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Bottom-up multiscale modelling of guard cell walls reveals molecular mechanisms of stomatal biomechanics

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Abstract. Stomata are dynamic pores on plant surfaces that regulate photosynthesis and are thus of critical importance for understanding and leveraging the carbon-capturing and food-producing capabilities of plants. However, our understanding of the molecular underpinnings of stomatal kinetics and the biomechanical properties of the cell walls of stomatal guard cells that enable their dynamic responses to environmental and intrinsic stimuli is limited. Here, we built multiscale models that simulate regions of the guard cell wall, representing cellulose fibrils and matrix polysaccharides as discrete, interacting units, and used these models to help explain how molecular changes in wall composition and underlying architecture alter guard wall biomechanics that gives rise to stomatal responses in mutants with altered wall synthesis and modification. These results point to strategies for engineering guard cell walls to enhance stomatal response times and efficiency.

KEYWORDS: plant cell wall; multiscale modelling; finite element modelling; stomatal guard cell; *Arabidopsis* thaliana.

1. INTRODUCTION

Stomata are pores on the surface of plant leaves that regulate gas exchange between the plant and its environment. In eudicot plants, stomatal opening and closure are controlled by two flanking guard cells, which are located on either side of the stomatal pore (Conklin *et al.* 2019). Guard cells can rapidly change their shape in response to changes in multiple environmental conditions, such as light, humidity and CO_2 levels, causing the stomatal pores to open or close (Meidner and Mansfield 1968).

Stomatal kinetics reflect the biomechanical manifestation of guard cell pairs as functions of internal turgor and the molecular architecture of the guard cell wall that determines its mechanics. The mechanical properties of the guard cell wall play a critical role in stomatal opening and closure (Marom *et al.* 2017; Rui *et al.* 2018; Yi *et al.* 2018; Chen *et al.* 2021). The guard cell wall is a multi-layered structure composed of cellulose microfibrils, hemicelluloses, pectic polysaccharides and proteins. The mechanical properties of each of these components, along with their interactions, are thought to contribute to the overall mechanical behaviour of the cell wall.

Recent studies of stomatal biomechanics have demonstrated that *in silico* models of stomatal complexes can be successfully used to quantitatively interrogate the contributions of the mechanical properties of guard cell walls to stomatal kinetics. These studies typically construct models of stomatal complexes *in silico* that parameterize the anisotropic and heterogeneous mechanical properties of guard cell walls and their contributions to stomatal opening and closing (Amsbury *et al.* 2016; Carter *et al.* 2017; Marom *et al.* 2017; Woolfenden *et al.* 2017, 2018; Rui *et al.* 2018, 2019; Yi *et al.* 2018, 2019; Chen *et al.* 2021). This approach reflects the circumferential arrangement of cellulose microfibrils in the guard cell wall (Galatis and Apostolakos 2004; Fujita and Wasteneys 2014; Sleboda *et al.* 2023), which during stomatal opening is expected to constrain guard cell widening and force guard cell elongation, which in combination with constraints on the height of the stomatal complex causes the stomatal pore to open (Meckel *et al.* 2007; Carter *et al.* 2017).

These studies implement the material properties of the guard cell wall with constitutive models. Such constitutive models are developed based on the principles of minimum potential energy and minimum complementary energy to predict the composite elastic moduli of guard cell walls based on given volume fractions of cellulose and cell wall matrix with presumed elastic properties. Although this approach can incorporate anisotropic behaviours of the guard cell wall, it does not allow for investigations into how the mechanical responses of guard cells emerge from the identity and arrangement of wall polysaccharide molecules.

Furthermore, while this is a valid approach when the guard cell wall is modelled as a homogeneous material, it does not allow for predicting localized strains or stresses at the molecular scale, where the cell wall components exist. This is because the underlying approximations of a constant stress or strain field violate the compatibility of deformation or local equilibrium, respectively (González *et al.* 2013). For example, the stress of cellulose microfibrils is expected to differ from the stress of the wall matrix. However, a homogenized stress–strain relationship predicts a continuous stress field across the guard cell wall, regardless of the constitution of the wall at a specific location. Additionally, this approach cannot consider clustering or size effects of cell wall components (Sekkate *et al.* 2022). These limitations make it difficult to connect the findings of constitutive computational models with the molecular regulation of wall assembly and patterning in plants because the guard cell wall is unrealistically represented as a continuous and homogeneous material.

On the other hand, guard cell shape and geometry also contribute to stomatal kinetics, e.g. changes in pore size when stomata open and close (Pautov *et al.* 2017; Yi *et al.* 2018, 2019). It is essential to understand the mechanical contribution of the overall shapes of the guard cells and the stomatal complex in addition to the mechanical properties of the guard cell wall to explain how plants regulate stomatal opening and closing. However, solutions to the mechanical problem of deformation in three dimensions are difficult to obtain, except for the simplest geometries. This is due to the governing equations of mechanics being partial differential equations that involve arbitrary functions rather than arbitrary constants (Bauchau and Craig 2009; Hibbeler 2018). Therefore, computational modelling accounting for irregular geometries has emerged as an essential tool in investigations of how guard cell geometry and wall architecture influence stomatal dynamics (Yi *et al.* 2018, 2019).

The aforementioned limitations can be overcome by implementing bottom-up, multiscale models of guard cell walls, in which component geometries and properties are explicitly modelled to construct simulated guard cell walls. In other words, such models can reflect the molecular structures of wall components, i.e. the species, quantities, interactions and arrangements that determine the mechanical properties of the wall (Ptashnyk and Seguin 2014; Jensen and Fozard 2015; Braybrook and Jönsson 2016). To that end, this study aimed to develop a multiscale model of the guard cell wall that encompasses the geometries of cellulose microfibrils, as well as the cell wall matrix including pectins and hemicelluloses.

The bottom-up multiscale modelling approach allows us to implement different material models for cell wall components. These can include a solid mechanics model for cellulose microfibrils behaving as an elastic material following Hooke's law and viscoelastic models for matrix components, and modelling the bonds between cellulose microfibrils and matrix components with relative deformation energetics equivalent to their hypothesized biochemical interactions (Cosgrove 2018).

This approach also permits the modelling of varying quantities, arrangements and interactions of major wall polysaccharides *in silico*. The predicted behaviour of these models of guard cell walls can be quantitatively compared with experimental results from different genotypes, elucidating the molecular origins of stomatal function. For example, it is hypothesized that the size and abundance of pectic homogalacturonan polymers in the *Arabidopsis* guard cell wall influence stomatal kinetics, i.e. reducing the molecular mass of pectins by overexpressing pectin-cleaving polygalacturonases, will reduce the strength of cellulose–pectin interactions, thereby facilitating cellulose rearrangements and accelerating stomatal opening (Rui *et al.* 2017). With a multiscale model of the guard cell wall, it becomes possible to model the hypothesized reduction in cellulose–pectin interactions and quantitatively compare predicted stomatal kinetics with experimental observations.

