1 **Short Title:** Cotton fiber cells grow through a diffusive model

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16 **Research Area:** Cell Biology

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Zoom in on Ca²⁺ pattern and ion flux dynamics to decode spatial and temporal regulation of cotton fiber growth

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One sentence summary

Confocal imaging of Ca²⁺ patterning and *in situ* microelectrode ion flux measurements demonstrate that, contrary to growing pollen tubes or root hairs, cotton fiber growth follows a diffusive, but not the tip growth, pattern.

43 **Footnotes**

44 **List of author contributions**

45 Y.-L.R. conceived the research; Y.-L.R. and S.S. supervised the research; J.-S.Y. and
 46 J.B. performed the experiments; All authors analysed the data; J.-S.Y., S.S. and
 47 Y.-L.R. wrote the article with inputs from J.B.

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68 Abstract

69 Cotton fibers are single-celled trichomes initiated from ovule epidermis prior to
70 anthesis. Thereafter, the fibers undergo rapid elongation for 20 d before switching to
71 intensive cell wall cellulose synthesis. The final length attained determines fiber yield
72 and quality. As such, cotton fiber represents an excellent single cell model to study
73 regulation of cell growth and differentiation, with significant agronomical
74 implications. One major unresolved question is whether fiber elongation follows a
75 diffusive or a tip growth pattern. We addressed this issue by using cell biology and
76 electrophysiological approaches. Confocal imaging of Ca^{2+} binding dye,
77 fluo-3 acetoxymethyl (Fluo-3), and *in situ* microelectrode ion flux measurement
78 revealed that cytosolic Ca^{2+} was evenly distributed along the elongating fiber cells
79 with Ca^{2+} and H^+ fluxes oscillating from apical to basal regions of the elongating
80 fibers. These findings demonstrate that, contrary to growing pollen tubes or root hairs,
81 cotton fiber growth follows a diffusive, but not the tip growth, pattern. Further
82 analyses showed that the elongating fibers exhibited substantial net H^+ efflux,
83 indicating a strong activity of the plasma membrane H^+ -ATPase required for energy
84 dependent solute uptake. Interestingly, the growing cotton fibers were responding to
85 H_2O_2 treatment, known to promote fiber elongation, by a massive increase in the net
86 Ca^{2+} and H^+ efflux in both tip and basal zones, while non-growing cells lacked this
87 ability. These observations suggest that desensitization of the cell and a loss of its
88 ability to respond to H_2O_2 may be causally related to the termination of the cotton
89 fiber elongation.

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93 **Introduction**

94 Cotton (*Gossypium*) fibers are single-celled trichomes initiated from a quarter to one
 95 third of ovule epidermal cells about 16 to 24 h prior to anthesis (Ruan et al., 2000;
 96 Wang et al 2021). Following their protrusion from the ovule epidermis, the fiber cells
 97 undergo rapid cell elongation for about 20 d before switching to intensive secondary
 98 cell wall cellulose synthesis that lasts for 15 to 20 d (Ruan, 2005; Ruan, 2007). By
 99 maturity, the fiber cells can reach ~3 and ~5 cm long, in the cultivated species of *G.*
 100 *hirsutum* and *G. barbadense*, respectively, rendering cotton fiber one of the longest
 101 cells in the plant kingdom (Kim and Triplett, 2001; Ruan et al., 2001). As such, cotton
 102 fiber represents an attractive single-cell model to study cell expansion (Ruan et al.,
 103 2001; Ruan et al., 2004; Shan et al., 2014; Yu et al., 2019), cellulose synthesis (Amor
 104 et al., 1995; Ruan et al., 2003) and cell patterning (MacHado et al., 2009; Walford et
 105 al., 2011; Tian et al., 2020; Wang et al., 2020). Apart from its significance in studying
 106 fundamental plant biology, cotton fiber is the most important source of cellulose for
 107 the global textile industry. Therefore, advance in fiber biology will help to design
 108 innovative approaches to improve cotton yield and quality using advanced breeding or
 109 gene technology.

