

Structural inhomogeneity of cellulose assembly in plant cell wall affecting anisotropic mechanical property

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Abstract

Cellulose microfibrils (CMFs) in plant cell walls are a major load-bearing component in plant primary cell walls, and their collective orientational alignment is known to be a key factor to determine the mechanical properties of the cell wall. Plant epidermis has been widely used as a model system for the primary cell wall to study the cellulose structure and tissue mechanics because of its ease of access for characterization. However, the structural information of CMFs in epidermal walls and their mechanics have often been interpreted assuming that CMFs are uniformly distributed in the whole tissue. Here, we report distinct CMF assembly patterns in the flat face region of the epidermal cell and the edge region of the cell where two cells meet. The vibrational sum frequency generation (SFG) imaging analysis found that the CMF orientation in the cell edges is preferentially aligned perpendicular to the anticlinal walls. Finite element analysis (FEA) was employed to test if the cell geometry and the discovered inhomogeneous CMF assemblies could explain the previously observed anisotropic mechanical properties of epidermal cell walls. Our study resolves discrepancies in microfibril structure obtained with different techniques and suggests that the distinct CMF assemblies in the edge region may contribute to tissue-level mechanical anisotropy of epidermal cell walls.

Introduction

Although the composition and structure of individual plant cell walls vary depending on their functions,^{1,} ^{2, 3, 4} it is generally accepted that cellulose microfibrils (CMFs) are the major load-bearing component that governs tensile mechanical properties of the cell wall.⁵ Each elemental CMF consists of 18 linear chains of b-1,4-linked glucose units synthesized from cellulose synthase complexes (CSCs).^{6, 7} The cellulose chains are assembled through inter-chain hydrogen-bonding interactions into sheets, and these sheets stack through van der Waals interactions forming a crystalline order.^{8, 9, 10} The mesoscale structural assemblies of these nanocrystalline CMFs with other polysaccharides in cell walls must be optimized to carry specific biological and physical properties at cellular or tissue levels that are harmonized for the survival and growth of the entire plant.^{1, 2, 11}

For a more accurate understanding of structure-property relationships of plant primary cell walls, a prerequisite is to know the structure of CMFs in the cell wall. Onion epidermal walls are considered a good model system for such studies because they are easy to prepare for characterizations.^{12, 13, 14, 15, 16} For example, the outermost periclinal wall of the onion epidermis is easily peeled off, revealing the most recently deposited CMFs on the cytoplasm side of the wall.^{17, 18} Those CMFs were imaged with atomic force microscopy (AFM) and field emission scanning electron microscopy (FESEM), which showed that CMFs are deposited in a crossed-polylamellate structure.^{16, 19, 20, 21} In each lamella, CMFs are loosely aligned along a preferential orientation, which varies by 30-90° among adjacent lamellae.¹⁶ Such variations of dominant CMF orientations among neighboring lamellae could result in the equal probability of all orientations if averaged over all lamellae inside the wall. A recent study using cryo-

electron tomography showed that microfibrils are distributed bimodally with broad angular distributions centered at $42^{\circ} \pm 8^{\circ}$ and $135^{\circ} \pm 10^{\circ}$ with respect to the longitudinal axis of the cell.²²

In contrast, spectroscopic studies suggested a different picture, namely 'anisotropic' orientational distribution. Most spectroscopic analyses of large-area samples have suggested that CMFs in the onion epidermis have a slightly anisotropic orientation with a preferential axis tilted toward the transverse direction of the cell.^{13, 14, 23} This anisotropic distribution of CMFs has been thought to be the origin of the larger mechanical extensibility of epidermal walls along the longitudinal direction than the transverse direction.^{24, 25} However, these results are inconsistent with the crossed-polylamellate structure in which the averaged CMF orientations across the wall thickness is expected to be nearly isotropic,^{14, 16, 23} or diagonally bimodal.²² Studies of onion epidermis stained with Congo Red and analyzed by polarization confocal microscopy reported net cellulose orientation varying from transverse to random to longitudinal depending on the growth stage of the scale, but generally running perpendicular to the major growth direction.^{15, 26}

