behavior is a collective response of multiple elements such as stomatal complex shape, guard cell shape, cell wall morphology, interactions between sister guard cells and their neighboring subsidiary or pavement cells, and the mechanical properties of guard cell walls that reflect their molecular structures, it is imperative to account for the implications of any assumptions, idealizations, and/or simplifications employed during analysis of experimental and modeling data. Reflecting this idea, this review highlights longstanding and recent findings, models, and hypotheses concerning cellular and cell wall mechanics in stomatal complexes, with a focus on the implicit and explicit assumptions of these investigations, and describes potential future strategies for achieving a better understanding of the biomechanics of stomatal guard cells.

1 Key Parameters that Determine Stomatal Dynamics

A stomatal complex consists of a pair of guard cells that are each an elongated pressure vessel, in which the internal pressure of the guard cell is contained by its cell wall. Changes in turgor give rise to deformation of the guard cell wall, resulting in widening of the stomatal complex and the enlargement of the stomatal pore that lies between the guard cells. In this process, a guard cell pair interacts with neighboring subsidiary or pavement cells, whose turgor pressure is also presumed to change (Meidner and Edwards, 1975; Franks *et al.*, 1995). Therefore, the driving force of stomatal opening is the load arising from interactions between changes in turgor in both guard cells and neighboring cells. Stomatal complex kinetics results from a combined biomechanical reaction encompassing how guard cell walls deform in response to these turgor changes, and how guard cells support this driving force as a pair of conjoined and elongated cells.

A stomatal complex can be conceptualized as a biomechanical structure consisting of a connected pair of guard cells that deform under loads (forces) arising from inside and outside the cells. In studying the biomechanics of a stomatal complex, key aspects to consider include turgor changes in both guard cells and neighboring cells, external loads exerted by neighboring cells, the geometry and strength of the connections between the guard cells, the shapes of the guard cells, and the mechanical properties and physical dimensions of the guard cell walls. In the following sections, we review experimental and modeling studies concerning each point.

1.1 Turgor Pressure as the Driving Force for Stomatal Dynamics

Turgor pressure has been identified as the major physiological driver of stomatal opening and closure. Although the importance of turgor pressure in regulating stomatal dynamics is widely accepted, quantitative measurements of turgor pressure in stomatal guard cells are still limited. So far, turgor pressure has been experimentally determined only in a few plant species. For example, Meidner and Edwards (1975) measured the change in pressure potential required to open and closed stomata of *Tradescantia virginiana*, reporting a required turgor pressure change of ~700 kPa between 100 and 800 kPa. More recently, Franks *et al.* (1998, 2001) reported that turgor pressure differences up to 5 MPa are required to open stomata in *Tradescantia virginiana L.*, *Vicia faba L.*, *Nephrolepis exaltata (L.) Schott*, and *Ginkgo biloba L.*, using an improved pressure probe and microscope. These experimental measurements of turgor pressure were conducted for relatively few cells, and these results have not been replicated for other species. Methods for high-throughput, quantitative measurements of turgor pressure in guard cells and neighboring epidermal cells are sorely needed as inputs for modeling tools that are now available to study the biomechanics of stomatal opening.

Turgor pressure change in *Arabidopsis thaliana* pavement cells has been measured using nanoindentation by Forouzesh *et al.* (2013). However, quantitative measurements of turgor pressure in *Arabidopsis* guard cells have not yet been reported. Methods to measure turgor pressure *in vivo* in *Arabidopsis* guard cells, combined with ever-expanding genetic tools in this species, will be a very powerful advance in resolving uncertainties about the precise, systematic mechanisms of how plants control stomatal dynamics. For example, Glinka (1971) and DeMichele and Sharpe (1973) showed that neighboring cells can dominate stomatal dynamics,

despite having a lower turgor pressure than guard cells as reported by Meidner and Mansfield (1968), because the neighboring cells have a mechanical advantage over guard cells due to their turgor acting over a larger surface area. However, few experimental studies address the question of whether the contributions of turgor changes in guard cells and subsidiary cells are equivalent, or if the turgor changes in neighboring cells, in fact, dominate stomatal dynamics.

In addition, it is important to note that the aqueous fluids inside guard cells are assumed to be incompressible liquids. More precisely, the volume change of water will be as small as 0.2% with a 5 MPa increase when the temperature is maintained at 25°C. Therefore, the increase in guard cell volume that occurs during stomatal opening (Meckel *et al.*, 2007; Tanaka *et al.*, 2007) happens not only because of an increase in turgor but also because of an increase in the amount of intracellular fluid. To maintain turgor as a guard cell enlarges during stomatal opening, fluid influx should be sustained at a high rate. The biomechanical signaling pathways that regulate and maintain increased turgor as the guard cell enlarges have yet to be elucidated.

Accepting this incompressibility assumption and the consequential independence of guard cell volume change from turgor change, one should not confuse the cell volumetric modulus (ε), as presented in Yang, Zhao, and Zhu (2011), with the biomechanical stiffness of the whole guard cell. As discussed by Cosgrove (1988), 'cell volumetric modulus (ε)' pertains to the water capacitance of a cell and not to its material stiffness.

