

Differential Cellulose Distribution Drives Polarized Growth of Cotton Fibers

Corresponding Author: Dr Zhaosheng Kong

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

Reviewer Comments:

The paper investigates the mechanism of polarized growth in cotton fiber cells, focusing on how the differential distribution of cellulose microfibrils (CMFs) drives the heterogeneity of the cell wall and directional expansion. The study found that the cellulose gradient is a key factor driving polarized growth, while pectin modification has a minimal impact on fiber elongation. These findings deepen the understanding of the mechanisms underlying polarized growth in plant cells, and provide new insights for improving crop fiber quality.

All my concerns have been addressed in this revision.

There are some minor comments need to be revised:

1 The bar is missed in Figure 5a, Figure 6a, Extended Data Fig.5b.

2 Some figures (Figure 1, Figure 5, Extended Data Fig.1, Extended Data Fig.4) does not indicate which groups were compared, and the asterisks are missing in the figure.

(Remarks on code availability)

This paper does not involve any code information.

Reviewer #4

(Remarks to the Author)

The authors examine the biomechanics of cotton fibres in the context of cotton fibre morphogenesis. I like the idea of using the cotton fibre to look at growth, especially because appears to be a blend of tip and diffuse growth, and has a relatively simple and highly anisotropic shape.

The authors have shown there is a gradient of cellulose, which they suggest translates into a gradient of stiffness that in turn results in growth gradient driving elongation. Unfortunately, they have no quantification showing the presence of growth gradients, and their method for measuring stiffness is not really suitable for the diffuse growing section, as it can only measure stiffness normal to the surface.

Without any growth measurements and stiffness measurements limited to the normal direction, it is difficult to be convinced of their main hypothesis.

General comments

I notice not much data is presented showing cotton fibres do indeed display both tip and diffuse growth, it seems to be just taken as given. It would be nice if the authors could point to some growth tracking data that does indeed show this. The

authors wrote a Current Opinion in 2011 on this subject, but did not present any tracking data there either. Does this mean it is not possible to track growth in cotton fibres? like people have done for trichomes and pollen tubes? In any case, if this is the state of the art, then statements of how they grow need to be presented more carefully as hypotheses, not fact. In addition, it would be good to know what the relative percentages are believed to be. Is it mostly at the tip? the flank? 50-50?

It is great that you have been able to track the cellulose synthase in this system with live imaging, so is there a reason you cannot track the growth as well? These things grow pretty fast, so one does not need to keep it happy that long.

Most of the results in relation to mechanical properties of the wall depend on AFM measurements. However, I am not sure that this technique is so useful to determine stiffness in diffuse growing areas, at least not in a way that is relevant for morphogenesis. In tip growing cells the wall at the apex has no cellulose, and thus growth there is mostly isotropic. In this case the AFM can likely be used to measure the stiffness that could be relevant for growth. In the diffuse part the wall is anisotropic because of the cellulose. Since the AFM can only measure in the direction normal to the surface, it cannot capture the directions that are important for growth. What is needed is an in-plane measurement of the stiffness, and in this case the longitudinal direction would be most important.

One could use potentially use the AFM to “see” cellulose bundles, as was done in Sampathkumar et al. 2014 using similar equipment. In that work the images look pretty convincing. Here the images appear very different, and I am not so convinced that these images visualize cellulose fibres. In order to show they do, the authors would need to show they are not just showing for irregularities in the surface shape, for example wrinkling from being plasmolysed. If a smooth patch of surface is found, with stiffer and softer sections, then this could indicate the presence of cellulose bundles. So it would be good to see both the surface topography and the stiffness map side by side.

In any case, the cellulose in this system is highly transversely oriented, so we have no way of knowing if more cellulose means more stiffness in the longitudinal direction. To access this, one would need a different method, such as stretching with an extensometer, or osmotic treatments. The system is too small for an extensometer, but what about osmotic treatments? One should be able to image the fibre, plasmolyse it, and then image again and observe the shrinkage. One could put fluorescent beads to make landmarks to track the deformation, again as has been done in trichomes and pollen tubes.

Another related issue throughout is the authors are stressing the importance of a cellulose gradient as a main driver for shape and morphogenesis in the cotton fiber. Since the cellulose is predominately transverse, would that not imply it primarily stop the cells from widening? And how is it that they don't get too wide towards the tip, where there is much less cellulose? especially since the stress is higher in the transverse direction. One has to wonder if this system mimics a bit pollen tube growth. In pollen tubes there is pure tip growth, but the tip has no cellulose, it appears displaced from the tip later on, mostly in a transverse pattern as here. Thus in pollen tubes, you get a very elongated shape from isotropic growth, that is limited to a small area at the tip. Cellulose in pollen tubes does not appear to be involved in creating the shape, but only appears in areas after the growth has stopped. The more tapered shape in cotton fibres that enters the region with cellulose suggests that diffuse growth is required, and that it cannot be solely tip growth

The authors frequently conflate the concepts of elasticity and extensibility. They are not the same, and just because a wall is more elastic (softer) does not mean it is more extensible. A wall needs to be elastic (have stretch) and have extensibility factors (ie expansins, etc) in order to grow. Without the latter the wall will not grow. It could be that there is a gradient of extensibility factors, rather than differences in elasticity, that are the primary drivers of growth gradients. Although expansin appears as the predominant extensibility factor in cell wall with cellulose, it is not clear what the extensibility actors are at the tip where growth happens without cellulose, since the usual pectin suspects have been apparently ruled out.

Not heard of the Turkey test (brings strange images to mind), perhaps the authors mean Tukey test?

Specific comments:

Line 40: GhCSI1 is mentioned in the abstract without saying what it is.

Line 52: What about callose?

Line 58: Technically speaking, CMFs create anisotropy in the wall that governs elastic behaviour, how the wall extends to make shape is a different matter, although they are usually, but not always, correlated.

Line 76: Deposition stops, but secondary cell wall is produced? Is that not still cell wall (ie cellulose) deposition?

Line 110: It is not clear what the authors mean by modulus and stiffness here. The authors only talk about stiffness in the methods. The fibres were dried, so I don't understand how they measure modulus if they say it relates turgor pressure. If modulus is being calculated from the stiffness with some sort of Herzian contact model, I am not sure why they should be so qualitatively different. This section needs substantial clean up, and needs to be made much more precise.

The max force for the AFM and indentation depth should also be reported.

Line 124: Again, you are conflating elasticity and extensibility. (also Lines 130,

Line 312: What existing paradigms about cell wall extensibility are being challenged?

Line 339: Do you really know what the gradient of extensibility looks like? If you have now time-lapse tracking, how do you know there is a gradient of growth. It could be the tip grows fast, and the whole shank grows more slowly, but the same. I don't think you can say this with any certainty.

Line 342: It is not known that microtubules respond to mechanical cues, it is a hypothesis, which so far has little direct experimental support.

Line 375: The idea that cellulose is the most important player for elongation, and that pectin is not important, is in obvious contrast with rapid growth at the tip. Again this stresses the necessity to have growth data in this system to support this hypothesis.

(Remarks on code availability)

The model seems pretty straightforward, more springs = slower growth, so the outcome is intuitive and I am sure it can be easily reproduced.

Reviewer #5

(Remarks to the Author)

The key messages of the study is a basipetal gradient of cellulose microfibril density from the apex to the shank. Apical regions are reported to be softer, while the shank is stiffer to support overall cell integrity. It make sense and is verified by computational simulation that CMF distribution influences strain patterns along the fibers. Primary wall during cotton fiber elongation is reported with 22% semi-crystalline cellulose fibrils, which are surrounded by matrix polymers including xyloglucan and pectin. So far, there has been a lot of evidence that presence and modification of the matrix polymer may regulate fiber elongation.

In this study, the focus is solely on cellulose accumulation and the main evidence by Raman spectroscopy lacks integrity of data and spectral interpretation. Only 3 spectra are shown from 10DPA and analysis is only based on the 380cm⁻¹ band, which is taken and interpreted as cellulose accumulation. Raman spectra of cellulose reflect also changes in cellulose orientation and crystallinity, which is not analysed and taken into account at all. Further, pectin and hemicellulose bands should not be overseen and discussed. Unfortunately only 10DPA is analysed with Raman, while data from AFM are from 3DPA and 7DPA and staining from 3DPA and 2DPA, cell wall synthesis 3DPA. The study would benefit from a more conclusive experimental design including Raman at different developmental stages (same as AFM) and mutants added. Additional the study will benefit from a more comprehensive Raman spectra interpretation, instead of plotting the intensity of one peak for cellulose content. That can only be done, if changes in orientation and crystallinity can be ruled out based on the spectral signatures.