In this study, we have used vibrational sum frequency generation (SFG) microscopy to reassess cellulose orientation in the onion wall in greater detail. SFG is known to selectively detect crystalline cellulose in plant cell walls, and its spectral features are sensitive to the nano-to-meso scale structural orders of cellulose.^{9, 27, 28, 29} Through sub-cellular scale imaging, the 'face' and 'edge' regions of cells in the epidermal peel could be distinguished.^{30, 31} Here, the edge is defined as the region where the periclinal walls of adjacent cells meet. The face is the flat and uniform-thickness region in the periclinal plane surrounded by the cell edges. The hyperspectral SFG imaging of epidermal walls of onion, *Arabidopsis* stem, and maize coleoptile showed that the microfibrils in the edge region are preferentially aligned perpendicular to the plane of anticlinal walls. Furthermore, finite element analysis (FEA) offered a novel explanation of the anisotropic tensile modulus of the onion epidermal wall.^{32, 33, 34}

Results

SFG imaging of CMF with subcellular resolution

Figure 1 shows the hyperspectral SFG images and full spectra of different regions of the fifth scale onion epidermis (also see Supplementary Fig. 1). Epidermal walls of *Arabidopsis* stem and maize coleoptile also show similar patterns (Supplementary Figs. 2 and 3). Although there are some variances among replica samples, common features can be found in hyperspectral images of these epidermal walls. In the face region, the cellulose-characteristic CH stretch peak can be identified around 2944 cm⁻¹, and it does not change upon rotation of the sample by 90°. The CH intensity is very weak and the 3320 cm⁻¹ OH stretch peak characteristic of cellulose is barely identifiable above the noise level. In the edge region, the SFG intensity is much stronger than in the face region (Supplementary Fig. 1–4). The 3320 cm⁻¹ / 2944 cm⁻¹ intensity ratio varies upon rotation of the sample by 90°. The ratio is larger when the edge region (dark lines in the optical image) is parallel to the *pps* laser incidence plane, and it is smaller when the

edge line is perpendicular to the laser incidence plane. Within the CH stretch region, the 2865 cm⁻¹ / 2944 cm⁻¹ and 2968 cm⁻¹ / 2944 cm⁻¹ intensity ratios also vary upon rotation of the sample by 90°, which is similar to the 3320 cm⁻¹ / 2944 cm⁻¹ ratio trend.

Anisotropic alignment of CMFs in edge regions

In previous SFG analyses of uniaxially aligned cellulose nanocrystals (CNCs), highly aligned CMFs in the G-layers of reaction woods and ramie fibers,^{35, 36} similar changes in the relative intensities of 2865 cm⁻¹, 2968 cm⁻¹, and 3320 cm⁻¹ peaks with respect to the 2944 cm⁻¹ peak were observed upon 90° rotation of the sample. In theoretical calculations of the SFG intensity of cellulose, it has been shown that when the chain axis of CNCs and CMFs are aligned with the electric field of IR beam (i.e., the third letter in the polarization combination), the 3320 cm⁻¹ OH intensity is enhanced and the 2944 cm⁻¹ CH intensity is relatively weak.³⁷ This means that, in the *pps*-SFG spectrum, the 3320 cm⁻¹ / 2944 cm¹ intensity ratio will be high when the CMF axis is aligned perpendicular to the laser incidence plane. In contrast, when CMFs are aligned parallel to the laser incidence plane, this intensity ratio becomes smaller as compared to its orthogonal orientation case. The relative intensities of the 2865 cm⁻¹ and 2968 cm⁻¹ peaks with respect to the 2944 cm⁻¹ peaks with respect to the 2944 cm⁻³ and 2968 cm⁻³ peaks with respect to the 2944 cm⁻¹ peak will show a similar trend for the highly anisotropic distribution case.³⁷

When this knowledge is employed to interpret the data in Fig. 1 and Supplementary Fig. 1–3, it is found that *CMFs in the edge region of the outermost periclinal wall are anisotropically aligned with the dominant axis perpendicular to the plane of the anticlinal walls*. This interpretation does not change even if the lamellae are gradually tilted out of the periclinal plane (Supplementary Fig. 5a-d) towards the anticlinal wall direction. If the distribution is modeled with a Gaussian function, the standard deviation is estimated to be around 30° or less from the dominant alignment direction (Supplementary Fig. 5e). Cross-polarization (CP) optical imaging also supported the high degree of CMF alignment in the edge region (Supplementary Fig. 6).