In this study, we present a bottom-up multiscale, multi-physics, *in silico* model of the guard cell wall. The bottom-up multiscale model predicts that the length of the cellulose microfibrils must be <1.5 μm to achieve an observed anisotropic behaviour of the guard cell wall. Similarly, the guard cell wall is predicted to behave anisotropically when cellulose abundance is more than 50% of wild type and cellulose microfibril modulus is larger than 10 GPa. On the other hand, the model predicts anisotropic behaviour in the guard cell wall with a wide range of moduli for the cell wall matrix, suggesting that the mechanical properties of the matrix can be modulated without losing the mechanical functionality of the guard cell wall for stomatal opening and closing. Comparing these predictions with hypothesized alterations in the properties of guard cell walls, the bottom-up multiscale model can leverage experimental studies on the molecular structure of guard cell wall and expand it to the cellular scale, where the contributions of wall architecture and cell shape can be considered.

2. A BOTTOM-UP MULTISCALE MODEL OF A SEGMENT OF A GUARD CELL WALL

The abundance and arrangement of the polysaccharide components in guard cell walls are thought to be ~30% cellulose microfibrils, 30% hemicelluloses and 30% pectins, with the remaining non-water mass being composed of proteins and glycoproteins (Albersheim *et al.* 2010). Reflecting this information and the established circumferential arrangement of cellulose in the guard cell wall, an encompassing segment of the guard cell wall in the form of a circular cross-section was modelled as a matrix containing elongated cellulose microfibrils.

The cross-sectional area of the modelled cell wall segment is 0.2 μm (thickness direction) by 0.075 μm (elongation direction), with a circular radius of 3 μm. The dimensions of the segment are consistent with previously reported guard cells of wild-type *Arabidopsis thaliana* plants (Rui *et al.* 2018; Yi *et al.* 2018). The proportion of cellulose embedded in the model is determined to account for one-third of the non-water mass using a crystalline cellulose density of 1.6 g/cm^3 (Sun 2005; Yano *et al.* 2018). Each cellulose fibril is modelled as an elongated fibre with a diameter of 15 nm, which corresponds to five bundled elementary cellulose microfibrils (Donaldson 2007; Fernandes *et al.* 2011; Lyczakowski *et al.* 2019).

The model simulates cellulose microfibrils arranged circumferentially and embedded within a matrix composed of hemicellulose and pectic polysaccharides (Albersheim *et al.* 2010). Different versions of the model were constructed with different lengths and amounts of cellulose fibrils embedded in the matrix at random locations. Models were meshed as tetrahedral volumes using gmsh 4.09 (Remacle and Geuzaine 2019). The tetrahedral volumetric representation of cellulose microfibrils and matrix produced contact surfaces between the cellulose fibrils and the matrix. Using contact surface descriptions, this model can predict the mechanical consequence of the properties, structural arrangements and interactions of the components of the guard cell wall.

The estimated length of cellulose spans a few hundred nanometres, as inferred from the velocity and duration of the cellulose synthase (CESA) complex movement during cellulose synthesis (Blaschek *et al.* 1982; Kamide *et al.* 1983; Kompella and Lambros 2002; Diotallevi and Mulder 2007; Hallac and Ragauskas 2011; Fujita *et al.* 2013; Sampathkumar *et al.* 2014; Liu *et al.* 2017; Hill *et al.* 2018; Jarvis 2018). Nonetheless, there has not been a definitive experimental observation regarding the length of cellulose fibres in native walls. In this study, three fragment models of guard cell walls with cellulose fibrils with length of 450 nm, 1.5 μm, and 4.5 μm were created to examine the effects of cellulose fibril length (Fig. 1). These models represent a complete circumferential segment of a guard cell wall comprising cellulose and cell wall matrix (hemicellulose and pectic polysaccharides) at the molecular scale.

3. MATERIAL PROPERTIES OF WALL COMPONENTS IN THE MULTISCALE MODEL

The overall mechanical properties of the multiscale model of the segment of the guard cell wall emerge from the combined mechanical behaviour of cellulose fibrils and wall matrix. Mechanical behaviours of cell wall components at the molecular scale are governed by their geometric arrangements and mechanical properties. In this study, cellulose fibrils and matrix components are modelled as elastic and viscoelastic materials, respectively.

Cellulose fibrils are modelled as a homogeneous elastic material, with Young's modulus and Poisson's ratio values taken from previous studies (Roberts *et al.* 1994; Nakamura *et al.* 2004, 2004; Cintrón *et al.* 2011; Li *et al.* 2011; Kobayashi *et al.* 2012). The effect of the anisotropic behaviour of a cellulose microfibril, i.e. large tensile modulus versus small lateral load-bearing capacity, arises from its elongated geometry.

$$\sigma = 2G\varepsilon + \lambda \mathrm{tr}(\varepsilon)\mathbf{I} \tag{1}$$

where G is the shear modulus, λ is the Lamé's first parameter, α is the stress tensor, $\boldsymbol{\varepsilon}$ is the strain tensor, $\boldsymbol{I}$ is the identity matrix and tr is the trace function.

It is generally accepted that the matrix in primary cell walls behaves mechanically as a viscous material. Recent studies using nano-indentation report such viscoelastic behaviours (Hayot *et al.* 2012; Digiuni *et al.* 2015; Chen *et al.* 2021). Reflecting this knowledge, the wall matrix (hemicelluloses and pectins) is modelled as a viscoelastic material (Dumais 2007; Hayot *et al.* 2012; Huang *et al.* 2012) as described in the form of the Prony series.

$$g_R(t) = 1 - \sum_{i=1}^{N} \bar{g}_i^P \left(1 - \mathrm{e}^{-t/\tau_i^G}\right) \tag{2}$$

where $\bar{g}_i^P$ is the ratio of shear traction modulus to relaxation modulus, τ_i^G is the relaxation time, and $\bar{g}_i^P$ is related to the instantaneous Young's modulus E_0 and the instantaneous shear modulus G_0.

Currently, few studies have quantitatively measured the mechanical properties of the wall matrix or its constituents. Because no definitive measured values of these parameters exist, we focussed on varying E_0 which accounts for the stiffness of the matrix in the guard cell wall. This study does not investigate time-delayed responses or varying levels of shear modulus under the assumption that deformation of the guard cell wall initiates over a relatively short duration of a few minutes.