In the Material and Method part of Raman experiments no information is given, but only the link to a paper which has nothing to do with Raman: 51) Liu, W. et al. Towards a better recording of microtubule cytoskeletal spatial organization and dynamics in plant cells. J Integr Plant Biol 61, 388-393 (2019). <https://doi.org/10.1111/jipb.12721>.

Please provide all necessary information or at least correct citation. And it would be interesting for the reader to comment why laser tweezers Raman microspectroscopy was chosen and not conventional Raman imaging using a Confocal Raman microscopy, which might give more detailed information including spatial changes in content and orientation.

Some questions also remain regarding AFM results: Why are peak force error images shown instead of stiffness and modulus images. They might not reflect changes in mechanical properties, but where the feedback system struggles to maintain the setpoint force. Stiffness and modulus are defined in a strange way, as "modulus refers to elasticity of the cell walls under turgor pressure, while stiffness reflects the overall resistance of the cell wall against deformation"(Line110-112). Why is modulus related to turgor pressure? Normally modulus is referred to the "intrinsic" material properties (taking geometry into account), while stiffness just refers to materials resistance to deformation under the applied force. In line 116 suddenly a conclusion is made on toughness, which is a mechanical parameter usually not gained by AFM data as no full strain-stress curve is acquired. Please take care about basic mechanical definitions.

In line 120 "a remarkable correlation between mechanical parameters and cotton fiber growth" is reported, but just due to the fact that the two cultivars showed differences in modulus. Based on the presented data it is not easy to follow and verify such correlation and statement! It could be a bit too far fetched to conclude from a lower modulus and stiffness in Hai fibers to a connection to greater final length (Line 123-126)! In Fig. 1, the modulus is similar in both cotton variants at 3DPA, but only the stiffness different. This raises again the question on how these values were derived. It would be more trustable to show at least in the supplementary the raw data – the acquired force curves of the two species, developmental stages and position. It would also be helpful to comment why the DMT model was used, as this is usually more recommended when adhesion forces are significant, like in hydrated conditions. Have you compared results to the Hertz model? How was the quality of the fit to the force-distance data? Was the decision for DMT model based on quality of the fit or other considerations? Have you measured wet cellulose fibrils and therefore chosen DMT? Please add this information in M&M. Some detailed comments:

Line 39-42: conclusion from microtubule perturbation to CSC cellulose distribution and cellulose gradient not clear and not shown. Or have there been any Raman and AFM measurements on the mutant? Figure1: b) why do peak force error images show mechanical features and which ones? Why not pictures of stiffness and modulus are shown? Line 774: what means differed developmental stages? 3 and 7? And are in c) the different positions mixed or is it the base or the shank or the tip? It is misleading that in c the different colors refer to different cotton species and in d) to different developmental stages. Are in the different regions (d) the species mixed or which species?

Supplementary Figure 1: what are "represent images"? Based on the pictures it is difficult to derive how height and width was

measured...please replace "represent images" by which images these are and mark the width and height of the measured fibre in the image.

Figure 2: a) Not necessary to show a cellulose standard. What is shown in the last? I guess it is the difference between all spectra, but at this size nothing is really visible. B) you talk about relative, so this means it is normalized? If so on what? Why only at 10DPA? c and d relevant for proofing a basipetal gradient of cellulose?

Supplementary Fig.1:

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The author has addressed all my comments and it seems to be accepted.

(Remarks on code availability)

Reviewer #4

(Remarks to the Author)

I am satisfied with the changes made by the authors.

The comments related to imprecise terminology in places have been resolved, along with the tempering of some statement where required to avoid overstatement. The addition of osmotic treatments is very nice, although it is a bit a pity they could not spatially resolve elongation with beads or similar, although I appreciate the technical challenges there.

My other comments were mostly related to growth tracking, which appears to be too technically challenging in this system, although I appreciate the effort there. I guess it is the same as longitudinal deformation tracking in osmotic treatments. It is too bad, because knowing where the growth, and the relative contribution between shank and tip, would strengthen their story, but it is what it is.

A few small points:

Line 112-116: This is OK, but I would go with "cell wall stiffness" or "cell wall elastic modulus" for the cell wall, and "apparent stiffness" or "apparent elastic modulus" for the AFM stiffness.

Line 360-363: The way this is worded, it sounds at first like you are talking about cell walls in general, maybe start with "In cotton fibres, our findings ..."

Line 908: I not sure what "mock" means here? You treated some fibres and not others? Normally this would be presented as a before/after experiment. A picture of the measurement before treatment (in water) and then after in Mannitol, ideally with the figure panel showing the same fibre.

(Remarks on code availability)

I looked at the code, but did not verify it in any way. The model is pretty simple, and does what one would expect. I don't think it would be hard to reproduce.

Reviewer #5

(Remarks to the Author)

Thank you for taking into account the mistakes in terminology and for correcting accordingly.

Instead of additional analysis and/or measurement of the sample spectra, non relevant reference spectra for hemicellulose and pectin have been added as Supplementary data as well four spectra of developmental stages without spectral interpretation. Supplementary reference spectra from my point of view can be removed as published elsewhere and the xylan spectrum as well as the pectin looks very strange and no clear material and methods from where these references were purchased. Same for 3 and 7DPA as long as they are not analysed as the author state them as "complex and deviate from the cellulose standard". Does this mean at this stage cellulose plays no role? In the current form adding the spectra is not beneficial as no conclusions are drawn and the idea to have data from the same developing stages from Raman and AFM still not fulfilled.

Based on the new spectra shown in Fig. 3a in the analysis of the 10DPA spectra it seems problematic when doing a min-max normalisation over the whole wavenumber region as in some spectra 380 will be max and in some 1095, which of

course influences the analysis of peak heights. I do not understand what the Pearson correlation with the cellulose standard should tell you? As it is higher in the tip and sup-tip, you have more cellulose there than in the shank? And no cellulose in 3DPA and 7DPA?

Based on the plotted data in Fig. 3b) it is difficult to follow that differences are really statistically significant! Data contradict the authors assumption that these bands can be interpreted as cellulose content as they give not the same results. For 1120band the shanke seems not really different anymore (statistical test correct?) and for 380 probalby some spectra were normalised on the 380band (see red spectra in a) and therefore high!.

Fig. 3a,c: I do not understand what the pearson correlation vs. cellulose should bring in 3DPA and 7DPA? Important in spectral analysis is not only correlation, but interpreting the peaks. It is logical that cellulose dominates as soon as present in crystalline form. I therefore still think that cellulose in such Raman data can not be analysed only by cellulose content, as the influence of crystallinity and orientation is tremendous on the height of the Raman signal! And even if contributions of hemicellulose and pectin are naturally less reflected in the spectra, they are obviously there and need to be taken into account. If this is not possible for the authors they have to use another method.

Fig. 3d): does not bring any information, it is evident from the spectra that they are different and will group accordingly. Interpreting bands of loading 1 and 2 would be informative. But please do not show a PCA without discussing loadings..than it is better not to show.

Fig. 3e and f: has nothing to do with the Raman results.

(Remarks on code availability)

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Point-by-point responses:

Reviewer #2 (Remarks to the Author):

Reviewer Comments:

The paper investigates the mechanism of polarized growth in cotton fiber cells, focusing on how the differential distribution of cellulose microfibrils (CMFs) drives the heterogeneity of the cell wall and directional expansion. The study found that the cellulose gradient is a key factor driving polarized growth, while pectin modification has a minimal impact on fiber elongation. These findings deepen the understanding of the mechanisms underlying polarized growth in plant cells, and provide new insights for improving crop fiber quality.

All my concerns have been addressed in this revision.

Response: We sincerely thank the reviewer for your thoughtful evaluation and kind words regarding our manuscript. We are very pleased to know that the revised version has fully addressed your previous concerns. We are grateful for your supportive comments on our work.

There are some minor comments need to be revised:

1 The bar is missed in Figure 5a, Figure 6a, Extended Data Fig.5b.

Response: We thank the reviewer for the careful inspection of the figures. For all conventional 2D images, we have checked and ensured that scale bars are now properly shown. For the 3D reconstruction panels, the images are rendered with an overlaid 3D grid, where each grid unit corresponds to the underlying voxel size. Adding an additional 2D scale bar would obscure the 3D rendering and is not recommended for this representation. To avoid confusion, we have now clarified the spatial scale of these 3D views explicitly in the figure legends.

2 Some figures (Figure 1, Figure 5, Extended Data Fig.1, Extended Data Fig.4) does not indicate which groups were compared, and the asterisks are missing in the figure.

Response: We appreciate the reviewer's comment. The asterisks have been replaced with exact p-values and group comparisons are now clearly stated in the figures.

Reviewer #4 (Remarks to the Author):

The authors examine the biomechanics of cotton fibres in the context of cotton fibre

morphogenesis. I like the idea of using the cotton fibre to look at growth, especially because appears to be a blend of tip and diffuse growth, and has a relatively simple and highly anisotropic shape.