110 As outlined above, one extraordinary feature of the cotton fiber cells is its fast
 111 and sustained elongation at the average rate of 1500 to 2500 μm per day for about 20
 112 d, with little or no increase in cell diameter that remains at around 10 to 15 μm (Ruan,
 113 2007). Given this remarkable cellular characteristic and the fact that the fiber length is
 114 a key determinant of cotton yield and quality, there has been intensive research on the
 115 regulation of cotton fiber elongation over the last three decades. To this end, early
 116 studies established that the microtubules in elongating fiber cells are arranged
 117 transversely along the longitudinal axis (Seagull, 1990), which guided cellulose

118 deposition accordingly, thereby forcing fibers to elongate unidirectionally (Ruan,
119 2007), a phenomenon confirmed recently using transgenic approach (Yu et al., 2019).
120 On the other hand, cell biology and gene expression analyses revealed a temporary
121 closure of plasmodesmata (PD), due to the deposition of callose at the fiber base,
122 which coincided with the maximal expression of the plasma membrane sucrose
123 transporter and expansin genes at the onset of rapid elongation from 10 d post anthesis,
124 DPA (Ruan et al., 2001; Ruan et al., 2004). These findings underpin a model of the
125 fiber elongation driven by the cell turgor and relaxed wall expansibility (Ruan, 2007).
126 A follow-up study revealed that the fiber PD gating is under a tight control of the
127 sterol-mediated callose degradation, and an increase in the duration of PD closure via
128 silencing a sterol carrier gene activated the expression of genes encoding both
129 H⁺-coupled sucrose transporters and -uncoupled clade III SWEETs (Zhang et al.,
130 2017b). Recent molecular genetic studies and genome sequencing have identified a
131 number of regulatory genes and networks underlying cotton fiber elongation (e.g.
132 Shan et al., 2014; Hu et al., 2019).

133 Despite of the aforementioned progress, major questions remain regarding the
134 spatial and temporal regulation of cotton fiber elongation. A key unresolved issue is
135 whether the fiber cells elongate evenly along the whole growth axis, or possess the
136 tip-based growth pattern as that found in morphologically-similar structures such as
137 root hairs or growing pollen tubes (Hepler et al., 2013; Mangano et al., 2018;
138 Nakamura and Grebe, 2018). In other words, does cotton fiber elongation follow a
139 diffusive pattern or a tip growth model? A related question is whether and how the
140 growth pattern may change during elongation. In this context, cotton fiber cells are
141 known lacking zonation of the endoplasmic reticulum, Golgi bodies and mitochondria
142 or vesicles in the tip region (Tiwari and Wilkins, 1995) that are characteristic of the

tip-based growth (Yang, 1998). This is indicative of no polarized deposition of the new cell wall material for the apex expansion. By imaging fibers expressing fluorescent-tagged cytoskeleton proteins, Yu et al. (2019) observed that microtubules are organized transversely during fiber elongation, instead of being in bundles in parallel to the growth axis as typically observed in tip growth cells such as pollen tubes (Yang, 1998), a finding consistent with an early report (Seagull, 1992). These results support a diffusive growth model of the cotton fiber proposed previously (Ruan, 2007).

However, counter arguments against the diffusive growth model arise from several Ca^{2+} imaging studies, where elevated levels of Ca^{2+} were observed at the tip region of 2-5 DPA fibers (Huang et al., 2008; Zhang et al., 2017a), which is consistent with a tip growth pattern observed in elongating pollen tubes (Chen et al., 2009; Qu et al., 2012; Gu et al., 2015; Suwińska et al., 2017) and root hairs (Monshausen et al., 2008; Fan et al., 2011). A close examination of those reports, however, revealed that the fluorescent Ca^{2+} signal was observed in a much extensive region of the cotton fiber tips, often spanning 100 to 500 μm from the edge of the tip (e.g. Huang et al., 2008), which does not match the geometric distribution of Ca^{2+} in either pollen tubes or root hairs that employ tip-based growth mechanism. Here, the hallmark of the tip growth is a localized elevation in the cytosolic free Ca^{2+} that is confined to only 5~ 25 μm region from the tip boundary (e.g. Fan et al., 2011; Gu et al., 2015; Gilroy et al., 2016). This, together with a lack of proper positive and negative controls and resolutions required to differentiate cytosol from other subcellular compartments such as vacuole in the previous reports on cotton fibers (Huang et al., 2008; Zhang et al., 2017a), raises a question of the validity of those findings on Ca^{2+} localization and the conclusions drawn (e.g. Qin and Zhu 2011). Also, reliance of fluorescence dyes alone

168 come with a caveat of the possible methodological issues of its loading in various
169 types of cells and/or regions that could be related to the properties of the cell walls.
170 This calls for a need to employ some other techniques to provide an unequivocal
171 answer to the above question.