1.2 Interaction with Pavement Cells and External Load From Pavement Cells

In either scenario of mechanical advantage or turgor differences being the dominant biomechanical mechanism underlying stomatal dynamics, interactions between stomatal guard cells and neighboring cells are important. Biomechanical interactions between guard cells and neighboring cells can include both support and constraints. This is important because these interactions influence the degree of deformation for guard cells, thus influencing pore dynamics. For example, the difference in the amount of potential deflection for a simply supported beam (Figure 1A) vs. a redundantly supported (fully clamped) beam (Figure 1B) is substantial. When differentially supported beams of identical geometry are subjected to identical amounts of pressure across the whole span of the beam, a simply supported beam undergoes five times the deflection of a fully clamped beam, even in the case of a relatively simple beam deflection model

such as Euler-Bernoulli beam theory (Timoshenko and Goodier, 1951) that predicts deflection for non-slender beams, e.g., its span length is smaller than 20 times its thickness.

In computational modeling studies, guard cell-neighbor interactions are modeled as distributed loads (pressure) from neighboring cells on the guard cells (Cooke *et al.*, 1976; Woolfenden *et al.*, 2017; Yi *et al.*, 2018) or are not explicitly considered (Marom *et al.*, 2017). Thus, there is no consensus on the magnitude and importance of the biomechanical influence of neighboring cells on guard cells during stomatal opening and closure. Woolfenden et al. (2017) state that epidermal pressure has a minor effect on stomatal opening, whereas Yi et al. (2018) showed that the same cell wall properties as those used in Woolfenden et al. (2017) result in different amounts of stomatal opening, depending on the presence or absence of constraints from surrounding pavement cells.

Again, experimental measurements of turgor pressure for guard cells and neighboring cells will help clarify this issue. It is likely that the middle lamella, which conjoins guard cells and neighboring cells, transduces force between the cells. Considering the impacts of support and constraints on the potential for cellular deformation (Figures 1 and 2), the biomechanical interactions between guard cells and neighboring cells might play an important role in the precise control of stomatal opening, especially because the neighboring cells continuously maintain contact with the guard cells during stomatal opening and closure and are potential sources or sinks for fluid that enters and leaves the guard cells. For example, when comparing the maximum deflection between a structure that is freely deflecting due to a distributed load (Figure 2A and 2C) and a structure that is supported by another deformable medium (Figure 2B and 2D), the latter will show much less deflection.

The developmental and physiological pathways that lead to and result from mechanical interactions between guard cells and neighboring subsidiary cells in grasses have been well defined. For instance, Raissig et al. (2017) demonstrated that subsidiary cells affect guard cell movements and stomatal physiology. Other studies examined possible mechanisms of active contributions of neighboring cells to stomatal dynamics through regulating shuttle transport (Raschke and Fellows, 1971; Chen *et al.*, 2017). However, the exact nature of the mechanical interactions between guard cells and pavement cells, and the dynamics of turgor pressure in

neighboring cells during stomatal opening and closure, have yet to be quantified (Franks and Farquhar, 2007).

1.3 Connections Between Sister Guard Cells

The way that structural elements are arranged, supported, and constrained influences any structural system's overall behavior, as illustrated in Figure 1. Likewise, the strength and flexibility of the junctions between guard cells, which are mediated by the middle lamella, as well as the areas of these stomatal junctions should play an important role in determining the amount of stomatal opening. The effects of the geometric and bio-mechanical configurations between sister guard cells have not been studied extensively. A recent finding of polar stiffening in stomatal complexes might be related to the need for junctional stiffening to achieve optimal stomatal dynamics (Carter *et al.*, 2017).

1.4 Guard Cell Geometry

Conceptualizing the stomatal complex as a biomechanically operated structural system, the ratio between guard cell length and diameter is important in that it constrains the degree of stomatal opening. For example, as shown in Figure 3, a narrower guard cell (Figure 3A) will be easier to deform laterally than a wider guard cell (Figure 3B).

An equally important aspect is the overall shape and size (geometry) of the stomatal complex. As demonstrated in Yi et al. (2018), even with identical mechanical properties in the cell wall, differences in the overall shape and size of stomatal complexes result in different stomatal opening under the same amount of turgor increase.

In an early computational study by Cooke et al. (1976), the stomatal complex was modeled as an elliptical torus. Despite recent advances in our ability to build computational models of complex geometries, seemingly idealized geometric shapes are often used to model stomatal complexes. For example, more recent computational modeling studies have used symmetric and well-defined geometric shapes (Cooke *et al.*, 2008; Marom *et al.*, 2017; Woolfenden *et al.*, 2017, 2018; Carter *et al.*, 2017). However, close observation of reconstructed 3D images of stomatal complexes reported in Zhao and Sack (1999), Meckel et al. (2007), and Yi et al. (2018) reveals that stomatal complexes are not all identical and are neither perfectly symmetrical nor perfectly elliptical or

toroidal. Recently, Yi et al. (2018) developed computational models directly from 3D confocal images of stomatal complexes that accurately reflect their irregular shapes. It is expected that similar approaches in developing computational models from real stomatal complexes will lead to new insights into the biomechanical implications of these geometric irregularities.

Another key property that directly influences the biomechanical behavior of stomatal guard cells, is the thickness of the guard cell wall. It is possible that varying cell wall thickness is an adaptation to cope with regions of the stomatal complexes that experience higher stress during stomatal opening. This is illustrated by walls that are thicker on the ventral sides of the guard cells, which abut the stomatal pore, than on the dorsal sides, which abut neighboring cells, and at the poles of the guard cells (Zhao and Sack, 1999). It has been hypothesized that a thicker ventral wall is essential for achieving stomatal opening (Ayolar et al. 1973; DeMichele and Sharpe, 1973), although this hypothesis has been questioned by Cooke et al. (1976) and Carter et al. (2017).