The authors have shown there is a gradient of cellulose, which they suggest translates into a gradient of stiffness that in turn results in growth gradient driving elongation. Unfortunately, they have no quantification showing the presence of growth gradients, and their method for measuring stiffness is not really suitable for the diffuse growing section, as it can only measure stiffness normal to the surface.

Without any growth measurements and stiffness measurements limited to the normal direction, it is difficult to be convinced of their main hypothesis.

Response: We thank the reviewer for the thoughtful and constructive evaluation of our work and for highlighting these two important limitations. We fully agree that direct, spatially resolved quantification of growth and in-plane measurements of wall stiffness would provide the most stringent test of a growth gradient model. In the present study, our experimental evidence and modelling are intended to support a tip-biased diffuse growth mode rather than to claim that we have directly measured a continuous growth gradient along the fiber. We have carefully revised the text to avoid overstating this point and to clarify the scope of our conclusions.

Technically, precise tracking of local growth rates in individual cotton fibres is challenging due to their small diameter, curvature, and movement on the ovule surface, and would likely require dedicated microfluidic or long-term immobilization setups that are beyond the scope of the current work. Nonetheless, we have tried our best to address the concerns by performing a simple tracking assay, which qualitatively supports faster elongation near the apex. Given the limited spatial resolution, these pilot data are summarized in this response letter but not included as a formal figure. In addition, we carried out osmotic deformation experiments to functionally probe regional differences in wall deformability. We refer to these new data in the Results and Discussion as supportive evidence for regional differences in growth potential, while still avoiding over-interpretation as a fully quantified growth gradient.

Regarding the stiffness measurements limitation, we agree that AFM does not directly measure the longitudinal stiffness that is most relevant for axial elongation. In our study, AFM-derived apparent modulus and stiffness are therefore used as comparative indicators of the local mechanical state of the cell wall, as is common in plant biomechanics studies, rather than as absolute elasticity parameters to directly evaluate plant cell growth rate. The new osmotic deformation data help bridge this gap by showing that regions with lower apparent stiffness and modulus are also more deformable under turgor-related perturbation. We have moderated the wording throughout to better reflect these limitations and the scope of our conclusions.

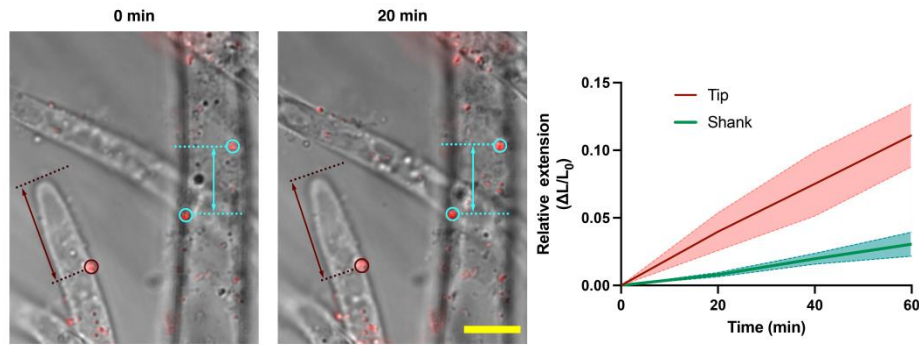
General comments

I notice not much data is presented showing cotton fibres do indeed display both tip and diffuse growth, it seems to be just taken as given. It would be nice if the authors could point to some growth tracking data that does indeed show this. The authors wrote a Current Opinion in 2011 on this subject, but did not present any tracking data there either. Does this mean it is not possible to track growth in cotton fibres? like people have done for trichomes and pollen tubes? In any case, if this is the state of the art, then statements of how they grow need to be presented more carefully as hypotheses, not fact. In addition, it would be good to know what the relative percentages are believed to be. Is it mostly at the tip? the flank? 50-50?

It is great that you have been able to track the cellulose synthase in this system with live imaging, so is there a reason you cannot track the growth as well? These things grow pretty fast, so one does not need to keep it happy that long.

Response: We appreciate this insightful comment and fully agree that direct tracking of growth dynamics would strengthen the support for the growth model of cotton fiber. In fact, we had considered and attempted growth-tracking experiments during the initial phase of this work. However, precise live tracking of cotton fiber growth remains technically challenging. At the rapid elongation stage, fibers are long, curved and frequently intertwined on the ovule surface, which makes it difficult to unambiguously identify and follow the apical regions over time. At earlier stages when fibers are shorter and less entangled, the instantaneous growth rate is relatively low, so that detectable length changes over experimentally accessible time windows are close to the resolution limit. In addition, we had to partially excise fiber bundles from the ovule and mount them in ovule culture medium under a coverslip; under these conditions, fiber growth is usually perturbed and the fibers tend to drift in the liquid, further compromising the stability required for accurate long-term tracking. By contrast, tracking the dynamic of CESAs and MT organization in live cotton fiber cells is more technically accessible. Because CESA particles provide bright, membrane-localized signals that can be followed without the need for long-term mechanical stability.

We have tried our best to perform a short-term tracking assay using fluorescent beads as fiduciary markers on the fiber surface. Although the beads did not allow homogeneous labelling along the entire fiber and their adhesion was not sufficiently stable for long-term recording, we followed several extension events from the apical and shank regions. A representative example is shown below, together with a plot of relative extension ($\Delta L/L_0$) over time (please see Responsive Figure 1). These preliminary data qualitatively support faster elongation near the apex compared with the shank, consistent with a tip-biased diffuse growth mode. As we felt that the spatial resolution and the measurement precision of these attempts were not yet sufficient for a robust quantitative analysis, we have chosen not to include them in the main text.



Responsive Figure 1. Left, representative bright-field images of fibers at 0 and 20 min, with fluorescent carboxylate-modified polystyrene beads (circles) used as fiduciary markers at apical (dark red) and shank (cyan) regions. Dashed lines indicate the tracked fiber segments. Scale bar 10 μm. Right, relative extension ($\Delta L/L_0$) over time for the apical (Tip, red) and shank (green) segments; lines indicate the mean trend and shaded areas show the confidence interval across tracked fibers.

Regarding the question about the relative contributions of the tip versus the shank (e.g. “50–50”), our current data are not sufficient to provide a robust quantitative partitioning of growth along the fiber. While the apical region clearly shows higher instantaneous extension potential, the shank spans a much larger fraction of the cell length, and its cumulative contribution to final length is likely non-negligible. The precise spatial profile of growth and the relative contributions of apex and shank remain to be quantified in future work. Such quantification will likely require dedicated microfluidic or immobilization setups that allow stable, long-term tracking of individual fibers. We have now clarified these technical limitations and moderated our statements in the Discussion accordingly. Please see Line 473-479.

Most of the results in relation to mechanical properties of the wall depend on AFM measurements. However, I am not sure that this technique is so useful to determine stiffness in diffuse growing areas, at least not in a way that is relevant for morphogenesis. In tip growing cells the wall at the apex has no cellulose, and thus growth there is mostly isotropic. In this case the AFM can likely be used to measure the stiffness that could be relevant for growth. In the diffuse part the wall is anisotropic because of the cellulose. Since the AFM can only measure in the direction normal to the surface, it cannot capture the directions that are important for growth. What is needed is an in-plane measurement of the stiffness, and in this case the longitudinal direction would be most important.

Response: We appreciate this important comment and fully agree that AFM indentation primarily probes the out-of-plane mechanical response of the wall and does not directly provide in-plane elastic constants, particularly in the longitudinal direction. Direct in-plane measurements at cellular resolution remain technically challenging in most plant systems, including cotton fibers.

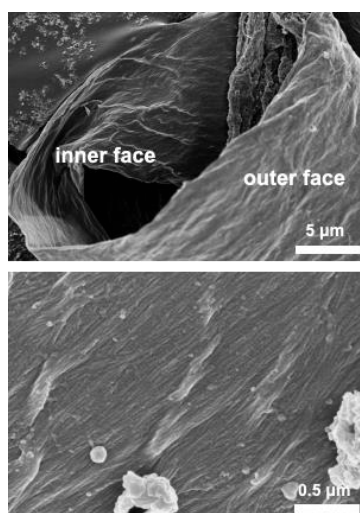
Nevertheless, AFM-based stiffness mapping reflects the composite mechanical state of

the wall and has been widely used to reveal spatial heterogeneity of wall mechanics relevant to morphogenesis in plant systems such as guard cells and the shoot apical meristem (Peaucelle, Braybrook et al. 2011, Sampathkumar, Krupinski et al. 2014, Carter, Woolfenden et al. 2017). In these contexts, regions with reduced stiffness are associated with higher wall deformation or growth, suggesting that local wall softening can accompany increased expansion potential. In this study, we follow the same logic and use AFM-derived apparent modulus and stiffness to provide a relative mechanical landscape that reports local differences in wall mechanical state, rather than as absolute in-plane stiffness values or direct measurements of growth rate.