One advantage of a bottom-up multiscale model of the guard cell wall is the ability to model interactions between its

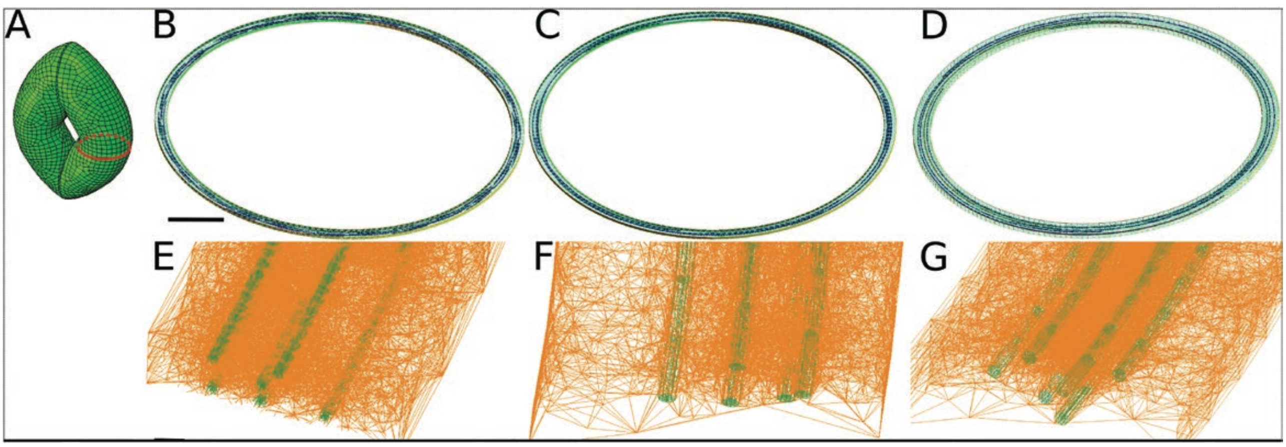

Figure 1. Models of a dotted red circular segment of guard cell walls in (A), with (B) 280 cellulose fibrils with lengths of 450 nm, (C) 76 cellulose fibrils with lengths of 1.5 μm and (D) 8 cellulose fibrils with lengths of 4.5 μm. The cellulose fibrils are modelled as bundled elementary cellulose microfibrils with a cross-sectional diameter of 15 nm, which represents five cellulose microfibrils (Brown 1999; Delmer 1999; Diotallevi and Mulder 2007). (E–G) Magnified cut-off sections of models (B–D). Purple lines represent matrix components, whereas blue and brown elements represent circumferentially deposited cellulose fibrils. Scale bar in the top row represents 1 μm; scale bar in the bottom row represents 10 nm. Circular models in (B–D) are shown in perspective.

components, i.e. interactions between cellulose and the wall matrix. In the current model, we represented such interactions as frictional contacts in which relative deformation between cellulose and matrix requires a linearly increasing force with the proportionality of k, which can be analogous to the frictional coefficient. These interactions between cellulose and matrix can be modelled with a non-linear relationship reflecting energetics as used in Nili *et al.* (2015); Zhang *et al.* (2021)). Here, this approach facilitates the examination of the effect of varying the strength of the interaction on the larger-scale mechanical properties and behaviours of the guard cell wall.

4. RESEARCH QUESTIONS USING THE MULTISCALE MODEL OF THE GUARD CELL WALL

With a bottom-up multiscale model of the guard cell wall, we can examine the specific contributions of different wall components to stomatal function. For example, it has long been proposed that cellulose is the major load-bearing component of plants cell walls and regulates their isotropy/anisotropy (Baskin 2005; Baskin and Jensen 2013; Fujita *et al.* 2013; Marom *et al.* 2017; Yi *et al.* 2018; Chen *et al.* 2021; Sleboda *et al.* 2023). However, how cellulose microfibrils contribute to regulating the anisotropy of the guard cell wall via their stiffness (Cintrón *et al.* 2011; Li *et al.* 2011; Kobayashi *et al.* 2012) and abundance (Rui and Anderson 2016; Yi *et al.* 2018) is poorly defined.

Previous studies have analysed mutants targeting pectic polysaccharides in the cell wall, e.g. studying the effects of altering pectin homogalacturonan. Specific to the guard cell wall, changes in de-methyl-esterified and calcium cross-linked homogalacturonan (Amsbury *et al.* 2016) and the molecular size of homogalacturonan with polygalacturonase and pectate lyase mutants (Rui *et al.* 2017; Chen *et al.* 2021) have been analysed and correlated with changes in stomatal function. However, quantitative causalities between altered pectin or hemicellulose structure and the mechanics of guard cell walls have yet to be established.

Finally, the mechanical consequences of the interactions between wall matrix components and cellulose have been a major research question in the field of plant cell wall mechanics. Recently, Zhang *et al.* (2021) demonstrated the effects of hydrogen bonding between cellulose microfibrils and between cellulose and hemicellulose using a coarse-grained model of a nanoscale segment of a simulated onion epidermal wall. This study provided quantitative insights into the nature of interactions between cellulose and wall matrix and the mechanical effects of these interactions. The bottom-up multiscale model developed here can leverage such quantitative knowledge and expand it to the cellular scale, where the contributions of wall architecture and cell shape can be captured.

5. CELLULOSE FIBRIL LENGTH HAS LIMITED EFFECTS ON THE ANISOTROPY OF THE MODELLED GUARD CELL WALL

The precise lengths of cellulose microfibrils *in situ* are difficult to measure. A microfibril length of 300–500 nm has been proposed based on the degree of polymerization (Diotallevi and Mulder 2007; Hallac and Ragauskas 2011; Jarvis 2018) and molecular mass (Blaschek *et al.* 1982) of cellulose chains. However, based on the speed of the cellulose synthase complex that averages about 300 nm/min (Liu *et al.* 2017) and its residence time in the plasma membrane of ~15 min (Sampathkumar *et al.* 2014), cellulose microfibrils might be as long as 4500 nm. Reflecting the range of proposed microfibril length, we constructed sets of models of guard cell walls with cellulose fibrils at lengths of 400, 1500 and 4500 nm.

Differences in radial and circumferential strains between the 400- and 1500-nm fibril models were not significant ($P > 0.05$, $n = 10$ models per fibril length, Wilcox test) (Fig. 2). However, strains in these directions were significantly smaller in the 4500-nm fibril models than in the 1500-nm fibril models ($P < 0.05$, $n = 10$ models per fibril length, Kruskal–Wallis test). Differences in longitudinal strain between models with different fibril lengths were all significant ($P < 0.05$, $n = 10$, Kruskal–Wallis test) with the largest strain value obtained from the 1500-nm fibril models.

The moduli in respective directions (Fig. 2D–F) indicate that the circumferential modulus is two orders of magnitude stiffer than the longitudinal and radial moduli. In the circumferential and radial directions, the modulus values of 4500-nm fibril models were significantly smaller than those of the 400- and 1500-nm models ($P < 0.05$, $n = 10$, Kruskal–Wallis test). In the longitudinal direction, only the modulus values of the 4500-nm models were significantly larger than the modulus values of the 1500-nm models ($P < 0.05$, $n = 10$, Kruskal–Wallis test).

The anisotropy ratios between circumferential or radial moduli and the longitudinal modulus for the 400-, 1500- and 4500-nm models were 1.3 and 70, 3.7 and 157, and 2.5 and 79, respectively. The higher ratios in the 1500-nm model imply that depositing this length of cellulose fibril enhances the anisotropy of the guard cell wall, but that even longer cellulose fibrils do not. In summary, increasing cellulose fibril length increases the magnitude of circumferential and radial strain, both of which are negative, upon simulated guard cell pressurization, implying that as single cellulose fibrils wrap further around the guard cell, they force the wall to become thinner upon guard cell pressurization.

