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ARTICLE ADDENDUM

Integrating cell biology, image analysis, and computational mechanical modeling to analyze the contributions of cellulose and xyloglucan to stomatal function

Yue Rui[a,b], Hojae Yi[c], Baris Kandemir[d], James Z. Wang[d], Virendra M. Puri[c], and Charles T. Anderson[a,e]

[a]Department of Biology; [b]Plant Biology Interdepartmental Graduate Program; [c]Department of Agricultural and Biological Engineering; [d]College of Information Sciences and Technology; [e]Center for Lignocellulose Structure and Formation, The Pennsylvania State University, University Park, PA, USA

ABSTRACT

Cell walls are likely to be essential determinants of the amazing strength and flexibility of the guard cells that surround each stomatal pore in plants, but surprisingly little is known about cell wall composition, organization, and dynamics in guard cells. Recent analyses of cell wall organization and stomatal function in the guard cells of *Arabidopsis thaliana* mutants with defects in cellulose and xyloglucan have allowed for the development of new hypotheses about the relative contributions of these components to guard cell function. Advanced image analysis methods can allow for the automated detection of key structures, such as microtubules (MTs) and Cellulose Synthesis Complexes (CSCs), in guard cells, to help determine their contributions to stomatal function. A major challenge in the mechanical modeling of dynamic biological structures, such as guard cell walls, is to connect nanoscale features (e.g., wall polymers and their molecular interactions) with cell-scale mechanics; this challenge can be addressed by applying multiscale computational modeling that spans multiple spatial scales and physical attributes for cell walls.

Abbreviations: CMF, cellulose microfibril; CESA, cellulose synthase; CSCs, Cellulose Synthesis Complexes; FEM, finite element model; FP, fluorescent protein; MT, microtubule

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Cell walls in stomatal guard cells

Stomata are microscopic pores surrounded by paired guard cells that serve as epidermal gateways for plant transpiration and gas exchange. Stomatal dynamics are driven by changes in guard cell turgor, and are regulated via stomatal patterning[2] and the unique mechanical properties of guard cells and their walls.[3] However, despite the detection of certain wall components[4,5] and functional analyses of pectic arabinans[7,8] in guard cells, the question of how guard cell walls are constructed to be both sufficiently strong to withstand high turgor and flexible enough to allow repeated stomatal movements remains largely unexplored.

Cellulose and xyloglucan regulate anisotropic guard cell expansion

Cellulose is produced by plasma membrane-localized cellulose synthesis complexes (CSCs), which are composed of multiple CELLULOSE SYNTHASE (CESA) proteins and travel along trajectories that co-align with underlying cortical microtubules (MTs).[9] Using spinning disk confocal microscopy of fluorescent protein (FP)-labeled CESAs in young tissues, we found that after stomatal closure, CSCs moved faster and there was less colocalization between CSCs and MTs.[1] We also detected drastically lower CSC density in guard cells in older tissues, suggesting that wall mechanical requirements for mature guard cells are largely fulfilled by existing cellulose that was previously deposited.[1]

Cellulose in guard cells is arranged radially,[11,12] and xyloglucan is a hemicellulose that modulates wall mechanics[13] and is present in guard cell walls.[4] To test whether cellulose and xyloglucan regulate the anisotropic deformation of guard cells during stomatal movements,[14,15] we analyzed mutants deficient in cellulose or lacking detectable xyloglucan, finding that cellulose restricts, whereas xyloglucan facilitates longitudinal expansion and contraction in guard cells, thus regulating stomatal aperture.[1] Cellulose and xyloglucan are also required for the reorganization of cellulose in guard cells between a diffuse pattern in the open state and extensive bundles in the close state.[1] The use of these mutants has allowed us to dissect the contributions of individual wall components to stomatal dynamics.

Automated detection of microtubules and CSCS using image analysis

Colocalization analysis for MTs and CSCs at different developmental stages and functional states can yield knowledge about the assembly of guard cell walls, since localization and physiological function are often associated for subcellular structures.[16] To gauge colocalization for MTs and CSCs, FP-CESA particles were detected using Imaris software (Bitplane, Zurich,