To further address this concern, we have now performed **osmotic deformation assays**. Under hyperosmotic treatment, the apical regions exhibit greater transverse shrinkage than the shank, consistent with a softer and more deformable wall at the apex. These results are in line with the AFM measurements and support the idea that regions with lower apparent stiffness have higher deformation potential under changes in turgor. We have incorporated these new data into the revised Fig1. We have also moderated our wording to emphasize that our AFM measurements and osmotic assays provide a relative mechanical context for tip-biased diffuse growth, rather than a direct quantification of longitudinal stiffness or a fully resolved growth field. Please see Line 114-123 and Line 142-148.

One could use potentially use the AFM to “see” cellulose bundles, as was done in Sampathkumar et al. 2014 using similar equipment. In that work the images look pretty convincing. Here the images appear very different, and I am not so convinced that these images visualize cellulose fibres. In order to show they do, the authors would need to show they are not just showing for irregularities in the surface shape, for example wrinkling from being plasmolysed. If a smooth patch of surface is found, with stiffer and softer sections, then this could indicate the presence of cellulose bundles. So it would be good to see both the surface topography and the stiffness map side by side.

Response: We appreciate this valuable comment and the reference to Sampathkumar et al. (2014) (Sampathkumar, Krupinski et al. 2014). The system used in that study was the *Arabidopsis* cotyledon epidermis, where cellulose microfibrils form a reticulate network on a relatively flat surface that is particularly well suited for AFM imaging. By contrast, our own data indicate that the primary walls of cotton fibers contain densely packed cellulose microfibrils that are highly aligned in a predominantly transverse orientation, rather than forming a crossed mesh. Importantly, scanning electron microscopy (please see Responsive Figure 2) and S4B staining both reveal densely packed, transversely oriented cellulose microfibrils on the fiber surface, consistent with the banded patterns in the AFM maps. Thus, the more parallel bands observed in our images most likely reflect a genuine biological difference in CMF organization.



Responsive Figure 2. SEM imaging of cotton fiber primary walls. **(Top)** Fractured cotton fiber showing the inner and outer faces of the cell wall. Transverse banding is observed on both the inner and outer faces. **(Bottom)** Higher-magnification view of the wall surface, revealing densely packed cellulose microfibrils aligned and transverse to the fiber axis.

From a technical standpoint, cotton fibers are slender cylindrical protrusions on the ovule surface, which makes it challenging to obtain large, perfectly planar imaging areas as in leaf epidermis. To minimize geometric artefacts, we therefore restricted our AFM scans to visually smooth segments of the fiber surface and used relatively small scan windows to reduce curvature effects. And all measurements acquired and fitted under identical conditions to ensure that relative differences are meaningful. Under these conditions, the apparent modulus maps provide a local readout of wall mechanics, which are used as comparative indicators of local wall mechanical state between regions and genotypes rather than as absolute *in vivo* stiffness values.

For the datasets shown, scan regions were selected only when the height images showed no obvious folds or detachment and the force–distance curves were stable and reproducible across the field. Under these conditions, the apparent modulus maps provide a robust readout of local wall mechanics, which is the quantity analyzed in this study. Representative topography images are provided in Supplementary Fig. S1, and SEM together with S4B staining (Responsive Fig. 2) independently confirm that the banded patterns reflect transversely aligned CMFs rather than surface wrinkling.

Following suggestions from another reviewer, we now show apparent modulus maps in the revised Fig. 1b, which more directly represent the mechanical contrast used in our analysis. The surface topography remain provided as 3D AFM height renderings in Extended Data Fig. S1, which were used for quantifying fiber morphology. Together with the SEM and S4B results, these maps support the interpretation that the AFM contrast reflects the underlying cellulose microfibril organization and wall mechanics, rather than artefacts from surface irregularities or plasmolysis.

In any case, the cellulose in this system is highly transversely oriented, so we have no way of knowing if more cellulose means more stiffness in the longitudinal direction. To access this, one would need a different method, such as stretching with an extensometer, or osmotic treatments. The system is too small for an extensometer, but what about osmotic treatments? One should be able to image the fibre, plasmolyse it, and then image again and observe the shrinkage. One could put fluorescent beads to make landmarks to track the deformation, again as has been done in trichomes and pollen tubes.

Response: We appreciate this insightful suggestion. In cotton fibers, cellulose microfibrils are predominantly oriented in the transverse direction, so their primary geometric role is indeed to resist circumferential expansion. At the same time, as the major load-bearing component of the wall, local cellulose accumulation is expected to influence the overall stiffness of the wall in both transverse and longitudinal directions through its interactions with matrix polymers. This view is consistent with recent theoretical treatments of the cell wall as an anisotropic composite material, in which cellulose reinforcement influences the overall stiffness of the wall in multiple directions (Coen and Cosgrove 2023). In our study, we therefore combine information on cellulose distribution, wall mechanics and modelling to infer how cellulose accumulation may shape deformation patterns along the fiber.

To provide the in-plane deformation readout suggested by the reviewer, we have now performed osmotic treatments on living 3-DPA fibers. Fibers were imaged after treatment with either 0.8 M mannitol or pure water, and changes in fiber width were quantified at the tip and the shank. Mannitol-induced plasmolysis caused a significantly larger transverse shrinkage at the tip than at the shank, whereas mock-treated fibers showed minimal changes (revised Fig. 1e,f). These data indicate that apical walls are more deformable under turgor-related perturbation, consistent with the softer wall mechanics at the tip and with the higher elongation potential predicted by our mechanical modelling. Although in the current experiments we quantified transverse shrinkage rather than axial shortening, the osmotic assay nevertheless provides an in-plane, turgor-driven measure of local wall compliance that complements our AFM and Raman analyses. We agree that fully resolving how transverse cellulose reinforcement translates into effective longitudinal stiffness will require more refined in-plane mechanical measurements. We have now discussed this point in the revised Discussion as an important direction for future work. Please see Lines 476-479.

Another related issue throughout is the authors are stressing the importance of a cellulose gradient as a main driver for shape and morphogenesis in the cotton fiber. Since the cellulose is predominately transverse, would that not imply it primarily stop the cells from widening? And how is it that they don't get too wide towards the tip, where there is much less cellulose? especially since the stress is higher in the transverse direction. One has to wonder if this system mimics a bit pollen tube growth. In pollen tubes there is pure tip growth, but the tip has no cellulose, it appears displaced from the

tip later on, mostly in a transverse pattern as here. Thus in pollen tubes, you get a very elongated shape from isotropic growth, that it limited to a small area at the tip. Cellulose in pollen tubes does not appear to be involved in creating the shape, but only appears in areas after the growth has stopped. The more tapered shape in cotton fibres that enters the region with cellulose suggests that diffuse growth is required, and that it cannot be solely tip growth

Response: We thank the reviewer for raising this important point. We fully agree that transversely oriented cellulose microfibrils primarily act to restrict radial expansion and thereby help maintain the anisotropic geometry of fiber cells during elongation. In the present study, however, our main focus is on how differential cellulose deposition and wall mechanics relate to axial elongation, rather than on providing a full mechanical description of fiber width and tip shape. To avoid overstating this aspect, we have now clarified this point more explicitly in our description of the growth model. Please see Lines 406-411.

We also wish to clarify that we do not propose purely tip growth in cotton fibers; instead, our data support a tip-biased diffuse growth mode in which both the apex and the shank elongate, but with higher elongation potential near the tip. Cotton fiber growth is therefore distinct from pollen tubes, which rely on highly localized, isotropic tip growth supported by actin-rich vesicle delivery. In our interpretation, the apical region with lower CMF density allows faster longitudinal wall expansion under turgor, while the remaining cellulose and matrix polymers still provide sufficient anisotropic reinforcement to limit excessive radial swelling. As a consequence, axial expansion at the tip outpaces lateral expansion, producing a narrow apex, and differential expansion between apical and subapical regions naturally generates a tapered shape. This differs from pollen tubes, where cellulose predominantly appears behind the growth zone and functions mainly as a stabilizer after expansion has ceased.

The detailed establishment of tip shape is likely to involve further biochemical and biomechanical mechanisms beyond the cellulose gradient itself. It has been implicated features such as local cytoskeletal organization and regional differences in wall polymers in shaping cotton fiber tips. And wall-modifying or structural proteins are also expected to fine-tune tip morphology through more complex mechanisms. These processes lie beyond the scope of the present work and will require future targeted studies. We now state this explicitly in the Discussion. Please see Line 406-411

The authors frequently conflate the concepts of elasticity and extensibility. They are not the same, and just because a wall is more elastic (softer) does not mean it is more extensible. A wall needs to be elastic (have stretch) and have extensibility factors (ie expansins, etc) in order to grow. Without the latter the wall will not grow. It could be that there is a gradient of extensibility factors, rather than differences in elasticity, that are the primary drivers of growth gradients. Although expansin appears as the predominant extensibility factor in cell wall with cellulose, it is not clear what the extensibility actors are at the tip where growth happens without cellulose, since the

usual pectin suspects have been apparently ruled out.