As shown in Fig. 2 (top row), longer fibrils tend to increase the magnitude of negative strain in radial and circumferential directions. However, such a trend is not observed in the longitudinal strain. Moreover, the magnitude of the longitudinal strain ranges between 0.15 and 0.17, which is close to the change in guard cell length reported in experimental measurements (Rui *et al.* 2018; Yi *et al.* 2018).

6. DIMINISHING CELLULOSE ABUNDANCE CAUSES ISOTROPIC MECHANICAL BEHAVIOUR IN THE MODELLED GUARD CELL WALL

Next, we investigated the mechanical effects of changing the abundance of cellulose in the guard cell wall by modelling wall segments with varying numbers of cellulose fibrils. To that end, models with different numbers of cellulose fibrils, each 1500 nm long, were constructed with 10 different random arrangements for each set of cellulose content. We constructed models with successively fewer cellulose fibrils with 75, 50 and 25 % of the cellulose in simulated wild-type walls (Figure 3). Specifically, the models with 50 % of the wild-type cellulose represent the cellulose-deficient walls of *cesa3^{je5}* guard cells (Yi *et al.* 2018).

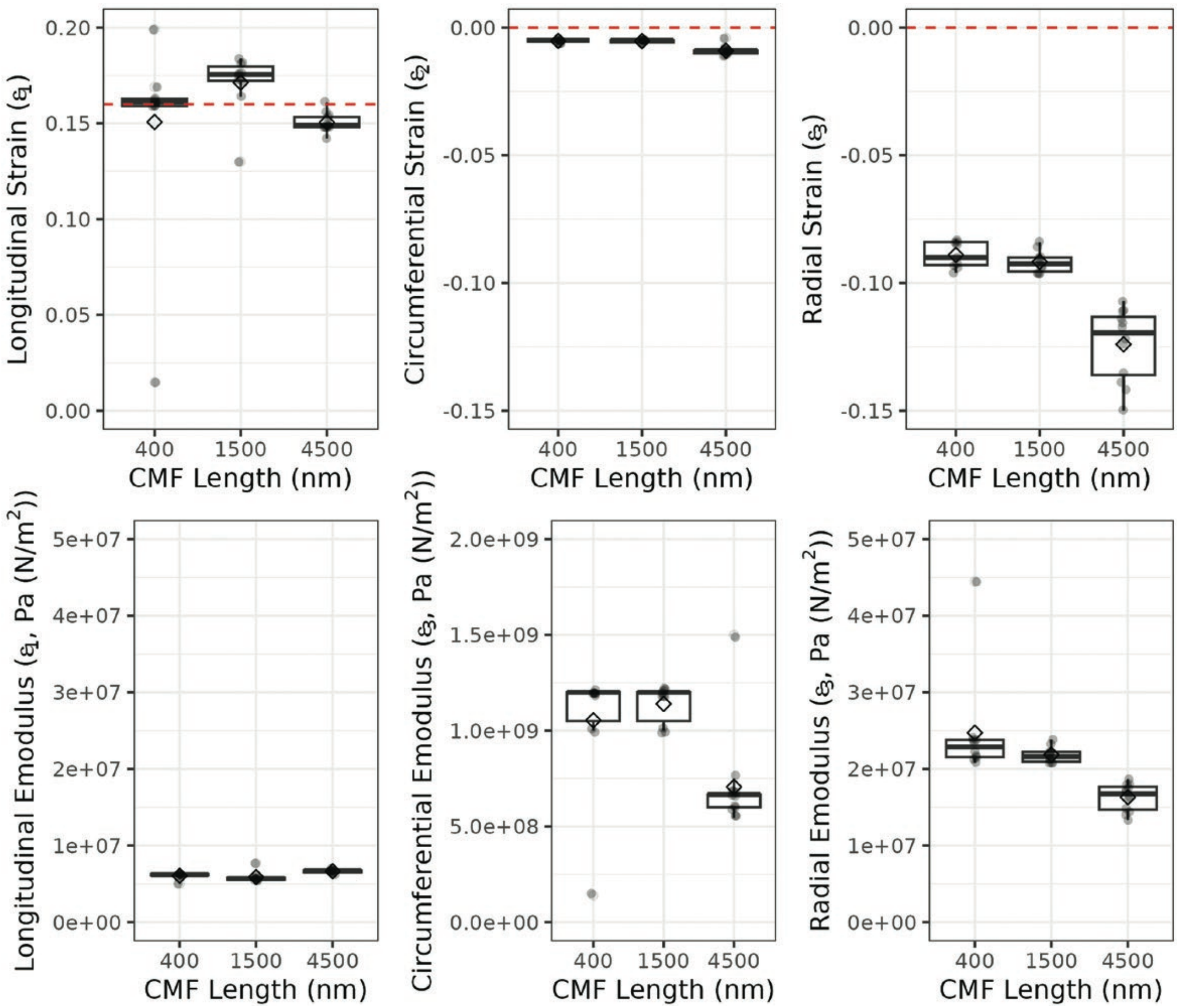

Figure 2. Effect of cellulose fibril length on strain in simulated guard cell walls under increased turgor (1 MPa): dashed red horizontal lines in the top row represent experimentally measured deformations of stomatal guard cells of *Arabidopsis thaliana* Col-0 when opened (Yi *et al.* 2018).

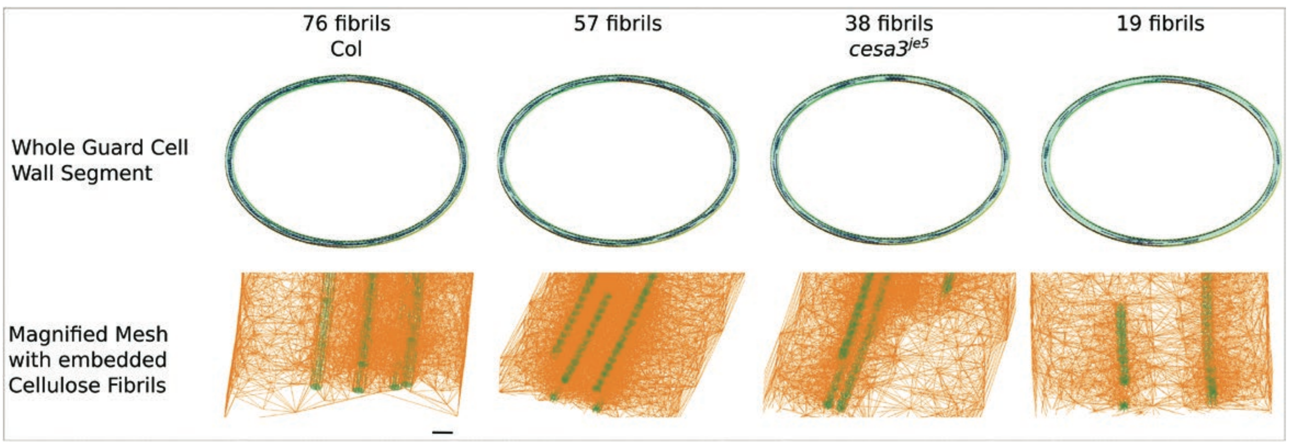

Figure 3. Multiscale guard cell segment models with 76, 57, 39 and 19 cellulose fibrils, each 1500 nm long. Cellulose fibrils (blue) are randomly arranged in the circumferential direction. A model with 76 cellulose fibrils represents wild type (Col), whereas a segment model with 38 fibrils represents the cellulose-deficient wall of cesa3^{je5}. Scale bar in the top row represents 1 μm; scale bar in the bottom row represents 10 nm. Circular models in the top row are shown in perspective.