CONTACT Charles T. Anderson cta3@psu.edu

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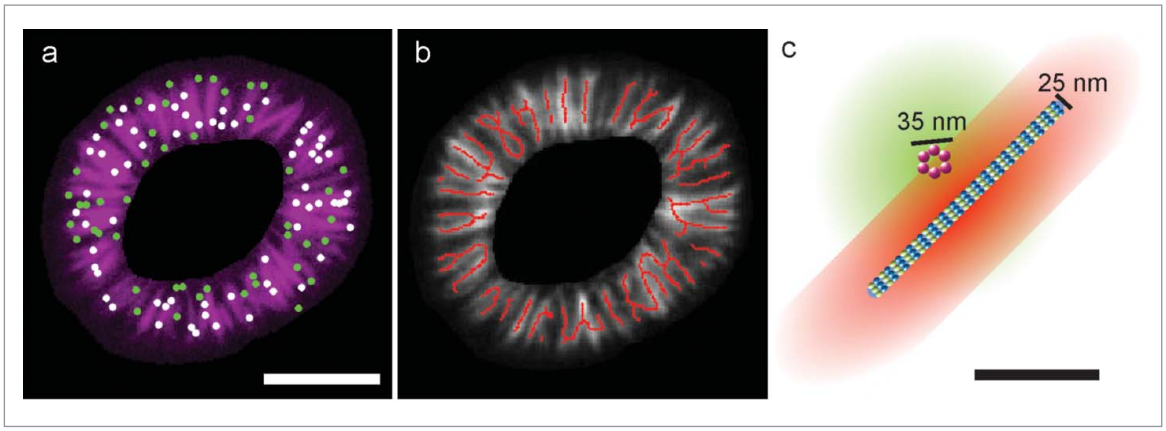

Figure 1. Automated CSC and MT detection, and the challenge of colocalization analyses. (a) shows automated detection of CSCs (circles) overlaid on top of MT image channel (magenta). White circles are judged by eye to overlap with MT signals, whereas green circles are judged not to overlap. In (b), red structures represent automatic MT detection through vesselness analysis and skeletonization. (c) depicts the uncertainty surrounding CSC (rosette structure 35 nm in diameter) and MT (linear structure 25 nm in diameter) localization (represented by green and red signal clouds, respectively, which approximate diffraction patterns for fluorescent signals) and the difficulty in colocalization analyses caused by this uncertainty, which can lead to apparent signal overlap when physical interaction does not occur. Scale bar in (a) = 5 μm; scale bar in (c) = 200 nm.

Switzerland) and the particles that overlapped with MT fluorescence were initially manually selected[1] (Fig. 1a). To reduce user bias and enhance quantitation, colocalization analyses can be automated through two basic approaches: pixel-based (signal overlap, intensity correlation), and object-based (spatial evaluation).[17] The latter is favored since it presents an opportunity for spatial exploration of the colocalized signal while being robust to noise and background fluorescence.[18]

In object-based colocalization analyses, MT detection can be improved by automatic extraction. For example, eigen analysis of nematic tensors (threadness measures) can be applied.[19] On the other hand, MTs have similar shapes to those of blood