Response: We appreciate this important clarification and fully agree that wall elasticity and extensibility are conceptually distinct. Our AFM measurements quantify apparent modulus and stiffness to describe the mechanical state of the fiber cell wall, rather than to directly measure growth rate or extensibility. At the same time, previous studies have reported that regions with lower apparent wall stiffness are spatially associated with zones of higher wall deformation or growth, suggesting that local wall softening can accompany increased expansion potential (Peaucelle, Braybrook et al. 2011, Carter, Woolfenden et al. 2017, Cosgrove 2018, Traas 2023). In this sense, elasticity provides a necessary physical background that can facilitate extension. We apologize that our earlier wording may have conflated these concepts. In the revised manuscript, we now make it explicit that the mechanical differences we observe between tip and shank are interpreted as differences in local wall compliance that may underlie differential deformation capacity, rather than as direct measurements of extensibility. Please see Lines 112-123

We also agree that biochemical wall-loosening factors are likely to play an important role in establishing local extensibility patterns. Factors such as expansins and XTHs may form spatial gradients that act in concert with cellulose-based mechanical heterogeneity to generate growth heterogeneity. However, the spatial distribution of these proteins in cotton fibers remains largely unexplored. We have now expanded the Discussion to acknowledge this possibility and emphasize that mapping wall-loosening factors will be an important direction for future work. Please see Lines 439-444.

Specific comments:

Not heard of the Turkey test (brings strange images to mind), perhaps the authors mean Tukey test?

Response: Thank you for pointing this out. We apologize for the typographical error. We have corrected it to Tukey test throughout the manuscript.

Line 40: GhCSI1 is mentioned in the abstract without saying what it is.

Response: Thank you for this comment. To avoid introducing an undefined gene name within the strict word limit of the abstract, we have removed GhCSI1 from the abstract. The full name and description of GhCSI1 are retained and defined in the main text.

Line 52: What about callose?

Response: Thank you for this helpful comment. Callose can transiently accumulate in specific developmental or stress-related contexts, but it is generally not considered a major, ubiquitous structural component of primary walls. To avoid confusion, we now explicitly state that primary cell walls are “**mainly**” composed of cellulose, hemicelluloses and pectins. Please see the revised sentence at Line 51.

Line 58: Technically speaking, CMFs create anisotropy in the wall that governs elastic behaviour, how the wall extends to make shape is a different matter, although they are usually, but not always, correlated.

Response: Thanks for this insightful remark. We agree that cellulose microfibrils primarily create anisotropy in the wall that governs its elastic behavior, and that how the wall extends to generate shape is a related but distinct issue. We have revised the sentence to clarify that wall heterogeneity leads to anisotropic mechanical responses to turgor pressure, which in turn bias deformation direction, rather than implying a direct control of growth. Please see Line 57-60.

Line 76: Deposition stops, but secondary cell wall is produced? Is that not still cell wall (ie cellulose) deposition?

Response: Thank you for pointing out this ambiguity. Our intention was not to imply that all wall deposition stops, but rather to refer to the end of the PCW dominated elongation stage. We have revised the sentence to avoid the misleading wording. Please see Line 77-79.

Line 110: It is not clear what the authors mean by modulus and stiffness here. The authors only talk about stiffness in the methods. The fibres were dried, so I don't understand how they measure modulus if they say it relates turgor pressure. If modulus is being calculated from the stiffness with some sort of Herzian contact model, I am not sure why they should be so qualitatively different. This section needs substantial clean up, and needs to be made much more precise.

The max force for the AFM and indentation depth should also be reported.

Response: Thank you for this helpful comment. We agree that our previous wording was not sufficiently precise and may have caused confusion. In the revised manuscript, we now state more clearly that both modulus and stiffness are AFM-derived descriptors of the elastic properties of the fiber cell wall surface, and that we use them as comparative measures of local wall mechanics rather than as direct readouts of turgor-related elasticity or growth. Please see Lines 112-123.

AFM measurements on intact cotton fibers are technically challenging due to curvature and surface roughness and, to our knowledge, comparable datasets are still scarce. Cotton fiber walls are biological composites that are not perfectly linearly elastic, and AFM measurements on fiber surfaces can be influenced by factors such as adhesion. Our initial analysis pipeline for modulus may not have fully accounted for these complexities. Because the fiber cell walls exhibit strong adhesion and nonlinear force-displacement behavior, In the revision, we have therefore re-analyzed the AFM data by directly using the stiffness and modulus values provided in the QNM maps (Hertz contact model with adhesion enabled). The overall trends, including softer apex versus shank and differences between cultivars, remain unchanged. Please see revised Fig.1. We thus treat AFM-derived modulus and stiffness as comparative indicators rather than absolute measurements of in vivo wall properties, and interpret these parameters as providing a relative mechanical landscape that reports local differences in wall

mechanical state.

The force setpoint and the typical indentation depth used in the AFM measurements are now reported in the Methods section.

Line 124: Again, you are conflating elasticity and extensibility. (also Lines 130,

Response: Thank you for pointing this out. We agree that our original wording at these lines could be read as conflating elasticity with extensibility. In line with our revised description of AFM-derived parameters as comparative indicators of wall mechanics rather than direct readouts of extensibility, we have now rephrased these sentences in terms of mechanical compliance and expansion potential. Please see Lines 123-128 and 131-134 in the revised manuscript.

Line 312: What existing paradigms about cell wall extensibility are being challenged?

Response: Thank you for pointing this out. We agree that our original wording was too strong and too vague. What we intended to convey is that the traditional view of wall extensibility has largely emphasized pectin modifications and matrix polysaccharides as the primary regulators of wall yielding, with cellulose often considered mainly as a structural scaffold. In our system, however, we find that differential cellulose deposition creates marked mechanical heterogeneity along the fiber and is closely associated with polarized elongation, whereas pectin modification appears to play a more limited role during the early rapid-elongation phase.

To avoid overstatement, we have revised the sentence to specify this point and to frame our results as “refining and extending”, rather than challenging the conventional view. Please see the revised text around Line 359-363

Line 339: Do you really know what the gradient of extensibility looks like? If you have now time-lapse tracking, how do you know there is a gradient of growth. It could be the tip grows fast, and the whole shank grows more slowly, but the same. I don't think you can say this with any certainty.

Response: Thank you for raising this important point. We agree that we do not directly measure a gradient of wall extensibility or growth, and that our data are insufficient to map a continuous growth field along the fiber. Our observations of CSC density and CMF distribution, together with the computational modelling and a short-term tracking assay that qualitatively supports faster elongation near the apex (see Responsive Figure 1), are consistent with a tip-biased diffuse growth mode, but they do not allow us to define a precise gradient of extensibility.

To avoid over-interpretation, we have revised the text accordingly. We now refer to a cellulose gradient to spatial variation in wall mechanical state and deformation potential, as inferred from AFM and osmotic assays. Please see Line 388-391. We have also added a discussion of future directions for directly resolving growth gradients in cotton fibers Please see Line 469-476.

Line 342: It is not known that microtubules respond to mechanical cues, it is a hypothesis, which so far has little direct experimental support.

Response: Thank you for this comment. We agree that the idea that microtubules respond to mechanical cues is still best regarded as a working hypothesis, rather than an established universal principle, and that the strength of experimental support varies between systems. Our intention was not to claim this as a fully proven mechanism, but to refer to a possible link between wall mechanics and MT organization. Please see Line 393-397.

Line 375: The idea that cellulose is the most important player for elongation, and that pectin is not important, is in obvious contrast with rapid growth at the tip. Again this stresses the necessity to have growth data in this system to support this hypothesis.

Response: Thank you for this comment. We would like to clarify that our data and interpretation do not imply that cellulose content is positively correlated with local growth rate. Rather, our conclusion is that differential cellulose deposition plays a key mechanical role in shaping the wall properties and anisotropy that underlie fiber elongation in our system. In our model, the apical region with lower cellulose density is mechanically softer and more compliant, which allows greater deformation under turgor provided that wall-loosening factors are present, whereas the gradual increase in cellulose toward the shank reinforces the wall and limits further extension. Thus, the observation of rapid growth at tip is not in contradiction with a central mechanical role for cellulose.

To avoid overstatement, we have softened the wording in the Discussion to emphasize that compared to pectins, cellulose emerges as a major mechanical contributor to fiber cell wall extensibility in our system. Please see Lines 435-436 in the revised manuscript.