Another supporting evidence for the highly anisotropic CMF distribution in the edge region can be found through direct imaging of CMFs exposed in the cytoplasm side of the cell wall, although this approach reveals the distributions in the top two layers only (sometimes up to 3 layers).¹⁶ Fig. 2a displays a low-magnification FESEM image of the abaxial epidermal wall of the fifth scale of onion after pectin removal through pectate lyase treatment and Fig. 2b-d show high-resolution images of CMFs in three distinct regions. In the face region (Fig. 2b), the crossed-polylamellate patterns of CMFs in the topmost and underneath lamellae can be seen clearly.¹⁶ Gradual changes in CMF orientations are observed in the region (Fig. 2c) between the face and edge region. In the edge region (Fig. 2d), CMFs appear to be highly aligned toward the anticlinal wall direction. This orientation is consistent with the preferential orientation found by the hyperspectral SFG analysis of CMFs in the edge region.

Angular distribution of CMFs in the face region

The absence of angular orientation dependence of the SFG spectral features in the face region is consistent with the nearly-isotropic CMF orientation distribution expected from the crossed-polylamellate structure or the diagonally bimodal distribution.^{16, 22} This is also supported by the low transmission intensity of the face region in the CP imaging (Supplementary Fig. 6). The OH SFG intensity of CMFs in the face region is quite weak, while the CH signal is still measurable (Fig. 1 and Supplementary Fig. 1–4). In the face region in primary cell walls, elementary fibrils are found as 'singletons' and they often merge together, forming 'bundles'.^{16, 19, 20} The elementary CMFs with ~ 3.5 nm diameter have only 18 chains of cellulose,^{2, 38} and about 44% of OH groups of cellulose are exposed at the CMF surface. The surface OH groups are readily converted to OD groups upon contact with D₂O which was used for hydration in SFG experiments. Another 33% OH groups are separated from the surrounding D₂O by only one glucose unit. If cellulose crystallinity is low or thermal stability of the surface glucan chain is not sufficiently high, the interior-side OH groups of 18-chain CMFs remain intact, which may explain the weakness of the OH SFG signal from CMFs in the face region.

Aligned polymer molecules can crystallize more easily than disordered molecules through inter-chain

binding.³⁹ The same could pertain to CMFs. If five CMFs are tightly bundled laterally through $(1 \ 1 \ 0)$ or (110) facets via hydrogen bonding interactions, then the interior OH group fraction increases to 74%. These interior OH groups can generate strong OH signals in SFG measurements of cell walls hydrated with D₂O since they will not be converted to OD.^{40, 41} Based on this argument, it could be inferred that CMFs packed along the preferential orientation direction in the edge region may be more highly bundled laterally than is the case for CMFs in the face region.

Sub-cellular variation in CMF assembly

Combining all information obtained from SFG microscopy, CP-transmission microscopy, and FESEM analyses, a comprehensive model describing the CMF arrangement in the outermost epidermal walls is constructed as Fig. 3. The face region has the crossed-polylamellate structure as documented previously.^{16, 20} On the other hand, in the edge region, CMFs are preferentially aligned perpendicular to the anticlinal plane and likely to be bundled more than those in the face region. Analysis of cross-sectioned samples by transmission electron microscopy (TEM) (inset of Fig. 3) confirms that lamellae in the edge region, except for a small portion near the cuticle side, gradually tilt away from the periclinal plane and transition toward the anticlinal plane. The anticlinal walls of epidermis are much thinner than the periclinal walls. Thus, it is likely that not all CMF-containing lamellae of the periclinal wall are continuously connected to those of the anticlinal wall.