1.5 Biomechanical Properties of Guard Cell Walls

The turgor pressure that results in stomatal opening is borne by the guard cell wall. Recent research has focused on understanding how plants construct guard cell walls that are strong enough to withstand high turgor pressure (measured as high as 5 MPa) and yet flexible enough to repeatedly expand and shrink. Most of these studies have focused on the thickness and mechanical properties of guard cell walls (Marom *et al.*, 2017; Woolfenden *et al.*, 2017, 2018; Carter *et al.*, 2017; Yi *et al.*, 2018). Studies concerning those two aspects are reviewed below, highlighting their biomechanical implications.

The specific mechanical properties of guard cell walls originate from their molecular structures. The functions of different wall polysaccharides in guard cell walls have been studied using *Arabidopsis* mutants (Rui and Anderson, 2016; Woolfenden *et al.*, 2017; Yi *et al.*, 2018). This approach has great potential to elucidate the genetic origins of stomatal function. To overcome the experimental challenge of manipulating and measuring the relatively small stomatal complexes of *Arabidopsis*, similar approaches could be applied to other plants with larger stomatal complexes, for example *via* genome editing.

Because guard cell deformation is reversible, guard cells and their walls are presumed to behave elastically, often assuming a linear response to biomechanical stimuli. The molecular origin of such reversibility has been explored and attributed in part to the flexibility of pectic polysaccharides in the wall (Jones *et al.*, 2003, 2005). However, the proportionality between turgor change and the amount of deformation in the stomatal complex may or may not remain linear over the entire range of deformation (Franks *et al.*, 2001). Since experimental observations of stomatal dynamics mostly include measurements of static "closed" and "open" states, this non-linearity may have been previously overlooked.

However, advances in imaging techniques have provided new insights into wall structure in guard cells (Majewska-Sawka *et al.*, 2002; Merced and Renzaglia, 2014; Rui and Anderson, 2016; Amsbury *et al.*, 2016; Shtein *et al.*, 2017) and stomatal dynamics (Rui *et al.*, 2017), drawing new connections between stomatal dynamics and the composition and organization of guard cell walls. Leveraging such knowledge, material models of guard cell walls can be parameterized and used to investigate the biomechanical attributes of their molecular structures.

For example, Woolfenden et al. (2017) used a two-phase hyper-elastic material model to match the nonlinear stomatal opening reported by Franks et al. (2001). Woolfenden et al. (2017) showed that strain-stiffening of guard cell walls is potentially independent of wall anisotropy. However, this study did not explore how this strain-stiffening is linked to the molecular structure of the guard cell wall.

One important point to note in modeling the biomechanics of stomatal complexes is that the amount of deformation during stomatal dynamics is large enough to change the configuration of the applied load. Therefore, deformation needs to be accounted for during calculations of the loads that result in the final geometry of the complex in response to stimuli. In a modern computational modeling environment, this is achieved by considering the geometric non-linearity of the stomatal complex (Marom *et al.*, 2017; Woolfenden *et al.*, 2017; Yi *et al.*, 2018).

Biomechanical anisotropy in the molecular structure of the guard cell wall is one key aspect of the wall. Wall anisotropy due to the patterning of cellulose, which is radially wrapped around the guard cell, is well-accepted. Most studies concerning wall mechanics in guard cells assume bidirectional anisotropy between the longitudinal and transverse directions. Several studies

employing a computational approach (Cooke et al., 1976; Marom et al., 2017; Woolfenden et al., 2017; Yi et al., 2018) considered the guard cell wall as bidirectionally anisotropic between the direction of cellulose wrapping and the orthogonal organization of wall matrix components that are intercalated between cellulose fibers.

Given that the cell wall is a three-dimensional structure with considerable thickness, mechanical behaviors in the thickness direction of the wall should also be considered. For a typical guard cell of *Arabidopsis*, the wall can be as thick as 2 μm, whereas the diameter of a guard cell is in the range of 5-10 μm (Yi et al. 2018). This is well above the recommended ratio (1:20) for assuming that the guard cell wall behaves as a "thin" or "slender" structural element. It is also important to consider whether the guard cell wall changes in thickness during stomatal opening. This question may relate to the unaddressed conundrum of why guard cell diameter does not change during opening, as reported in (Rui and Anderson, 2016).

Thus, when considering the anisotropic biomechanical behavior of the guard cell wall, three-dimensional anisotropy, rather than bidirectional anisotropy, can be presumed. For example, wood, which contains anisotropic layers of cellulose and anisotropic cell shapes, is often modeled as a three-dimensional orthotropic material at the macroscopic scale (Gillis, 1972). Such an approach can reflect the specific architectural arrangements of wall components in guard cells (Woolfenden *et al.*, 2017; Yi *et al.*, 2018), such as the highly anisotropic, radially aligned cellulose that encircles guard cells (Fujita *et al.*, 2013), plus the arrangements of matrix components between cellulose and the spacings and differential orientations between wall lamellae.

In addition to anisotropy in the composition and architecture of the wall, the spatial distribution of wall properties, including thickness and biomechanics, across guard cells should be recognized. For example, Carter et al. (2017) reported a non-uniformity of biomechanical properties in the guard cell wall and found that polar regions exhibit elevated stiffness. The authors proposed that polar stiffening of guard cells is mechanically more important than radial thickening for the regulation of stomatal opening. This observation corroborates the “importance of the micellation on the polar section,” which was proposed by Aylor et al. (1973).