Regarding the need for growth data, we fully agree that direct measurements of growth distribution provide an important test of any mechanistic model. As detailed in our responses to earlier comments, we have now added osmotic deformation assays and a short-term tracking experiment, and we have revised the Discussion to frame our conclusions as being consistent with a tip-biased diffuse growth mode. Future work will be required to quantitatively map growth gradients along the fiber. Line 469-479.

Reviewer #4 (Remarks on code availability):

The model seems pretty straightforward, more springs = slower growth, so the outcome is intuitive and I am sure it can be easily reproduced.

Response: We thank the reviewer for this positive assessment.

Reviewer #5 (Remarks to the Author):

The key messages of the study is a basipetal gradient of cellulose microfibril density from the apex to the shank. Apical regions are reported to be softer, while the shank is stiffer to support overall cell integrity. It make sense and is verified by computational simulation that CMF distribution influences strain patterns along the fibers. Primary wall during cotton fiber elongation is reported with 22% semi-crystalline cellulose fibrils, which are surrounded by matrix polymers including xyloglucan and pectin. So far, there has been a lot of evidence that presence and modification of the matrix polymer may regulate fiber elongation.

Response: We thank the reviewer for the clear and positive summary of our study and are pleased that the overall framework, which is differential CMF distribution is associated with wall mechanical heterogeneity and polarized elongation in cotton fibers, is considered mechanistically sensible. We fully agree that previous work has established important roles for matrix polymers such as pectins and hemicelluloses in regulating primary wall extensibility. In the present work, our focus is specifically on how differential cellulose deposition contributes to wall mechanical heterogeneity and polarized elongation during the early elongation of cotton fiber development. We have clarified this scope more explicitly in the revised manuscript. And in the following responses we address the detailed comments on the Raman analysis and AFM-based mechanical measurements.

In this study, the focus is solely on cellulose accumulation and the main evidence by Raman spectroscopy lacks integrity of data and spectral interpretation. Only 3 spectra are shown from 10DPA and analysis is only based on the 380cm⁻¹ band, which is taken and interpreted as cellulose accumulation. Raman spectra of cellulose reflect also changes in cellulose orientation and crystallinity, which is not analyzed and taken into account at all. Further, pectin and hemicellulose bands should not be overseen and discussed. Unfortunately only 10DPA is analyzed with Raman, while data from AFM are from 3DPA and 7DPA and staining from 3DPA and 2DPA, cell wall synthesis 3DPA. The study would benefit from a more conclusive experimental design including Raman at different developmental stages (same as AFM) and mutants added. Additional the study will benefit from a more comprehensive Raman spectra interpretation, instead of plotting the intensity of one peak for cellulose content. That can only be done, if changes in orientation and crystallinity can be ruled out based on the spectral signatures.

Response: We thank the reviewer for these detailed and very helpful comments on the Raman analysis. In the revision, we have extended our laser-tweezers Raman measurements to additional developmental stages and to wall-polymer standards, and we have clarified the scope of what we infer from the spectra.

In the revised manuscript we now show spectra from 3, 7 and 10 DPA fibers together with spectra of cellulose, pectins and hemicellulose (Xylan) standards. Across development, the overall spectral shape of the fibers progressively converges towards that of crystalline cellulose, consistent with an increasing contribution of cellulose to the wall signal. However, the spectra at 3 and 7 DPA differ from all three standards: they show broader features and additional bands that likely reflect a mixture of wall

and intracellular components, especially given the thin walls and relatively large cytoplasmic volume at early stages. Although spectra within each stage are reproducible, their interpretation in terms of individual wall polymers is not straightforward. For this reason, we now explicitly state that the 3/7 DPA Raman data are used only qualitatively to illustrate the developmental trend towards more cellulose-like spectra, and not for quantitative comparison of cell wall composition (please see Line 179-193). We also apologize that our sampling strategy was not made sufficiently clear in the original figure. The three 10-DPA traces represent averaged spectra, not single measurements: for each position, multiple spectra were collected from several fibers, background-corrected and normalized to their maximum, and then averaged. We now state these numbers explicitly in the figure legends.

To address the concern that our original analysis relied on a single band at $\sim 380\text{ cm}^{-1}$, we have added a second cellulose-associated window around $\sim 1120\text{ cm}^{-1}$. After background correction and normalization of each spectrum to its maximum intensity, we quantify the relative peak intensities at ~ 380 and $\sim 1120\text{ cm}^{-1}$ along 10 DPA fibers. Both bands report a consistent increase in cellulose-related signal from tip to sub-tip to shank (revised Fig. 3 and Supplementary Fig. 4), supporting our conclusion of a basipetal enrichment of cellulose. In the text we now emphasize that the 380 and 120 cm^{-1} band is used as a convenient representative peak for this trend, rather than as the sole basis of our interpretation. Please see Line 193-198

We also have explicitly considered the contributions of pectins and hemicelluloses. Pectin and xylan standards display characteristic bands that are clearly distinct from the cellulose standard. The fiber spectra do not show strong changes in the pectins or xylan-associated bands. On this basis we infer that the dominant Raman differences along the fiber axis most likely reflect changes in cellulose content. At the same time, we now clearly state in the Results that the absence of distinct pectin/xylan bands in our spectra does not imply that these polymers are absent from the wall; rather, their signals are probably masked by the stronger cellulose deposition or fall below the sensitivity of our current laser-tweezers setup. Please see Line 199-208.

We agree that Raman spectra of cellulose also contain information about crystallinity and molecular orientation. A rigorous analysis of these aspects would require polarization-resolved measurements on well-defined wall geometries and a more extensive spectral deconvolution than is feasible within the scope of this study. Our primary goal was to use Raman spectroscopy as a strength tool to assess relative cellulose enrichment along the fiber axis, rather than to perform an exhaustive, spectroscopy-driven analysis of all structural parameters. Moreover, how changes in crystallinity and microfibril orientation quantitatively influence cotton fiber development is still not well understood. We therefore use the Raman data to inferring relative cellulose enrichment along the fiber axis, and we avoid making claims about changes in cellulose orientation or crystallinity. In addition, we note that Raman analyses specifically focused on cotton fibers are still very scarce. A more exhaustive

spectral interpretation, including systematic analyses of mutants and a detailed changes of crystallinity and molecular orientation, would be an interesting follow-up direction building on the current work. Please see Line 208-216.

Together with the CFW and S4B staining and the distribution of cellulose synthase complexes, the revised Raman analysis provides a more coherent basis for our conclusion that cellulose content increases basipetally along elongating fibers. Corresponding changes and clarifications have been made in the Results.

In the Material and Method part of Raman experiments no information is given, but only the link to a paper which has nothing to do with Raman: 51) Liu, W. et al. Towards a better recording of microtubule cytoskeletal spatial organization and dynamics in plant cells. *J Integr Plant Biol* 61, 388-393 (2019). <https://doi.org/10.1111/jipb.12721>. Please provide all necessary information or at least correct citation. And it would be interesting for the reader to comment why laser tweezers Raman microspectroscopy was chosen and not conventional Raman imaging using a Confocal Raman microscopy, which might give more detailed information including spatial changes in content and orientation.

Response: We thank the reviewer for pointing out this issue. The previous citation in the Raman Methods section was indeed incorrect and could be resulted from a reference mismatch during manuscript merging. We have now replaced it with two appropriate Raman references describing a similar laser-tweezers Raman micro-spectroscopy approach (Lu, Chen et al. 2020, Lu, Li et al. 2022) and we have substantially expanded the description of our experimental conditions.

Regarding the choice of laser-tweezers Raman versus confocal Raman imaging, our laboratory is currently only equipped with an LTRS system but not with an imaging module. In combination with CFW and S4B staining, which independently reveal the spatial distribution of cellulose in the fiber wall, these point spectra from LTRS. have provided sufficient information to support our conclusion of a basipetal increase in cellulose content. We now note that future work using confocal Raman imaging on fiber samples would be valuable for more detailed mapping of spatial changes in wall composition and orientation. Please see Line 211-214.

Some questions also remain regarding AFM results: Why are peak force error images shown instead of stiffness and modulus images. They might not reflect changes in mechanical properties, but where the feedback system struggles to maintain the setpoint force. Stiffness and modulus are defined in a strange way, as “modulus refers to elasticity of the cell walls under turgor pressure, while stiffness reflects the overall resistance of the cell wall against deformation” (Line110-112). Why is modulus related to turgor pressure? Normally modulus is referred to the “intrinsic” material properties (taking geometry into account), while stiffness just refers to materials resistance to deformation under the applied force. In line 116 suddenly a conclusion is made on toughness, which is a mechanical parameter usually not gained by AFM data as no full

strain-stress curve is acquired. Please take care about basic mechanical definitions.