The mechanical deformation of the guard cell wall during stomatal opening was modelled as that caused by a turgor increase of 1 MPa (Chen *et al.* 2021) from a baseline turgor to simulate the closed state of a stomatal complex. Strain and stress in the models were analysed to examine the effects of diminishing cellulose amount on the mechanics of the guard cell wall.

Strain values in the radial, circumferential and longitudinal directions indicate that a reduction in the number of cellulose fibrils increases the radial and circumferential strains. However, longitudinal strain decreases slightly with reductions in cellulose fibril number (Fig. 4). These data suggest that circumferentially arranged cellulose fibrils have a limited contribution to the longitudinal deformability of the guard cell wall.

It is notable that there exists an abrupt increase in the radial and circumferential strain when the number of cellulose microfibrils decreases from 100 to 50 % of the wild type, which corroborates experimental observations of stomatal responses in *cesa3*^{je5} mutants, where the stomatal pore opens wider than in Col plants in response to light stimulus (Yi *et al.* 2018). The reversal of longitudinal strain from positive to negative, when the number of cellulose fibrils diminishes from 100 to 75 % of the wild type, indicates that the guard cell wall loses its anisotropy with an insufficient number of cellulose fibrils, expanding radially and shortening upon pressurization rather than elongating. This result contradicts the experimental observation of *cesa3*^{je5} stomata opening by elongation, just less so than Col stomata. This

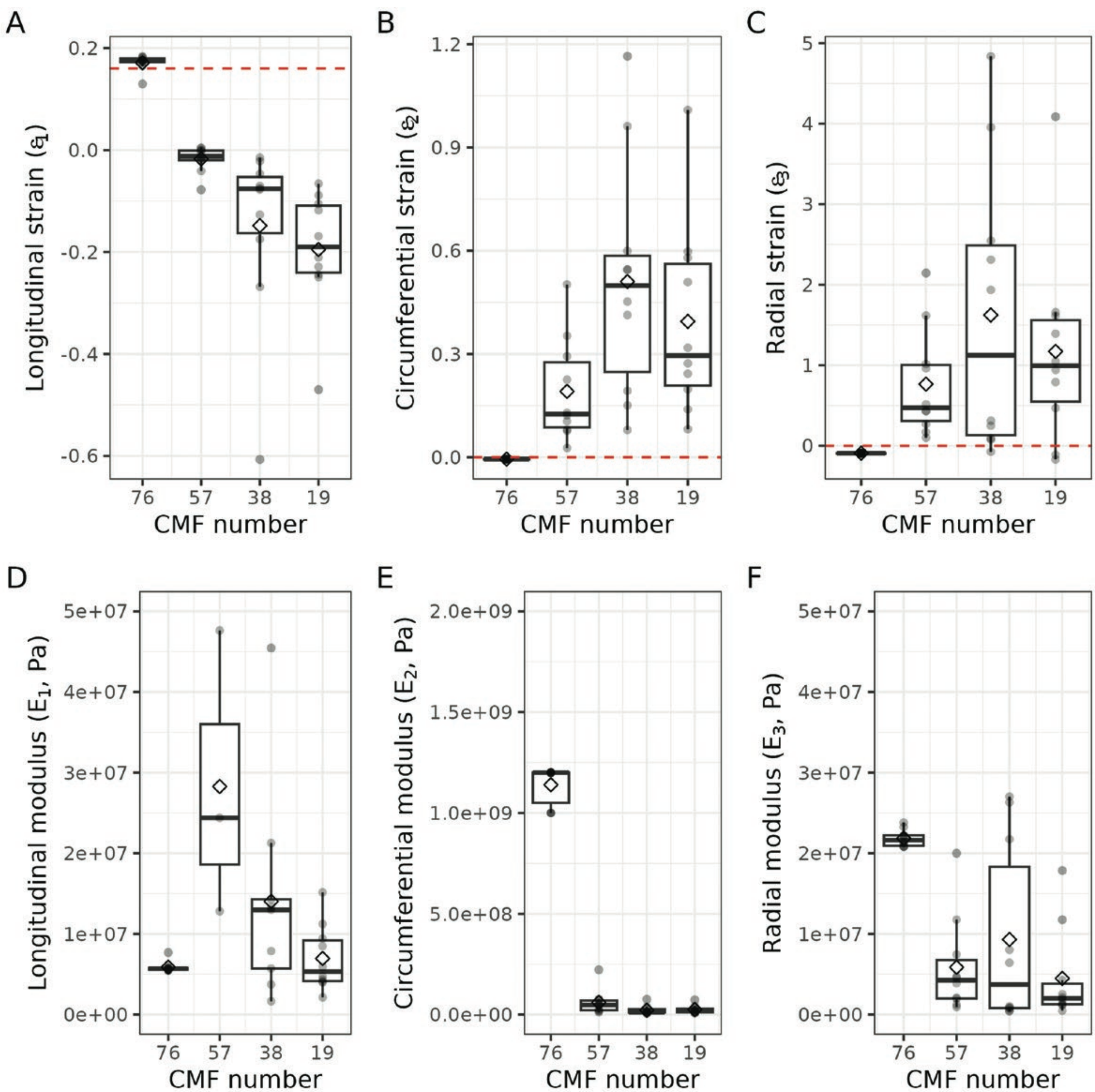

Figure 4. Effects of cellulose microfibril abundance (CMF number) on strain (A–C) and modulus (D–F) of the guard cell wall in longitudinal, circumferential, and radial directions when simulated turgor is increased by 1 MPa for models with cellulose microfibrils that are 1500 nm long.

observation suggests that the *cesa3^je5^* guard cell wall may have an alternate pathway to rescue anisotropic mechanical behaviour to compensate the loss of cellulose.

Since the modulus of the guard cell wall is inversely related to strain, the modulus of the guard cell wall decreases sharply in the radial (thickness) and circumferential directions when the cellulose fibrils diminish from 100 to 75 % of the wild type (Fig. 4). The longitudinal elastic modulus, which is in the direction of contraction (negative strain), increases up at 75 % of wild-type cellulose amount, then diminishes with further reductions in cellulose abundance. This change in the elastic modulus is the result of reversed strain in the longitudinal direction. When cellulose microfibrils decrease from 100 to 75 % of wild type, the wall modulus increases in the direction of elongation.