It is also possible that this observation is related to guard cell geometry rather than or in addition to the biomechanics of guard cell walls. Because the mechanical measurements of Carter et al. (2017) were performed on leaf blocks, it is possible that their observations are attributable at least in part to the geometric properties of guard cells rather than solely to the biomechanical properties of their walls. These experiments were carried out with atomic force microscopy (AFM)-based nano-indentation and data analyses that were developed to measure material properties in flat samples. However, some limitations of the technique are likely to affect the interpretation of data. For example, if the sample is not flat, either due to the shape of the object itself or from it being imperfectly laid on a substrate, the structural configuration of the sample will affect the measurements. In addition, mechanical contributions from underlying cell layers and the mechanics of the guard cell protoplast might obfuscate measurements of cell wall mechanics *per se* using this approach.

Despite these potential limitations, computational modeling combined with mechanical experiments using AFM-based nano-indentation is a very promising approach in advancing our understanding of the biomechanical origins of stomatal behaviors. Among the assumptions embedded in computational modeling studies, accurate values for turgor pressure changes in guard cells and neighboring cells in genetically manipulatable species might be the most impactful new information. Improved microscopy and image analysis will also eliminate uncertainties in the interpretation of experimental results by producing more accurate geometric parameters for guard cells and neighboring cells, including spatially varying wall thickness, cross-sectional areas, sizes and shapes of guard cell junctions, guard cell asymmetries, and overall shapes of stomatal complexes.

2 Mechanisms Underlying Stomatal Dynamics

Putting together all of the elements of the stomatal complex, this section reviews the potential mechanisms that underlie stomatal dynamics. Essentially, stomatal opening is achieved by specific deformations of guard cells. Assuming this deformation is initiated by turgor increase, guard cell enlargement and deformation is likely to be a major driving mechanism of stomatal kinetics. Based on the apparent shapes of un-deformed and deformed guard cells during stomatal opening and closure, mechanical behavior similar to that of a structural beam, which supports

imposed forces by flexural bending resistance (Figure 4A), has been hypothesized to be the mechanism of stomatal opening.

An early experimental study by Aylor *et al.* (1973) proposed that constraints on the junctions between paired guard cells, the circumferential arrangement of cellulose reinforcements in guard cell walls, and reinforcement of ventral walls, which surround the stomatal pore, contribute to stomatal opening. The main idea of Aylor *et al.* (1973) is that flexural bending of guard cells arises from an unsubstantiated force along the "constrained ventral wall." A similar idea, that the existence of a neutral axis located closer to the ventral region as the basis for the development of a bending moment, was proposed by DeMichele and Sharpe (1973) and Shoemaker and Srivastava (1973). Simply put, this misalignment between the geometric center of the guard cell and the neutral axis gives rise to coupled forces, creating a bending moment (Figure 4A). To substantiate this conjecture, one should experimentally demonstrate the existence of such a neutral axis on the guard cell. To that end, an experimental study using fiducial markers as used in Kim *et al.* (2015) deposited on the guard cell wall could be conducted to monitor subcellular deformation patterns during stomatal opening and closure. Such an experimental study would also be useful to validate subcellular deformations calculated in computational modeling studies.

Another important aspect of the conjecture that guard cell bending is the origin of stomatal pore opening is the origin of the forces that act on the guard cells and give rise to the bending moment. DeMichele and Sharpe (1973, 1974), Wu and Sharpe (1978; 1979), and Wu *et al.* (1985) explain that the bending moment arises from "forces exerted on the cell walls" by increased turgor. This is because the isotropic and uniform turgor pressure of the cytoplasm is contained and supported by the cell wall, which is not structurally uniform due to its spatially varying thickness and composition. DeMichele and Sharpe (1973) and Shoemaker and Srivastava (1973) hypothesized that this variance creates a pair of forces along the guard cell at a distance, resulting in a bending moment and causing a flexural deflection of guard cell and eventual stomatal opening.

In addition, neighboring cells acting as a uniformly distributed lateral load on the dorsal walls of the guard cells make them act as beams under a distributed load and further promote guard cell deformation and stomatal opening (Figure 2). The "mechanical advantage" of guard cells over

neighboring cells is thought to amplify the amount of guard cell deformation, allowing stomatal opening (DeMichele and Sharpe, 1973). As a result, the guard cell will flexurally bend toward the neighboring cells. Similarly, Aylor *et al.* (1973) and Shoemaker and Srivastava (1973) hypothesized that guard cells bend when turgor increase introduces an axial force along the geometric axis of a guard cell, which does not coincide with its neutral axis.

Some studies have attributed the driving mechanism of stomatal dynamics to axial elongation of guard cells arising from anisotropic biomechanical behavior of their cell walls (Meckel *et al.*, 2007; Woolfenden *et al.*, 2017; Yi *et al.*, 2018) or localized stiffening of the cell or wall (Carter *et al.*, 2017). Meckel *et al.* (2007) reported that the elongation of guard cells is the origin of stomatal opening and argued that such elongation might happen near the guard cell junctions. However, hypotheses of stomatal dynamics involving the flexural bending of guard cells have neither been experimentally substantiated nor rejected. Recent advances in technologies that allow for mechanical manipulation and measurements of microscopic or nanoscale materials might provide the requisite quantitative data. In particular, direct mechanical experiments on guard cells or cell walls, combined with simultaneous measurements of turgor pressure changes *in vivo* in stomatal complexes, would be especially helpful in advancing our understanding of how plants regulate stomatal function.