The sub-cellular inhomogeneity in CMF assembly sheds light on the discrepancy between micro- imaging of the face region with AFM and FESEM and macro-scale spectroscopic studies of epidermal peels. In the previous SFG analyses of large-area samples encompassing walls of multiple cells, the average orientation of CMFs was considered to be transversely biased.¹⁴ Due to the elongated cell shape, the

longitudinal edge fraction is larger than the transverse edge fraction in a given area. In other words, transversely aligned CMFs in the longitudinal edge region are sampled more in the large probe area analysis. This sampling bias effect can be seen in the area-averaged SFG spectra shown in Fig. 1e and f.

The preferentially aligned CMFs in the edge region may also contribute to the anisotropic extensibility of onion epidermal peels with nearly isotropic or diagonally distributed CMF orientations. A polarized-IR microscopy study of onion epidermis found that the average orientation angle of CMFs in the face region is ~ 54° with respect the longitudinal cell axis, which could be interpreted as random (since the value is close to the 54.7° magic angle) or a slight preferential orientation toward the transverse direction (since the value is larger than 45°).²³ In that study, it was suggested that CMFs in the face region are likely to be tilted more toward the transverse direction, based on a ~ 20% larger extensibility along the longitudinal direction for a given mechanical load. However, the microscopic SFG (Fig. 1) and CP-transmission (Supplementary Fig. 6) data of this study indicate CMF orientations across the entire wall thickness of the face region consistent with nearly isotropic or diagonally bimodal distribution.²² Then, the 54° angle found in the polarized-IR microscopy analysis should be interpreted as equal probability of all possible orientations. Assuming this is the case, the anisotropic extensibility may originate from the anisotropic alignment of CMFs in the edge region (Fig. 3).²³ When the preferentially aligned CMF organization in the edge is included, the fraction of the more extensible cell wall, in which CMFs are aligned perpendicular to the stretch direction, is larger when stretched in the longitudinal direction than in the transverse direction due to the elongated cell shape.

Explaining anisotropy in tissue-level modulus

The stress-strain curves of epidermal cell walls show, in general, a relatively linear stress response in a small strain followed by a strain stiffening and yielding behavior at larger strains,^{5, 34, 42} even though there are some variances due to differences in sample preparation, tissue thickness, and clamping method. The fifth-scale abaxial epidermis of onion also shows the same nonlinear behavior (Fig. 4a). It is interesting to note that the modulus appears to be higher along the longitudinal direction at a small strain (< 6%; Supplementary Fig. 8) but larger along the transverse direction at a large strain (> 12%). This suggests strain stiffening along the transverse direction. The elastic modulus of the fifth-scale onion epidermis in the small strain regime was reported to be about 25% higher in the longitudinal direction (E_1) than in the transverse direction (E_T) .³⁴ In Fig. 4a, the calculated elastic modulus ratio (E_I/E_T) in the small strain (0–3%) was ~ 1.2 (15.3 \pm 4.0 MPa and 12.9 \pm 1.2 MPa). This is in qualitative agreement with the previous literature.³⁴ Even though the species or tissues are different, several other studies with plant epidermis have reported that the elastic modulus in the small strain is larger in the longitudinal direction.^{32, 33, 43} Also, the second-scale abaxial epidermis of onion was stretched by \sim 13% in the longitudinal direction and ~ 11% in the transverse direction under the same tensile stress of ~ 4 MPa.²³ Such tissue-level anisotropy is difficult to explain if the entire cell wall has a uniform CMF structure.^{34, 43} Thus, some studies have suggested anisotropic net orientation of CMFs within the periclinal cell wall,^{23,} ⁴³ which is possible if the distribution of CMFs in the crossed-polylamellate structure or diagonally

bimodal distribution in the periclinal wall is slightly skewed into one direction. As an additional factor or alternative explanation, we propose that the anisotropic alignment of CMFs in the edge region (Fig. 3) could contribute to the tissue-level anisotropy.