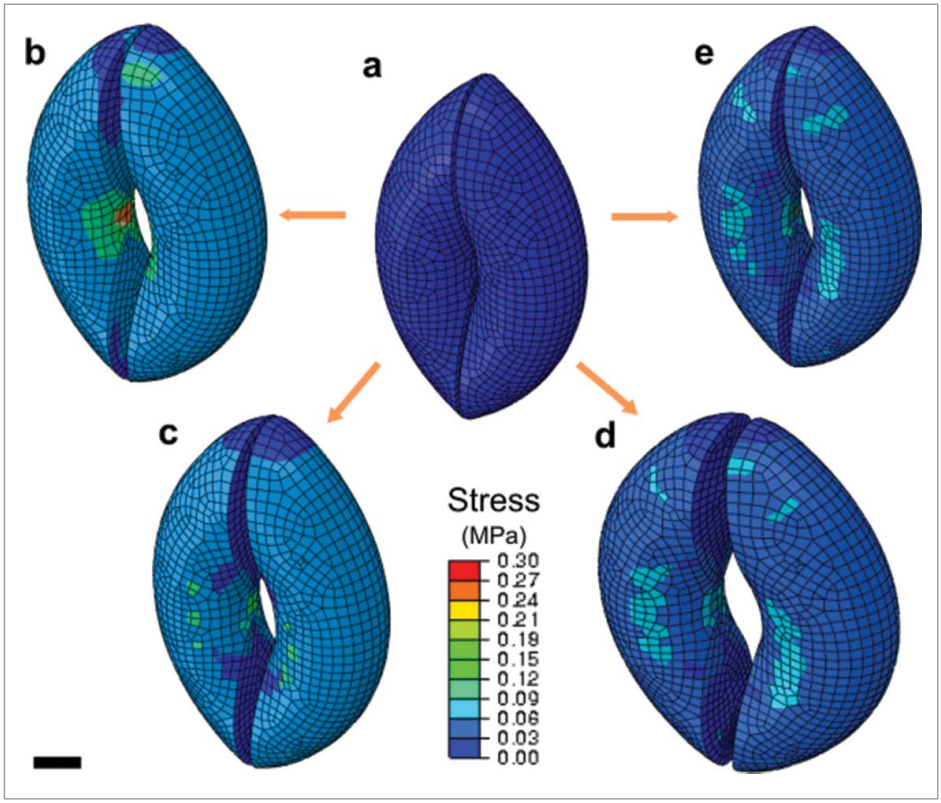

Figure 2. FEMs of Col, cellulose-deficient, and xyloglucan-deficient stomata. (a) FEM of a stomatal guard cell pair using shell elements based on measured Arabidopsis stomatal dimensions[1] and guard cell wall thickness.[6] (a) shows a FEM of a closed stomate, whereas (b) shows the result of simulated opening of a stomate with uniform cell wall thickness caused by 5 MPa turgor. (c) When the thickness of the ventral region of guard cell wall is increased,[6] overall stress also increases but concentrated stress near the opening decreases, suggesting feedback between mechanical stress and wall deposition to reinforce the high stress area. (d) FEM with decreased wall stiffness (e.g., due to cellulose deficiency) results in a larger stomatal aperture after pressurization. (e) However, when stiffness reduction is anisotropic (e.g., in the absence of xyloglucan[10]), the stomatal opening does not increase as much after pressurization. Scale bar = 5 μm.

vessels. Thus, vessel segmentation methods[20,21] can be leveraged to detect MTs. Tubular structures in images can be modeled through eigen analysis of Hessian images, using a multidirectional, second order derivative matrix to obtain a vesselness map.[22] Noise due to point spread functions can be reduced via vessel enhancing diffusion and low rank representation.[23,24] Post-processing on vesselness maps can reveal MT positions (Fig. 1b). MT segmentation maps can then be quantitatively compared to CSC positions detected by Imaris. One remaining problem is the uncertainty about the real locations of CSCs and MTs due to the resolution limits of optical imaging systems. Even after structure segmentation, "uncertainty clouds" for object positions must be accounted for when measuring colocalization (Fig. 1c).

Multi-scale mechanical modeling of wild type, cellulose-deficient, and xyloglucan-deficient guard cell walls

To extend the findings of Rui and Anderson,[1] we developed a finite element model (FEM) of guard cell pairs (Fig. 2) that integrates several aspects of guard cell geometry and dynamics, including aperture,[1] pore area, surface area, and volume changes.[15,25] In this model, the guard cell pair is constructed with anisotropic shell elements, including mechanical interactions with neighboring cells. This model enables quantitative investigation of changes in turgor and of interactions between guard cells and surrounding cells during stomatal movements.[3]

From the quantitative results of our computational model, we have gained new insights into the importance of the morphology and composition of guard cell walls. For example, instead of modeling uniform wall thickness (Fig. 2b), modeling differential thickness across the guard cell wall[6] decreases stress concentration near the stomatal pore after simulated opening (Fig. 2c). In addition, we modeled a mutant guard cell pair with altered mechanical properties due to cellulose deficiency. After pressurization, stomatal aperture is noticeably larger in this stomate (Fig. 2d). Because xyloglucan is thought to act as a linker or spacer between cellulose microfibrils (CMFs),[13] which are highly oriented in guard cell walls,[12] the loss of xyloglucan in *xxt1 xxt2* mutants might have anisotropic effects on wall stiffness. When we introduced anisotropic changes in wall stiffness, the model predicted slightly smaller stomatal apertures after turgor increase (Fig. 2e). However, this aperture value is close to that of a "wild type" modeled cell wall (Fig. 2c,e), suggesting that compensatory changes in the wall, e.g., CMF bundling,[26] might buffer the mechanics of *xxt1 xxt2* guard cell walls.

Conclusions

Advances in imaging resolution and computational image analysis should allow the molecular organization of guard cell walls and the cellular machinery that constructs them to become clearer. We now have evidence that pectins, xyloglucan, and cellulose all contribute to regulating stomatal dynamics,[1,8] implying that multiple components of guard cell walls contribute to their mechanical properties. Future studies will be required to determine how these and other wall components work in concert to enable guard cells to open and close repeatedly without mechanical wear, and might uncover sensing, maintenance, and repair mechanisms that enable these cellular hydraulic devices to function over the lifetime of a plant tissue.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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