2.1 Deformation of Guard Cells During Stomatal Dynamics

When one considers flexural bending as a potential driving mechanism of stomatal opening, not all of a guard cell cross-section, i.e., a section orthogonal to the longitudinal axis of the guard cell, is presumed to be in a compressive or tensile stress state. This is because the intracellular fluid is assumed to be incompressible and there should not be a stress gradient across this fluid, according to Pascal's law. Thus, compressive or tensile stress only develops in the solid walls of guard cells, and this fact reduces the likelihood that a flexural bending moment is solely responsible for guard cell deformations during stomatal dynamics. In addition, the intrinsically arched shape of a guard cell reacts differently to lateral forces compared to a traditional beam, which has a straight profile when it is not loaded. For an arch-shaped structure (Figure 4D), lateral forces are supported by an axial compressive stiffness (Karnovskiĭ, 2012). Therefore, axial deformation, i.e., elongation, might act as a dominant stomatal opening mechanism

(Meckel *et al.*, 2007). This hypothesis can also explain the reported volume increase in stomatal guard cells during stomatal opening (Franks *et al.*, 2001; Meckel *et al.*, 2007), wherein the guard cell width does not increase (Rui and Anderson, 2016; Yi *et al.*, 2018).

2.2 Do Changes in the Cross-sectional Shapes of Guard Cells Occur During Stomatal Dynamics?

Another notable potential mechanism for stomatal opening is deformation in the guard cell cross-section. Cooke *et al.* (1976) argued that stomatal opening is caused by changes in the cross-sectional shape of a guard cell when it is pressurized, progressing from an ellipse with a major axis parallel to the leaf surface to an ellipse with a major axis perpendicular to the leaf surface. To support this model, there must be a substantial amount of out-of-plane bulging of guard cells during stomatal opening, which has not been experimentally substantiated (Meckel *et al.*, 2007). Also, it should be noted that the cross-sectional shapes of guard cells in both open and closed states are not always consistent and not geometrically simple, as shown in (Zhao and Sack, 1999).

2.3 Temporal Considerations for Stomatal Dynamics

Stomatal response time affects how well a plant can adapt to fluctuating environmental conditions by regulating the gas exchange rate at the plant surface (Franks and Farquhar, 2007). In addition, the biological time scales of stomatal opening and closing influence experimental measurements of guard cell responses. However, the impacts of time scales on stomatal dynamics have not been studied as extensively as other influences on stomatal dynamics.

Moreover, studying the temporal responses of stomatal dynamics can provide novel insights into how plants regulate stomatal function as shown in Rui *et al.* (2017). The challenge in interpreting the contributions of time-dependent biophysical processes (Chen *et al.*, 2012; Hills *et al.*, 2012) and biomechanical processes (Rui *et al.*, 2017) to guard cell responses is that they are poorly defined. In particular, the biomechanical underpinnings of time-dependent responses in guard cells have yet to be quantified. For example, it is well known that grass stomata exhibit faster opening and closing responses than eudicot stomata (Chen *et al.*, 2017). The dumbbell shapes of grass guard cells and the particular arrangement of their subsidiary cells are often hypothesized

to be the origin of such rapid stomatal responses. However, the exact contributions of guard cell shapes, and the physiological or mechanical interactions between guard cells and subsidiary cells, to the time scales of stomatal dynamics remain to be elucidated.

3 Future Directions and Unanswered Questions

Despite reports of guard cell elongation during stomatal opening (Meckel *et al.*, 2007), the uniformity or localization of such elongation has not been substantiated. Intuitively, the shapes of stomatal complexes and the anisotropic mechanical responses of guard cells walls to turgor changes should both contribute to stomatal dynamics. Quantitative studies of changes in guard cell geometry during stomatal opening and closure (Meckel *et al.*, 2007; Rui and Anderson, 2016; Amsbury *et al.*, 2016; Woolfenden *et al.*, 2017; Yi *et al.*, 2018) suggest that anisotropic guard cell deformation upon turgor increase seems to be a key requirement of stomatal kinetics, but these observations have not yet been extended into three dimensions at the cell wall scale while accounting for the molecular-scale architecture and biomechanical properties of guard cell walls to elucidate how plants construct guard cell walls to achieve their uniquely elastic behaviors during repeated rounds of stomatal opening and closing.

In addition, one of the major impediments for studying the biomechanics of stomatal complexes is the lack of direct methods for characterizing wall mechanics and turgor pressure simultaneously on a dynamic basis. Recent developments in nanotechnology and imaging are beginning to provide useful tools toward this goal. There have been many studies using AFM to examine the structure and biomechanics of plant cell walls (Peaucelle *et al.*, 2011; Hayot *et al.*, 2012; Digiuni *et al.*, 2015; Yakubov *et al.*, 2016). In particular, Sampathkumar *et al.* (2014) used AFM and confocal microscopy to image stress distributions and microtubule arrangement patterns in stomatal guard cells and pavement cells. Similarly, Carter *et al.* (2017) used AFM to image stiffness distributions in the *Arabidopsis* epidermis and reported higher levels of mechanical resistance near stomatal poles. Furthermore, Forouzesh *et al.* (2013) estimated turgor pressure by combining nanoindentation measurements with finite element modeling, and this approach could prove useful in determining how turgor pressure changes might initiate and/or sustain stomatal dynamics. It should be noted that AFM can also be used for imaging in addition

to probing cell wall mechanics, thus providing both geometric and mechanical information (Ding and Himmel, 2006; Kafle *et al.*, 2013; Zhang *et al.*, 2014, 2016; Sampathkumar *et al.*, 2014).