A closer look at the longitudinal stiffness trend with respect to the number of cellulose fibrils confirms that a reduction in the number of cellulose fibrils does not soften the guard cell wall in the longitudinal direction ($P > 0.05$, $n = 10$, Kruskal–Wallis test). A reduced number of cellulose fibrils softens the guard cell wall in circumferential and radial directions only between 19, 38 and 57 fibrils in comparison to 76 fibrils ($P < 0.05$, $n = 10$, Kruskal–Wallis test). Reducing the number of cellulose fibrils below 57 has a non-significant trend of softening the guard cell wall in the circumferential and radial directions ($P > 0.05$, $n = 10$, Kruskal–Wallis test).

7. INCREASING CELLULOSE MODULUS CAUSES MORE ANISOTROPIC MECHANICAL BEHAVIOUR IN THE MODELLED GUARD CELL WALL

It is widely accepted that cellulose microfibrils are the major load-bearing component in the cell wall (Albersheim *et al.* 2010; Atalla and Isogai 2010; Cosgrove 2014). However, their actual mechanical properties have yet to be experimentally determined. On the other hand, advances in molecular dynamics and quantum mechanics modelling have produced quantitative estimates of the mechanical properties of cellulose (Cintrón *et al.* 2011; Li *et al.* 2011; Kobayashi *et al.* 2012). Predictions of cellulose modulus range from 50 GPa (Zhang *et al.* 2021) to over 100 GPa (10^{11} Pa) (Cintrón *et al.* 2011; Li *et al.* 2011). Here, we examined the implications of varying the modulus of cellulose fibrils on the mechanics of the guard cell wall.

The simulation results (Fig. 5) indicate that increasing the modulus of cellulose two or more orders of magnitude from a baseline of 10^6 Pa significantly reduces the magnitude of strain in the longitudinal (ε_1) and circumferential (ε_2) directions ($P < 0.05$, $n = 10$, Kruskal–Wallis test). The change in the radial strain is not significant ($P > 0.05$, $n = 10$, Kruskal–Wallis test), but there exists a notable decrease in variability for the predicted strains. The decreasing variability when cellulose modulus increases is also observed in longitudinal and circumferential strains.

The modulus of the guard cell wall increases significantly in the longitudinal and circumferential directions when cellulose modulus is increased by two orders of magnitude from 10^8 Pa to 10^{10} Pa ($P < 0.05$, $n = 10$, Kruskal–Wallis test), but the wall modulus shows a non-significant increase in the radial direction ($P > 0.05$, $n = 10$, Kruskal–Wallis test). More importantly, when the cellulose modulus is higher than 10^{10} Pa, the magnitude of longitudinal, circumferential and radial strain becomes comparable with experimental observations (Yi *et al.* 2018), which are represented as dotted red lines in Fig. 5A–C. It is noticeable that the magnitude of strains in all directions when the cellulose modulus is $<10^9$ Pa becomes close to or >0.1, which indicates a more isotropic volumetric expansion of the guard cell, i.e. ballooning rather than elongating.

8. EFFECT OF WALL MATRIX STIFFNESS ON GUARD CELL WALL STRAIN

Hemicelluloses and pectins are major components of the primary plant cell wall and are defined as matrix polymers. The contributions of hemicelluloses to the mechanical integrity of cell walls had been widely proposed and studied (Keegstra *et al.* 1973; Fry 1986; Hayashi 1989; Talbott and Ray 1992; Ha *et al.* 1997; Cosgrove 2000, 2001; Bruce 2003; Thompson 2005; Kha *et al.* 2007; Dyson and Jensen 2010; Dyson *et al.* 2012; Yi and Puri 2012; Nili *et al.* 2015). The importance of pectins in cell wall mechanics has been highlighted more recently, including studies of methyl-esterified homogalacturonan (Haas *et al.* 2020, Haas et al. 2021), de-methyl-esterified and cross-linked pectic homogalacturonan (Amsbury *et al.* 2016), and pectinase mutants such as PGX3 or *PLL12* by altered elastic stiffness or time-dependent behaviour (Rui *et al.* 2017; Chen *et al.* 2021).

While it is typically stated that cellulose microfibrils are the major load-bearing components in the plant cell wall, the mechanical contribution of the wall matrix, which is mainly composed of hemicelluloses and pectins, cannot be ignored. Especially for the guard cell wall, controlled compliance of the wall matrix is thought to be critical for achieving the optimal magnitudes and patterns of guard cell deformation leading to proper stomatal function. To examine the roles and contribution of the wall matrix to stomatal mechanics, the mechanical responses of the multiscale model of the guard cell wall were simulated with the matrix modelled as a viscoelastic material with a modulus ranging from 75 kPa to 75 MPa (Williams *et al.* 2007; Kiemle *et al.* 2014).

The predicted strain of the guard cell wall becomes comparable with experimental observations (Yi *et al.* 2018) in all directions when the matrix modulus is >1 MPa (Fig. 6A–C). This result suggests that there exists a minimum stiffness of the wall matrix that is required to achieve the appropriate levels of guard cell deformation in the longitudinal and circumferential directions.

When the corresponding wall modulus values are compared (Fig. 6D–F), it is notable that the longitudinal modulus achieves a comparable magnitude to the circumferential modulus when the matrix modulus is 75 MPa ($P < 0.05$, $n = 10$, Kruskal–Wallis test). The cell wall modulus in the circumferential direction does not change significantly ($P > 0.05$, $n = 10$, Kruskal–Wallis test) when the matrix modulus is >1 MPa. The wall modulus in the circumferential and radial directions show inconsistent trends. These data suggest that stomatal opening can be achieved as long as the matrix modulus is larger than a certain threshold, e.g. 7.5 MPa.

9. DISCUSSION

9.1 Validation of the bottom-up multiscale model of a segment of the guard cell wall

The simulation results of the bottom-up multiscale model of a segment of the guard cell wall corroborate previous research on