To study the potential impact of the anisotropic alignment of CMFs in the edge region on the tissue-level anisotropy of tensile modulus in the small strain region, finite element analysis (FEA) was conducted with a simple elastic model. The true elastic modulus of the edge region with the varying thickness could not be modeled; thus, the effects of modulus and thickness changes are lumped into a single 'effective' modulus for simplification (see inset of Fig. 4b). Since CMFs are aligned anisotropically in the edge region, the effective modulus in the direction perpendicular to the edge (E_a^{eff}) could be different from that in the direction parallel to the edge (E_b^{eff}). Then, FEA calculated the possible E_a^{eff}/E_{face} and E_b^{eff}/E_{face} solutions for given E_L/E_T ratios for elastic strain along the longitudinal and transverse directions (Fig. 4b).

Here, the most plausible solution is the area where $E_a^{eff} > E_b^{eff}$ (region-I, right side of the diagonal dotted line in Fig. 4b). Assuming CMFs are the major load-bearing component,⁵ the E_a^{eff} along the preferential alignment direction of CMFs is expected to be larger than the E_b^{eff} perpendicular to the CMF alignment direction. In this case, the edge region modulus (E_b^{eff} , E_b^{eff}) should also be larger than the face region modulus (E_{face}). This is consistent with the higher SFG intensity in the edge region than in the face region, which suggests more crystalline CMFs or a higher degree of CMF bundling as mentioned above.

The solution with $E_{face} < E_a^{eff} < E_b^{eff}$ (region-II in Fig. 4b) is mathematically possible but physically inconceivable because the elastic modulus of cell walls in which microfibrils are along the stretch direction (E_a^{eff}) is expected to be higher than that of cell walls in which microfibrils are perpendicular to the stretch direction (E_b^{eff}).⁵ When cell walls are stretched in the direction perpendicular to the microfibrils, microfibrils are separated and curved, carrying a little load.⁵ If CMFs are not the main load-bearing component in the edge region because of the deviation of the lamella plane from the tensile stress axis (see TEM image in Fig. 3), then E_a^{eff} could be smaller than E_{face} (region-III in Fig. 4b). This would be the case where compression or shear of the pectin-rich region between adjacent lamellae are the main strain response under small stress.

In the large strain regime, CMFs are definitely the main load bearing component.⁵ In this case, the stretch along the preferential alignment direction of CMFs would be more difficult than that in the orthogonal direction. Due to the anisotropic shape of the cell, there are more longitudinal edges than transverse edges in a given square-shape area (Fig. 1d). Thus, there is a larger fraction of CMFs that are aligned along the transverse direction than the longitudinal direction (Fig. 3). This can explain why the strain at the large stress is smaller along the transverse direction (Fig. 4a).²³ This may be the main reason that the

strain stiffening behavior is more prominent along the transverse direction, leading to a crossover from $E_L > E_T$ in the small strain regime to $E_L < E_T$ in the large strain regime.

In summary, sub-cellular SFG imaging of CMF assemblies in the outmost periclinal walls of epidermal cells of onion bulb, *Arabidopsis* stem, and maize coleoptile revealed that CMFs are preferentially aligned perpendicular to the anticlinal plane in the cell edge region, while CMFs are nearly isotropic or diagonally distributed in the cell face region. This finding was further corroborated with CP-transmission microscopy and FESEM analysis of onion epidermis. The anisotropic CMF assembly in the edge region of the periclinal wall may contribute to the mechanical anisotropy of the epidermis.