Mechanical indentation tests were first developed to determine the mechanical properties of materials under the assumption that the indentation tip geometry and indentation depth are much smaller than the test sample. This is an important assumption of Oliver and Pharr (1992), which has been widely used in estimating mechanical properties from AFM measurements. For a soft film material, a test sample is usually mounted on a rigid surface to ensure that indentation only occurs in the sample material at the point of contact and does not involve large-scale deformation of the sample. However, the effect of the mechanical properties of the mounting surface should be accounted for (Oliver and Pharr, 1992). It is difficult to prepare samples of plant cell walls to meet these requirements. Although a nano-indentor or AFM tip can be very small in size (2-10 nm), the indentation depth used for mechanical measurements is often larger than 500 nm, which becomes comparable to an entire *Arabidopsis* guard cell (5-10 μm in diameter). Moreover, even though one can assume that the cytoplasm is incompressible, indentations comparable to the thickness of the cell wall (~2 μm in *Arabidopsis*) will induce overall deformation of the guard cell in addition to the local indentation, which will complicate the interpretation of the force measurements. Incorporating geometrically accurate finite element modeling into the interpretation of AFM data is one possible way to address these issues (Forouzesh *et al.*, 2013).

Scarcelli *et al.* (2015) demonstrated the ability of Brillouin spectroscopy to map the longitudinal moduli of living animal cells. Similarly, Elsayad *et al.* (2016) used Brillouin spectroscopy to measure mechanical properties and fluorescence of *Arabidopsis* epidermal cell walls at the sub-micrometer scale. Brillouin spectroscopy analyzes the scattering of light from the long-wavelength thermal, acoustic modes in a solid and from random thermal density fluctuations in a liquid or gas (Dil, 1982). From this measurement, one can estimate elastic constants, bulk modulus, and bulk viscosity from the hypersonic transport coefficients determined by viscoelastic properties of the scattering materials. Since Brillouin spectroscopy is a non-contact measurement and has subcellular resolution, this approach appears to be a promising experimental method. However, in Brillouin spectroscopy, mechanical properties are estimated from the dynamic responses of a material. This means that the estimations are inherently indirect and include assumptions specific to a particular range of force-displacement responses.

Therefore, compared to mechanical experiments that impart forces or measure displacements, e.g., experiments using a nano-indentor or AFM, the relevance of estimated mechanical properties using Brillouin spectroscopy to static or long-timescale elastic moduli should be carefully interpreted from a biomechanical standpoint.

4 Conclusion

These are exciting times for research into the biomechanical mechanisms by which stomatal guard cells achieve their amazingly durable and responsive behaviors in plants. Future work delving into the molecular architecture of the guard cell wall, in combination with new measurements of the dynamic mechanics of guard cell walls and the hydraulics of stomatal complexes and neighboring cells, plus the refinement of modeling approaches to capture and predictively quantify these data, will open up new avenues of understanding and provide engineering strategies to optimize stomatal responsiveness, allowing plants to manage water and maximize photosynthesis to produce food, materials, and bioenergy for human use. By unlocking the biomechanical puzzle box that is embodied by stomatal complexes, we can also potentially develop new architectural strategies to build robust macro-scale structures that mimic the unique strength and flexibility of these amazing cellular machines.

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Figure Legends

Figure 1. The configuration of end supports of structural elements can affect the deformation of those elements. Balls on left sides depict roller supports that allow horizontal and rotational movement of a beam on that end. Triangles and balls on right sides depict hinge supports that allow rotational movements only. End supports of a beam are similar to the middle lamellae at guard cell junctions (C and D). Light blue middle lamellae depict softer supports, whereas dark blue middle lamellae depict stiffer supports. Undeformed guard cells in light green and deformed guard cells in dark green are overlapped in the righthand column to highlight predicted differences in stomatal pore opening due to end support conditions. A. A simply supported beam will deflect when loaded. B. When both ends are rigidly supported and are not allowed to move or rotate, the overall deformation of the beam will be less than that for a simply supported beam. C. Similarly, if stomatal junctions allow for movements of guard cells near the junction area, guard cells are freer to deform when turgor increases. D. On the other hand, if stomatal junctions are constrained during stomatal opening, the degree of stomatal opening will be limited.

Figure 2. Underlying support of structural elements will limit the deformation of those elements. The configurations of end supports are the same as shown in Figure 1A. Undeformed guard cells in light green and deformed guard cells in dark green are overlapped in the righthand column to highlight predicted differences in stomatal pore opening. A. A simply supported beam will deflect when loaded. B. When the same structure overlies continuous support, the amount of deflection is limited. C. Analogously to a simply supported beam, when guard cells are modeled without lateral support, they are freer to deform when turgor increases. As a result, the stomatal opening (hatched area) is overestimated. D. When constraints from pavement cells are considered, guard cell deformation is limited. As a result, the stomatal opening (hatched area) gets smaller. E. Projections of confocal z-stacks of propidium iodide (PI)-stained *Arabidopsis thaliana* stomatal complexes. Because each complex is surrounded by neighboring pavement cells as depicted with yellow arrows, accounting for biomechanical interactions between

stomatal guard cells and pavement cells is important for accurately modeling stomatal dynamics. Bars = 5 μm.

Figure 3. The shapes of structural elements affect their ability to deform. The configurations of end supports are the same as shown in Figure 1A. Undeformed guard cells in light green and deformed guard cells in dark green are overlapped in the righthand column to highlight predicted differences in stomatal pore opening. A. When a structural element is slender, i.e., its length is much larger than its thickness, its deflection is supported by a combination of compressive stiffness on the upper side and tensile stiffness on the lower side. B. For a thicker structural element, support also comes from the thickness direction that coincides with the lateral load direction. This means that shear resistance also limits the deformation of the beam under load. Similarly, when modeling, slender guard cells (C) and wider guard cells (D) should use an appropriate mechanical model that considers shear stiffness. The same consideration should be applied to guard cell walls. Where the guard cell wall is significantly thick, e.g., when it is larger than 1/20 of the length of a guard cell, as occurs in *Arabidopsis*, the effect of shear should be accounted for.