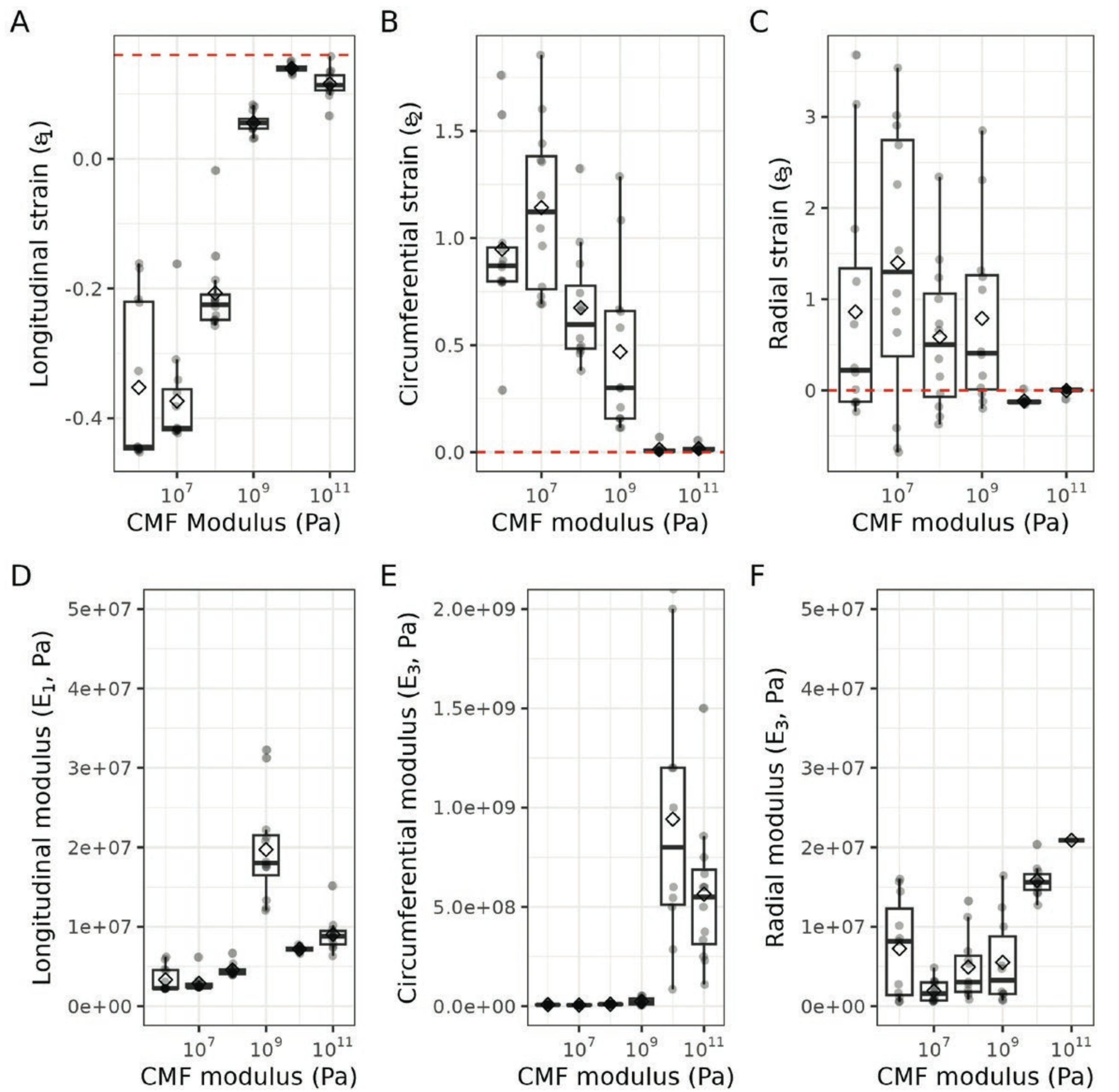

Figure 5. Effect of cellulose modulus on the strain (A–C) and the elastic modulus (D–F) of the guard cell wall in longitudinal, circumferential and radial directions when turgor is simulated to increase by 1 MPa.

the mechanics of *Arabidopsis thaliana* stomatal guard cells. Most notably, the ratio between longitudinal and circumferential stiffness is predicted to range between 2 and 8 when the predicted longitudinal strain matches with experimental observations. This anisotropy of moduli between longitudinal and circumferential directions includes previously predicted values (Yi *et al.* 2018; Chen *et al.* 2021). This observation supports the hypothesized contribution of circumferentially arranged cellulose microfibrils to stomatal function in that they help drive preferential deformation of the guard cell, i.e. elongation, with water influx and increased turgor.

The elastic modulus in radial and circumferential directions decreases rapidly when the amount of cellulose decreases relative to the estimated density of cellulose in the simulated cell walls of wild-type *Arabidopsis* guard cells. This result suggests that a minimum amount of cellulose microfibrils must exist in the guard cell wall to limit radial and circumferential strain. This observation also indicates that the mechanical anisotropy of the guard cell wall emerges when cellulose microfibrils are deposited in the circumferential direction at a level of at least 50 % of the cellulose abundance in the wild-type guard cell wall. In addition, achieving the appropriate stiffness of cellulose also seems critical, because excessively soft cellulose fibrils are predicted not to achieve appropriate stiffness and anisotropy in the guard cell wall.

9.2 Origins of anisotropy in the guard cell wall

Anisotropy of mechanical properties induces displacement or motion of different magnitudes in different directions when