Methods

Materials Fresh white onion bulbs (*Allium cepa*, cv. Cometa) were purchased from a local grocery store. The fresh hydrated scale immediately appearing after removal of the dried scales was numbered as first, and the outermost single layer of cell walls (abaxial epidermis) of the fifth scale was analyzed. *Arabidopsis Thaliana* (ecotype Columbia-0) was obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. The seed growth condition is described in the previous publication.⁴⁴ After 10 days, seedlings were transferred to pots containing soil and grown in a growth chamber (Percival, Perry, GA, USA) at 22°C under a 16-h-light and 8-h-dark cycle for 7 weeks. Hybrid maize seeds (*Zea mays*, 5480GENVT2PRIB) from SEEDWAY, LLC. (Hall, NY) were soaked in water for 30 min, sown on an absorbent paper cloth saturated with water, and placed in a plastic box wrapped in aluminum foil. Seeds were incubated in the dark at 28°C for 4 days.

Preparation for SFG analysis The fifth scale onion epidermis was peeled and rinsed with deionized water several times. The details of this protocol are described in previous publications.^{14, 17, 18, 45} The maize coleoptile epidermis was peeled approximately 0.5 cm from the tip. The *Arabidopsis* stem epidermis was peeled approximately 15 cm from the bottom using branched, and the single layer of cell wall at the end of the peel was cut and used. The excised and rinsed peels were immersed in D₂O with 0.02% sodium azide overnight. The peel was mounted on a slide glass with the plasma membrane side facing up. After a few drops of D₂O were applied, a coverslip was placed on the sample. After removing any overflowing liquid, the coverslip edges were sealed with nail polish to prevent sample dehydration.

Vibrational SFG microscopy system A broadband SFG system with 800 nm pulses (pulse width ~ 85 fs with 2 kHz repetition rate) was utilized for this experiment. The detailed description of the microscopic SFG system can be found in previous publications.^{46, 47} For the analysis of the onion epidermis, a 15× reflective objective was used which produced the Gaussian-like beam shape with ~ 5.4 µm along X-axis, ~ 7.9 µm along Y-axis, and ~ 26 µm along Z-axis in the lab coordinate (See **Fig. 1**).⁴⁶ For the analysis of the maize coleoptile epidermis, *Arabidopsis* stem epidermis and cross-sectioned onion epidermis, a 36× reflective objective was used which generated the Gaussian-like beam shape with ~ 2.4 µm along X-axis, ~ 4.1 µm along Y-axis, and ~ 15 µm along Z-axis. ⁴⁶ Note that this dimension is based on the one-sigma standard deviation of the Gaussian beam shape; the tail of the beam spread much larger than this

dimension. The SFG spectra of fully hydrated samples in D_2O were collected at two different sample mount geometries (0° and 90° with respect to the laser incidence plane) to study the preferential orientation of CMFs. The hydration with D_2O instead of H_2O was to avoid the attenuation of IR probe beam in the OH stretch band region. The polarization combination used for data collection was p for SFG signal p for 800 nm, and s for IR (which will be called pps hereafter). The effect of CMF orientation on SFG intensities of cellulose characteristic peaks was simulated using the theoretical algorithm fully described in the previous publication.³⁷

Field Emission Scanning Electron Microscope (FESEM) The fifth scale abaxial epidermal strips from the middle of the convex surface were treated with pectate lyase to remove the pectin. Then, the samples were undergone critical point drying through Leica EM CPD 300, and the inner side (plasma membrane side) of the epidermis was imaged by Zeiss Sigma FESEM. The detailed experimental procedure is described in previous publications.^{16, 48}

Transmission Electron Microscope (TEM) The fifth scale onion epidermis was undergone a high-pressure freezing through (Leica, Wetzlar, Germany) EM HPM 100 and media substitution through Leica automatic freeze substitution (AFS). The tissue blocks were trimmed, and the cross-section side was imaged by FEI Tecnai Spirit G2 TEM (FEI, USA). The detailed procedure was described in the previous publications.¹⁶

Finite Element Analysis (FEA) A two-dimensional repetitive volume element (RVE) was modeled using Abaqus CAE (Dassault Systèmes, Simulia Corporation). The idealized cell shape and parameters used for RVE were based on the previous study by Zamil et al. (120 µm long, 30 µm and 60 µm short and wide width).³⁴ The width of cell edge region was set to 10 µm. The elastic and isotropic modulus of the face wall area was assumed to be 15 MPa.⁵ For the edge regions, various values of orthotropic and elastic 'effective' modulus were assumed, and the tissue-level longitudinal and transverse moduli were calculated with the periodic boundary condition.⁴⁹ The Poisson's ratio of 0.48, which is a typical value for incompressible biological materials, was used for the face region for the stability criterion and to reduce the convergence issue in Abaqus CAE.³⁴ The same Poisson's ratio and shear modulus in the face region was assumed for the edge region.