Figure 4. Studies hypothesize flexural bending or elongation of guard cells to be driving mechanisms of stomatal dynamics. The configurations of end supports are the same shown as in Figure 1A. A pair of forces acting in the opposite direction at a certain distance creates a moment (A). When two forces act in a downward direction at each end of a beam and the center is supported by a hinge (B), the resultant force at the center and the force at each end act in the opposite direction and create a bending moment. C. When moments act on a beam, that makes the beam flexurally deflect, therefore these are called ‘bending moments’ (C). D. Applying a bending moment model to guard cells, internal turgor pressure applies forces at each end along the midline of the guard cell (dashed line), whereas the reactive force of the guard cell wall is near the ventral side due to the ventral wall being thicker than the dorsal wall. These two forces and their misalignment creates a bending moment (DeMichele and Sharpe, 1973; Aylor *et al.*, 1973; Shoemaker and Srivastava, 1973). E. Forces on the dorsal wall are hypothesized to act in an outward direction due to the mechanical advantage of guard cells over neighboring cells, and this force is transferred to the ventral side due *via* circumferentially arranged cellulose, resulting in ventral deformation (DeMichele and Sharpe, 1973). F. Considering that closed guard cells

remain arched, the guard cell wall can be hypothesized to act as an axially loaded arch where radial (cell wall thickness) expansion is limited by circumferentially arranged cellulose, whereas axial extension drives changes in arch curvature due to wall anisotropy. This explanation is consistent with the observations of Meckel et al. (2007) and Yi et al. (2018).