Response: We thank the reviewer for these careful comments on the AFM analysis and for drawing attention to the mechanical terminology. We fully agree that peak force error images mainly reflect feedback performance and are not the most direct representation of mechanical properties. We apologize for this inappropriate choice in the initial version. In the revised Fig. 1b we now show apparent modulus maps, which directly correspond to the parameter used for our quantitative analysis.

We also agree that our earlier wording about modulus and stiffness was not sufficiently precise and could be misleading. In the revision, we explicitly state that both quantities are AFM-derived descriptors report the intrinsic elastic properties of the wall material, rather than as direct measures of in vivo elasticity under turgor or of growth rate. Previous studies have reported that regions with lower apparent wall stiffness are spatially associated with zones of higher wall deformation or growth, suggesting that local wall softening can accompany increased expansion potential (Peaucelle, Braybrook et al. 2011, Carter, Woolfenden et al. 2017, Cosgrove 2018, Traas 2023). Although our AFM measurements cannot be taken as absolute in vivo stiffness values, they provide a relative mechanical landscape that is informative for understanding cotton fiber elongation. We have rewritten the relevant sentences accordingly. Please see Lines 116-123.

We further agree that it is not appropriate to infer “toughness” from our AFM data, as a full stress-strain curve is not obtained. We have therefore removed the previous statement referring to toughness and no longer use this term in the revised manuscript. The stress-strain curve will require future in-plane mechanical measurements and high-resolution strain mapping, which is currently not applicable. Instead, we have performed osmotic deformation assays. The apical regions exhibit significantly greater transverse shrinkage than the shank under hyperosmotic treatment, in contrast to minimal changes in water-treated controls. These results are consistent with the AFM measurements and support the idea that regions with lower apparent stiffness have higher deformation potential under changes in turgor. We have incorporated these new data into the revised Fig. 1e,f. Therefore we interpret AFM-derived modulus and stiffness in combination with the osmotic shrinkage assays for inferring cell wall are mechanically compliant, rather than as standalone, absolute elasticity values. Please see Line 131-146 and Line 142-148.

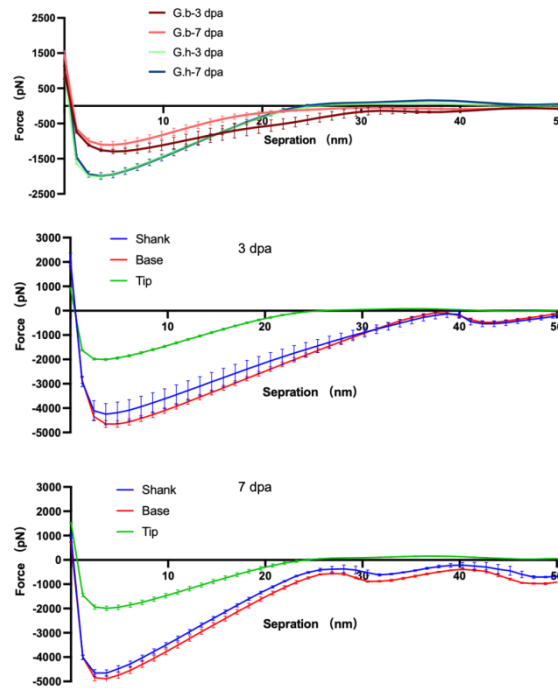
In line 120 “a remarkable correlation between mechanical parameters and cotton fiber growth” is reported, but just due to the fact that the two cultivars showed differences in modulus. Based on the presented data it is not easy to follow and verify such correlation and statement! It could be a bit too far fetched to conclude from a lower modulus and stiffness in Hai fibers to a connection to greater final length (Line 123-126)! In Fig. 1, the modulus is similar in both cotton variants at 3DPA, but only the stiffness different. This raises again the question on how these values were derived. It would be more trustable to show at least in the supplementary the raw data – the acquired force curves

of the two species, developmental stages and position. It would also be helpful to comment why the DMT model was used, as this is usually more recommended when adhesion forces are significant, like in hydrated conditions. Have you compared results to the Hertz model? How was the quality of the fit to the force-distance data? Was the decision for DMT model based on quality of the fit or other considerations? Have you measured wet cellulose fibrils and therefore chosen DMT? Please add this information in M&M.

Response: We thank the reviewer for these thoughtful comments on the AFM analysis and the interpretation of the mechanical parameters. We agree that our previous wording was too strong. In the revised manuscript, we now describe this only as a possibility: we note that the cultivar with lower apparent wall modulus and stiffness also develops longer fibers. This co-occurrence raises the hypothesis that early wall mechanics may influence final fiber length. If this potential link could be tested in future studies using larger cotton populations, it might provide a useful mechanical indicator for fiber-quality breeding. Please see Line 132-136.

AFM measurements on intact cotton fibers are technically challenging due to curvature and surface roughness, and cotton fiber walls are biological composites that are not perfectly linearly elastic; in addition, adhesion at the wall interface can influence the fitted parameters. Therefore, we re-analyzed the AFM datasets by re-selecting regions of interest and directly using the stiffness and modulus values provided by the instrument software. The overall trends, including softer apex versus shank and differences between cultivars, remain unchanged. Please see revised Fig.1. We thus treat AFM-derived modulus and stiffness as comparative indicators rather than absolute measurements of *in vivo* wall properties, and interpret these parameters as providing a relative mechanical landscape that reports local differences in wall mechanical state.

We now provide representative averaged force-indentation curves for both cultivars, and different positions. Please see Responsive Figure 3. These curves display smooth, physically reasonable force-distance behavior and are consistent with the lower apparent stiffness of fiber apex regions as reported in Fig 1. If the editor and reviewers consider it useful for readers, we would be happy to include this figure as a supplementary figure.



Responsive Figure 3. AFM force-indentation curves from cotton fiber primary cell walls. Mean force-indentation responses (lines with error bars) are shown for different cultivars, TM-1 (*G.h*) and Hai7124 (*G.b*), developmental stages (3 and 7 DPA) and positions along the fibre (tip, shank and base), acquired under identical probe and imaging conditions. These curves are consistent with the relative stiffness differences between fiber tip and shanks.

Because the fiber cell walls exhibit strong adhesion and nonlinear force-displacement behavior, in our re-analysis, all quantitative stiffness and modulus values reported in Fig. 1 are taken directly from the QNM maps, as calculated by NanoScope Analysis 1.8 using the built-in Hertz contact model with adhesion enabled. As our analysis focuses on relative differences between regions or cultivars, rather than on absolute modulus values, we did not perform a systematic comparison with alternative models. The description has been corrected accordingly in the Methods.

Some detailed comments:

Line 39-42: conclusion from microtubule perturbation to CSC cellulose distribution and cellulose gradient not clear and not shown. Or have there been any Raman and AFM measurements on the mutant? Figure1: b) why do peak force error images show mechanical features and which ones? Why not pictures of stiffness and modulus are shown? Line 774: what means differed developmental stages? 3 and 7? And are in c) the different positions mixed or is it the base or the shank or the tip? It is misleading that in c the different colors refer to different cotton species and in d) to different developmental stages. Are in the different regions (d) the species mixed or which species?

Supplementary Figure 1: what are “represent images”? Based on the pictures it is difficult to derive how height and width was measured....please replace “represent

images” by which images these are and mark the width and height of the measured fibre in the image.

Response: We thank the reviewer for these careful comments. In this study, the microtubule-perturbation experiments as well as the *csi1-cr* lines were designed to test the coupling between cortical microtubules and CSC distribution, rather than to directly re-measure the cellulose gradient or wall mechanics in these backgrounds. We have therefore revised the Abstract to make this clear. We did not perform AFM or Raman measurements on drug-treated ovules or mutants, because short-term microtubule perturbations in cultured ovules are unlikely to produce robust, spatially resolved changes in cellulose deposition that can be captured by these techniques. Moreover, perform AFM on fiber samples as well as interpretation of Raman spectra remain technically challenging. A rigorous characterization of cellulose gradients and wall mechanics under such perturbations would therefore require a dedicated, technically optimized study in future.

We agree that peak force error images mainly report feedback performance and are not the most direct representation of mechanical properties. We apologize for this earlier choice. In the revised Fig. 1b, we now show the apparent modulus maps, which correspond to the parameter used for analysis.

Regarding the legend for Fig. 1c, d, we apologize for our previous wording could be confusing. In Fig. 1c, only apical regions of fibers at 3 and 7 DPA are analyzed, and the two colors represent the two cultivars (TM-1 and Hai7124). This is now explicitly stated in the legend. In Fig. 1d, the analysis of different positions (tip vs shank) is performed on TM-1 fibers at 3 and 7 DPA. Here the colors indicate developmental stages (3 and 7 DPA), not cultivars. We have clarified in the legend that TM-1 is used in this panel.