Tensile testing of onion epidermal walls Onion abaxial epidermal wall strips (10 mm × 3 mm × 7 μ m) were peeled from the center region of the fifth scale of onion bulbs. Wall strips peeled from longitudinal (along long axis of onion cells) and transverse directions were stretched at the speed of 5 mm/min on a custom-built stretching device where the position of the clamp and applied loading were recorded simultaneously.¹⁸ The extensometer and stretch experiment procedure were described in the previous publications.^{20, 50} The stress was calculated by dividing the applied force by the initial cross-sectional area of the wall strip (3 mm × 7 μ m). Strain was the amount of extension divided by the initial gauge length of 5 mm. Modulus was calculated as the slope from 0 to 3% strain by linear regression (n = 11 and 9 for the longitudinal and transverse directions, respectively).

Cross-polarized (CP) optical microscopy The cross-polarized (CP) optical microscopic imaging of onion epidermis was carried out with Olympus BX61 compound microscope equipped with a UPLFL 10× objective (NA = 0.3), a polarizer, and a U-AN360 analyzer. Images at different sample orientations were captured with a fixed exposure time under identical conditions.

Cross-polarized (CP) optical microscopy intensity calculation The optical response of CMFs in the transmission CP imaging was modeled theoretically.⁵¹ Cellulose has a positive birefringence with the main optical axis along the cellulose chain axis. The refractive indices associated with the two short axes are similar.⁵² In the theoretical calculation, the birefringent refractive index of cellulose determined with Muller matrix ellipsometry was used: n_e = 1.6538 and n_o =1.4847 at the wavelength of 600 nm.⁵³ The degree of rotation and the attenuation of the polarized input beam by a single crystal at different azimuth angles was calculated as in Supplementary Fig. 9. The cellulose crystal was modeled as a thin slab with a thickness of 3.5 nm, and the input beam was divided into two components – parallel (E) and perpendicular (E_{\perp}) to the cellulose long axis (Supplementary Fig. 9a). The transmission coefficients for E and E_{\perp} are calculated using Eq. 1⁵¹:

$$t_{123} = \frac{t_{12}^* t_{23}^* \text{Exp}[-i\beta]}{1 + r_{12}^* r_{23}^* \text{Exp}[-i2\beta]}$$

1

where t_{nm} and r_{nm} are the transmission and reflection coefficients at the interface of two media 'n' and 'm', and b is the phase difference between the two beams reflected from the interface between the first and second media and the interface between the second and third media along the beam propagation direction. In the calculation, the mediums 1 and 3 are water, and the medium 2 is cellulose. b is calculated with the tilt angle (q) of near 90° using Eq. 2:

$$\beta = \frac{2\pi \mathrm{d}}{\lambda} \mathrm{n}_2^* \mathrm{cos} \theta$$

2

The parallel and perpendicular components have different transmission coefficients due to the different refractive indices.⁵³ The resultant E_{\perp} and E_{\perp} are used to calculate the polarization direction (y) and the amplitude after passing through the crystal using Eqs. 3 and 4:

$$\psi{=} an^{-1}({
m E}\ /{
m E}_{ot})$$

3

$$|\mathrm{E}_{\mathrm{t}}| \!=\! \sqrt{\mathrm{E} \,\, \mathrm{E}^{*} + \mathrm{E}_{\perp} \mathrm{E}_{\perp}^{*}}$$

4

where ψ is the polarization direction of the beam with respect to the Y-axis in Supplementary Fig. 9a. The square of the E_t after the projection to the second polarizer axis is the final polarization intensity.

$$\mathrm{I}{=}(|\mathrm{E}_{\mathrm{t}}|*{\mathrm{sin}}\psi)^2$$

5

This calculation can be repeated 100 times to calculate the overall CP-transmission of the polarized light after passing through 100 cellulose crystals. 100 crystals with specific angular distributions were generated according to the four possible organizations of cellulose microfibrils in plant cell walls, and the polarization direction and amplitude after the 100th crystal were calculated as in Supplementary Fig. 9d-f.