Figure 1

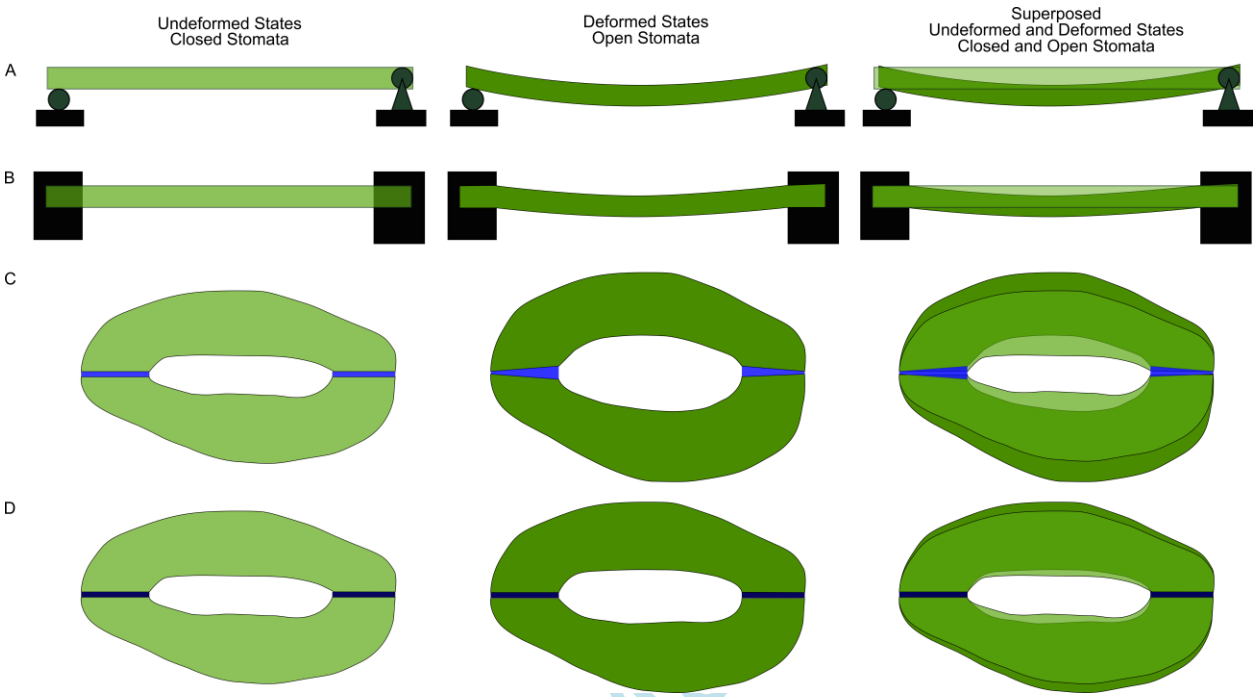

Figure 2

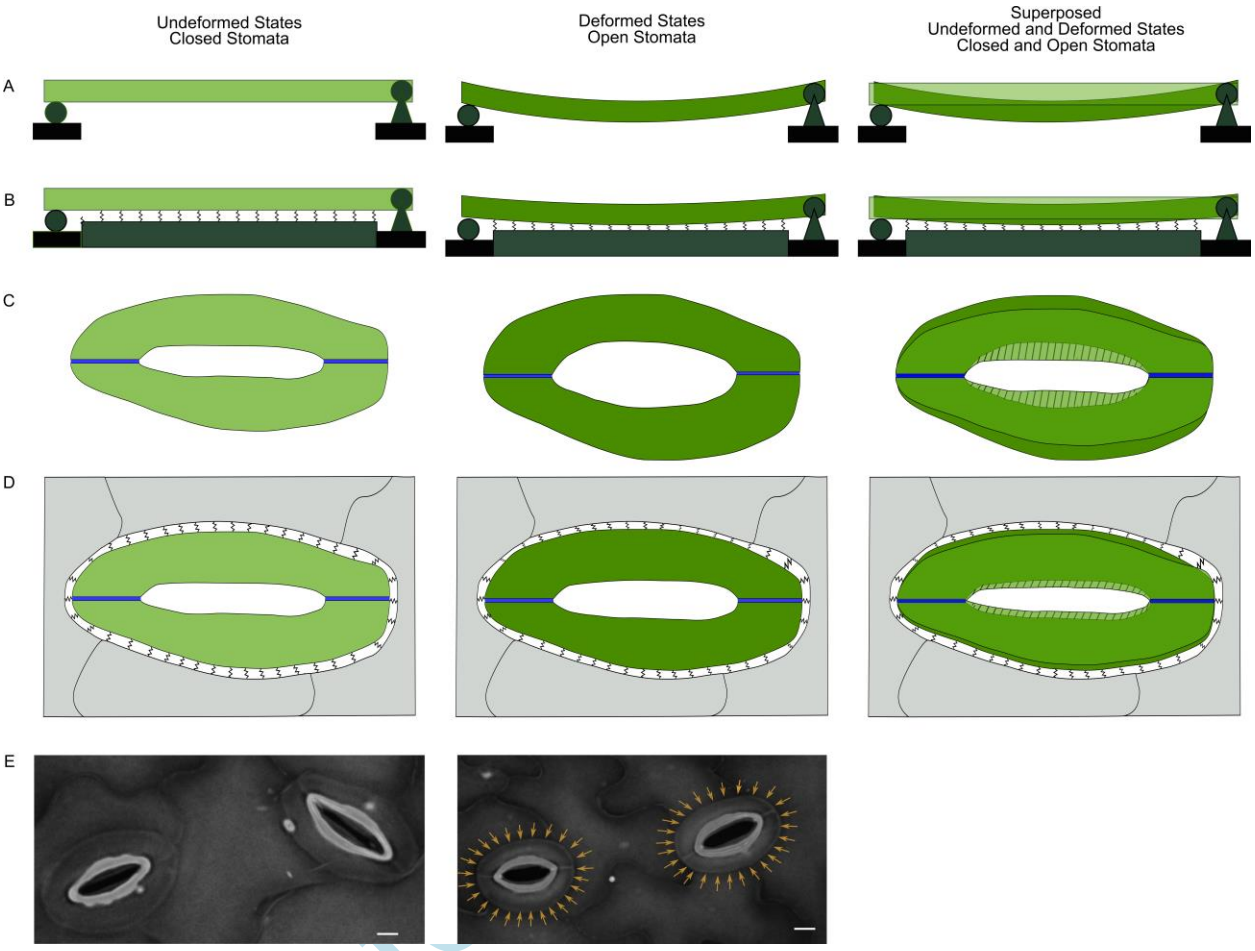

Figure 3

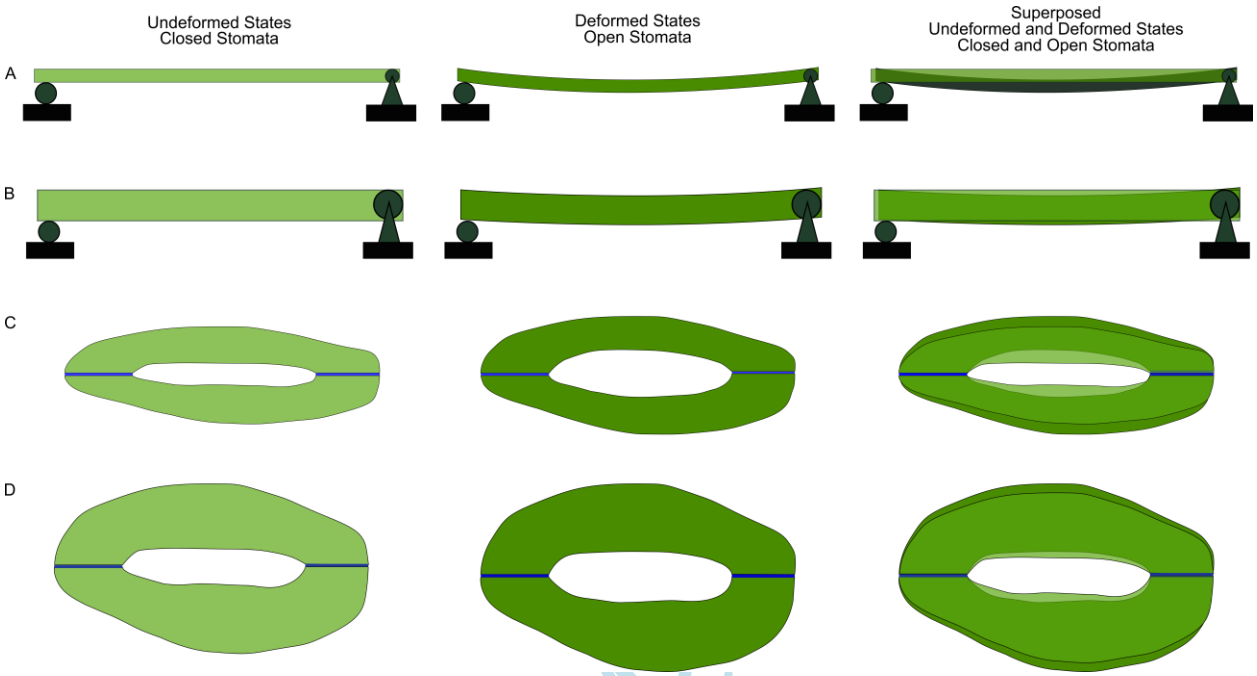

Figure 4