In Supplementary Figure 1, we have revised the legend with a more precise description of the samples shown. On each 3D AFM height renderings, we have added a double-head arrow indicating the line along which the height profile was taken. The CMF width and height were obtained by extracting the height profile along this line and fitting it with a Gaussian function. Full width and peak height from the fit were taken as the apparent width and height of the CMF.

Figure 2: a) Not necessary to show a cellulose standard. What is shown in the last? I guess it is the difference between all spectra, but at this size nothing is really visible. B) you talk about relative, so this means it is normalized? If so on what? Why only at 10DPA? c and d relevant for proofing a basipetal gradient of cellulose?

Response: We thank the reviewer for these helpful comments on the Raman analysis and presentation. In the revised version, the cellulose, pectins and xylan standards have been moved to a Supplementary Figure 4.

And we now display the mean spectra from tip, sub-tip and shank of 10 DPA fibers in

Fig 3 together with the mean spectra from 3 and 7 DPA. Because the measurements from 3 and 7 DPA fibers show more complex and deviate from the cellulose standard, likely reflecting a mixture of thin primary wall and intracellular contributions, we now use these spectra only to illustrate the developmental evolution of the fiber Raman signature. The description has been corrected accordingly in the Main text. Please see Line 185-193.

All Raman spectra were processed by Savitzky-Golay smoothing, polynomial baseline subtraction, and min–max normalization. On this basis, the peak heights at $\sim 380\text{ cm}^{-1}$ and $\sim 1120\text{ cm}^{-1}$ are used as representative cellulose-associated bands to evaluate relative cellulose enrichment. We now explicitly state it in the Methods and figure legend. This is now explicitly stated in the Methods and figure legend.

The CFW stainings are intended as independent imaging evidence for a differential enrichment of cellulose, complementary to the Raman data. In the same figure, the isoxaben inhibition results are included as functional assays showing that cellulose deposition is essential for normal wall formation and fiber elongation. Although these perturbations are not used to reveal the differential cellulose deposition, they support the important role of cellulose in cotton fiber growing. So we consider it appropriate to present them together with the Raman results.

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Reviewer #2 (Remarks to the Author):

The author has addressed all my comments and it seems to be accepted.

Response: We thank Reviewer #2 for the positive evaluation of our revised manuscript. We are pleased that the revisions have addressed all of the reviewer's concerns.

Reviewer #4 (Remarks to the Author):

I am satisfied with the changes made by the authors.

Response: We thank Reviewer #4 for the positive evaluation and for recognizing the improvements made in this revision.

The comments related to imprecise terminology in places have been resolved, along with the tempering of some statement where required to avoid overstatement. The addition of osmotic treatments is very nice, although it is a bit a pity they could not spatially resolve elongation with beads or similar, although I appreciate the technical challenges there.

My other comments were mostly related to growth tracking, which appears to be too technically challenging in this system, although I appreciate the effort there. I guess it is the same as longitudinal deformation tracking in osmotic treatments. It is too bad, because knowing where the growth, and the relative contribution between shank and tip, would strengthen their story, but it is what it is.

Response: We are pleased that the imprecise terminology has been corrected and that statements have been appropriately tempered to avoid overstatement. We also appreciate the reviewer's comments on the osmotic treatments. We agree that spatially resolved elongation tracking would further strengthen the manuscript. However, as the reviewer notes, implementing robust high resolution tracking in this system is technically challenging. We are also looking forward to emerging approaches those may enable spatially resolved deformation and elongation mapping in cotton fibers.

A few small points:

Line 112-116: This is OK, but I would go with "cell wall stiffness" or "cell wall elastic modulus" for the cell wall, and "apparent stiffness" or "apparent elastic modulus" for the AFM stiffness.

Response: Thanks for pointing this out. We agree and have revised the

manuscript to clearly distinguish intrinsic cell wall elastic modulus/stiffness from AFM indentation-derived apparent elastic modulus/apparent stiffness, and we now consistently use the “apparent” terminology when referring to AFM-derived values throughout the text.

Line 360-363: The way this is worded, it sounds at first like you are talking about cell walls in general, maybe start with “In cotton fibres, our findings ...”

Response: Thanks for pointing this out. We agree and have revised the sentence to explicitly anchor the statement in cotton fibers and to clarify that the “conventional view” is largely based on evidence from other plant systems. The revised text:

“In cotton fibers, our findings refine and extend the conventional view, largely derived from other plant systems, that cell wall extensibility is primarily regulated by pectin modifications ...”

Line 908: I not sure what “mock” means here? You treated some fibres and not others? Normally this would be presented as a before/after experiment. A picture of the measurement before treatment (in water) and then after in Mannitol, ideally with the figure panel showing the same fibre.

Response: We thank the reviewer for pointing this out. We agree that “mock” was unclear and have replaced it with “water condition”. The medium was exchanged from water to mannitol while imaging/measuring the same ovule region. Because fibers are densely packed, individual fiber pairing cannot be reliably ensured, so we report a within-region before/after comparison. We have added this statement in the legend.

Reviewer #4 (Remarks on code availability):

I looked at the code, but did not verify it in any way. The model is pretty simple, and does what one would expect. I don't think it would be hard to reproduce.

Response: We thank Reviewer #4 for checking the code availability. We are pleased that the model implementation is clear and should be straightforward to reproduce.

Reviewer #5 (Remarks to the Author):

Thank you for taking into account the mistakes in terminology and for correcting accordingly. Instead of additional analysis and/or measurement of the sample spectra, non relevant reference spectra for hemicellulose and pectin have been added as Supplementary data as well four spectra of developmental stages without spectral interpretation. Supplementary reference spectra from my point

of view can be removed as published elsewhere and the xylan spectrum as well as the pectin looks very strange and no clear material and methods from where these references were purchased. Same for 3 and 7DPA as long as they are not analysed as the author state them as “complex and deviate from the cellulose standard”. Does this mean at this stage cellulose plays no role? In the current form adding the spectra is not beneficial as no conclusions are drawn and the idea to have data from the same developing stages from Raman and AFM still not fulfilled.

Based on the new spectra shown in Fig. 3a in the analysis of the 10DPA spectra it seems problematic when doing a min-max normalisation over the whole wavenumber region as in some spectra 380 will be max and in some 1095, which of course influences the analysis of peak heights. I do not understand what the Pearson correlation with the cellulose standard should tell you? As it is higher in the tip and sup-tip, you have more cellulose there than in the shank? And no cellulose in 3DPA and 7DPA?

Based on the plotted data in Fig. 3b) it is difficult to follow that differences are really statistically significant! Data contradict the authors assumption that these bands can be interpreted as cellulose content as they give not the same results. For 1120band the shanke seems not really different anymore (statistical test correct?) and for 380 probalby some spectra were normalised on the 380band (see red spectra in a) and therefore high!.

Fig. 3a,c: I do not understand what the pearson correlation vs. cellulose should bring in 3DPA and 7DPA? Important in spectral analysis is not only correlation, but interpreting the peaks. It is logical that cellulose dominates as soon as present in crystalline form. I therefore still think that cellulose in such Raman data can not be analysed only by cellulose content, as the influence of crystallinity and orientation is tremendous on the height of the Raman signal! And even if contributions of hemicellulose and pectin are naturally less reflected in the spectra, they are obviously there and need to be taken into account. If this is not possible for the authors they have to use another method.

Fig. 3d): does not bring any information, it is evident from the spectra that they are different and will group accordingly. Interpreting bands of loading 1 and 2 would be informative. But please do not show a PCA without discussing loadings..than it is better not to show.

Fig. 3e and f: has nothing to do with the Raman results.

Response: We sincerely thank Reviewer #5 for the careful and technically informed assessment of our Raman spectroscopy experiments. We apologize that our presentation of the Raman-related data caused confusion.

We fully understand the reviewer's expectation for a more detailed interpretation of Raman features across developmental stages. We also agree that Raman band intensities and spectral shapes can be influenced by factors such as crystallinity, microfibril orientation, and cellular/background contributions. We have tried our best to collect Raman spectra from 3 and 7

DPA fibers. However, we noted that the spectra were complex and likely influenced by cellular/background contributions, making robust interpretation challenging in our hands. Assigning and interpreting cellulose-related Raman features across stages would require reference datasets about these factors, which are not yet well established for cotton fiber cell walls. In the previous version, we therefore used Pearson correlation primarily to describe broad similarity trends across stages rather than as a direct measure of cellulose content.

In view of these complexities, and following the editor's guidance, we have removed the Raman dataset in its entirety from the manuscript and Supplementary Information, together with all Raman-related analyses and claims. We have accordingly refocused the study on AFM-based mechanics, cellulose labeling, live-cell imaging and mathematical modeling. Importantly, the central conclusions are unchanged and are now supported solely by these independent lines of evidence.