Declarations

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Author Contributions

J.L. conceived the paper. J.L., J.C., L.F., J.Y., Y.Z., and Y.L. collected and analysis the data and drafted the manuscript. S.S. and Q. Z. provided *Arabidopsis thaliana* and maize coleoptile samples. All authors edited the manuscript and contributed to data analysis and interpretations.

Competing interests

The authors declare no competing interests.

Additional information

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Figures

Figure 1

Microscopic SFG analysis of onion epidermal cell wall. (a) Microscopic SFG analysis (300 $^{\prime}$ 300 μ m²) of an abaxial epidermis of the fifth scale onion hydrated in D₂O. The aspect ratio of cells is 3.6±0.2. The sample is lying in the XY plane, and the laser incidence is in the XZ plane. The polarizations of the probe beams are *p* for SFG, *p* for 800 nm, and *s* for IR. (b,c) Hyperspectral images of the 3320 cm⁻¹ SFG signal superimposed on the optical image. (d) Regions from which full spectra are extracted. (e,f) SFG spectra averaged over the longitudinal and transverse edge regions and the face region. The error bar is standard

error of mean at the peak position. In (e,f), also shown are the averaged SFG spectra of the whole area of (b,c). The data shown in (b,e) and (c,f) are when the long axis of the cell is perpendicular and parallel to the laser incidence plane, respectively. In (b,c), note that the lowest intensity area in the contour plots was adjusted to be transparent to show the underlying optical images. The color scale bars next to the contour plots were also adjusted accordingly. The black scale bars in the images are 20 µm. Similar features are observed in two additional datasets (Supplementary Fig. 1).



Figure 2

FESEM images of the onion epidermal cell wall. (a) Low magnification image showing both the edge and face regions. The inset in (a) shows the entire cell, and the marked box is (a). Also shown are high magnification images of the (b) face, (c) transition, and (d) edge regions of the epidermal cell wall. Arrows in the images show the directions of microfibrils. Note that wrinkles running across the entire image of each panel are due to the shrink and collapse of the cell wall during the sample drying. A similar trend was observed in three replicates (Supplementary Fig. 7a-1).



Figure 3

Inhomogeneous cellulose microfibril assemblies in onion epidermis. (a) Schematic drawing of the outermost periclinal wall of epidermis with the illustration of CMF assemblies in the edge and face regions. The TEM image is the edge region of the cross-sectioned fifth scale onion epidermis (scale bar = $2 \mu m$). A similar trend was observed in three replicates (Supplementary Fig. 7m-o).



Fig. 4 Anisotropic nonlinear stress-strain behavior of onion epidermis. (a) stress-strain curve from uniaxial tensile tests of the abaxial wall of the fifth scale onion in the longitudinal and transverse directions. (b) Isoline map of the calculated E_L/E_T ratio as a function of E_a^{eff}/E_{face} and E_b^{eff}/E_{face} for an elastic (small) strain along the longitudinal and transverse directions. Insets show how effective modulus along the direction perpendicular and parallel to the inter-cellular edge line (E_a^{eff} and E_b^{eff}) is defined, and the cell-scale RVE model. The longitudinal and transverse dimensions of the individual cell (L=120 µm, W₁=30 µm, W₂=60 µm, W₃=10 µm) were adapted from the previous FEA study by Zamil et al.³⁴ The solid isolines are marked at $E_L/E_T = 1.0$, 1.19 (the calculated value from Fig. 4a), and 1.25 (study by Zamil et al.³⁴).

Figure 4

See image above for figure legend.

Supplementary Files

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