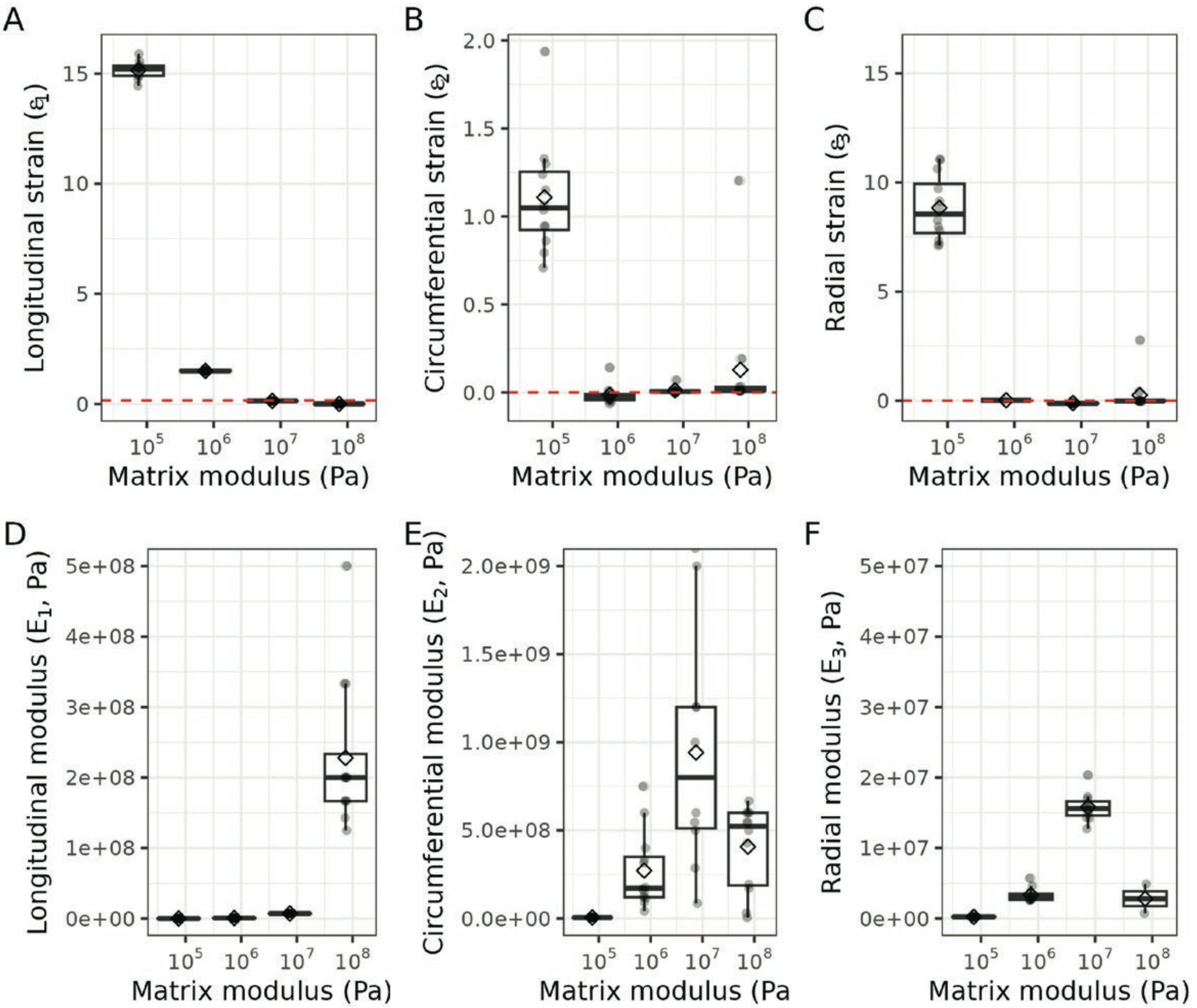

Figure 6. Effect of changing wall matrix modulus on the strain (top row) and the total modulus (bottom row) of the simulated guard cell wall in longitudinal, circumferential and radial directions when turgor is simulated to increase by 1 MPa.

a material is subjected to the same load in all directions. In the case of the *Arabidopsis* guard cell wall, such an isotropic loading condition arises when turgor in the guard cell increases as a result of water influx. If the mechanical properties of the guard cell wall are isotropic and the guard cell pair is not constrained with a preferential direction, the guard cell wall will expand isotropically. However, guard cell diameter does not change during stomatal opening (Woolfenden *et al.* 2017; Yi *et al.* 2018). This observation suggests that mechanical anisotropy is one of the essential characteristics enabling stomatal kinetics through water exchange.

The orientation of cellulose is believed to be the major contributor to achieving such anisotropy. The question then arises as to the amount of cellulose needed for a plant to generate functional stomatal guard cells. It was observed that cellulose-deficient *cesa3*^{je5} plants develop functional guard cells, although the stomatal complex is larger in this mutant than in the wild type (Rui and Anderson 2016). The size difference is thought to originate from the deficient amount of cellulose, leading to less stiff guard cell walls throughout development. Our results also suggest that a noticeable mechanical anisotropy emerges with as little as 25 % of the cellulose predicted to exist in wide-type plants. Also, anisotropy is achieved by rapidly increasing stiffness in the circumferential and radial (thickness) direction, while the contribution of additional cellulose to longitudinal stiffness in the guard cell wall remains limited.

This observation suggests that the amount of cellulose in the guard cell wall provides mechanical redundancy with respect to the anisotropic deformation of the wall. From the almost logarithmic increase in stiffness in the circumferential and radial (thickness) directions, it can also be hypothesized that the optimal stiffness of the guard cell wall has a narrow range, but the control of cellulose abundance is not as tight since the rate of increase diminishes when the cellulose amount increases beyond a certain point.

9.3 Range of stiffness of guard cell wall

From the effect of the wall matrix modulus on the guard cell wall modulus, it can be deduced that plants may need to regulate wall matrix stiffness within a narrow allowable range to achieve the appropriate level of longitudinal elongation of guard cells. This requirement, along with the plasticity of cell wall composition and the ability of plant cells to sense and optimize cell wall integrity, could be a reason for the alterations in stomatal kinetics, but usually without a complete loss of stomatal function, observed in hemicellulose and pectin-related mutants. For example, a change in pectin composition in response to cellulose deficiency has been hypothesized to compensate for defective cell wall structure (His et al. 2001). Previous experimental studies of stomatal function in various genotypes, including a cellulose deficient mutant (*cesa3^je5^*), a hemicellulose deficient mutant (*xxt1 xxt2*) and altered pectin-related mutants (*PGX1*, *PGX3* and *pll12*), demonstrate the partial maintenance of stomatal function (Rui and Anderson 2016; Carter *et al.* 2017; Rui *et al.* 2017; Yi *et al.* 2018). Based on the prediction of the multiscale model, such a robust recovery is possible not because the required mechanical properties of the guard cell wall are forgiving, but because multiple pathways exist to maintain those properties. The question then becomes how plants sense deficiencies in the guard cell wall and activate compensatory mechanisms to preserve stomatal function; wall integrity sensing from a mechanical perspective is only beginning to be studied in plants (Vaahtera *et al.* 2019). Additionally, further research could explore whether alternate combinations of guard cell wall composition and water exchange mechanics exist across the plant kingdom that maintain or improve stomatal functionality from the perspective of water and nutrient use. It should be noted that genotypes investigated in the above-mentioned studies are, by necessity, ones that have suitable compensatory mechanisms to prolong the lifespans of the plants enough to be studied. Therefore, the effects of alterations in cell wall components on stomatal function need to be interpreted with such potential biases in mind.

9.4 Future studies

The presented bottom-up multiscale guard cell wall model can generate relevant research questions concerning how plants regulate stomatal guard cell wall mechanics to achieve stomatal function. Combined with advances in imaging techniques, such as super-resolution microscopy (Haas *et al.* 2020), this model can examine the biomechanical and functional implications of the orientation, arrangement and distribution of cell wall polysaccharides. For example, cellulose-deficient or pectin-altered mutants tend to have thinner cell walls (Yi *et al.* 2018). Currently, it is challenging to measure changes in the dimensions of guard cell walls *in vivo*. Nonetheless, the described model can help quantify the implication of such variations, including different turgor pressure increments, non-circular cross-sections of guard cell walls traced from micrographs of different plant species and cellulose microfibrils of varying abundance and length distribution.

Furthermore, this model enables quantitative investigation of the implications of the interactions between cellulose and wall matrix components, including hydrogen bond potentials (Lennard–Jones potential) as investigated by Zhang *et al.* (2021) or altered interactions between matrix components and cellulose in cellulose-, hemicellulose-, and pectin-deficient mutant plants. The findings from this study can also be extended to investigate inelastic mechanical behaviours in plants, e.g. stress stiffening or stress softening at the tissue or whole-plant scales connecting the molecular structure of the plant cell wall to macroscale plant dynamics such as growth (Moulia *et al.* 2015; Bou Daher *et al.* 2018; Bidhendi and Geitmann 2019; Piatnitski and Ptashnyk 2020).

Given that the nature and contribution of cellulose–matrix interactions are considered critical for proper plant cell functions, this quantitative research tool has the potential to lead to new discoveries through *in silico* experiments and identification of exciting experimental research questions.

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CONTRIBUTIONS BY THE AUTHORS

H.Y. and C.T.A. conceived the project; H.Y. developed the model and prepared the manuscript; C.T.A. edited the manuscript.

CONFLICT OF INTEREST STATEMENT

None declared.

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