172 Physiologically, intracellular Ca^{2+} concentration is determined by Ca^{2+} influx
173 and efflux across cellular membranes, with tip-based growing cells characterized with
174 pronounced Ca^{2+} oscillation in the tip, but not in the shank region (Gilroy et al., 2016).
175 Similar oscillatory patterns have been reported for pollen tubes (Holdaway-Clarke et
176 al., 1998; Damineli et al., 2017) that also employ tip-based growth mechanism. There
177 have been no studies thus far on the spatial behaviour of Ca^{2+} oscillation along the
178 longitudinal axis of the growing cotton fibers.

179 To determine whether the cotton fiber cell follows a tip or diffusive growth
180 pattern and to better understand the spatial and temporal regulation of fiber elongation,
181 we have combined two advanced complementary techniques, namely fluorescent
182 imaging using Ca^{2+} binding dye, fluo-3 acetoxymethyl (Fluo-3 thereafter) (Zhang et
183 al., 1998; Zhang et al., 2015) and non-invasive ion-selective microelectrode technique,
184 MIFE (Microelectrode Ion Flux Estimation, Shabala et al., 1997; Wu et al., 2020).
185 Our analyses revealed that cytosolic Ca^{2+} was evenly distributed along the elongating
186 fiber cells, with Ca^{2+} and H^+ oscillations occurring in both apical and basal regions of
187 the elongating fibers at 5 and 10 d post anthesis (DPA), a phenomenon absent in
188 elongated fibers at 20 DPA. These findings demonstrate that cotton fiber growth
189 clearly follows the diffusive but not tip-based growth pattern. Pharmacological
190 experiments using various Ca^{2+} channel blockers on cultured cotton ovules indicate
191 the involvement of both voltage-dependent and -independent Ca^{2+} channels in the
192 cotton fiber elongation. Our analyses further uncovered several novel patterns

193 including findings that (i) the elongating, but not elongated, cotton fibers exhibited
194 massive H^+ efflux with strongest observed at 10 DPA, the onset of rapid fiber
195 elongation, indicating strong plasma membrane H^+ -ATPase activity; (ii) H_2O_2
196 stimulated Ca^{2+} and H^+ efflux from elongating, but not elongated, fibers and (iii) H_2O_2
197 application promoted K^+ uptake into and efflux from growing and non-growing fibers,
198 respectively. This data is discussed in the context of regulation of cell expansion by
199 cellular energy status, ion homeostasis and H_2O_2 -mediated signal transduction.

200

201

Results

Validation on visualization of Ca^{2+} in the cytosol of cotton fiber cells

To visualize Ca^{2+} signals in growing cotton fiber cells, cotton seeds with fibers attached were harvested on day of anthesis (0 DPA) and pre-loaded with ester form of Fluo-3 to produce the intracellular Ca^{2+} -binding fluorescent probe, Fluo-3, following the cleavage of the ester group by the cytosolic esterase (Zhang et al., 1998; Zhang et al., 2015). A set of experiments was firstly conducted to ascertain that the Fluo-3 fluorescent signals were emitted from the binding of cytosolic Ca^{2+} , but not from that of other subcellular compartments.

Confocal imaging of the free-hand sections counterstained with Calcofluor White for cellulose revealed green fluorescent signals of the Ca^{2+} -binding Fluo-3 in the region between cell walls and vacuoles (Fig. 1, A and B). Here, the Calcofluor White-labeled cell walls exhibited blue fluorescence only, whereas the vacuoles (asterisks in Fig. 1, A) showed no fluorescence, flanked by the Fluo-3 green fluorescence in the middle. The observation indicates that no Fluo-3 was produced in the cell wall and vacuolar compartments in our procedure, which could happen due to the presence of esterase in the extracellular matrix or leakage of tonoplasts (Zhang et al., 1998). To determine if the green fluorescence of Fluo-3 was sensitive to changes in Ca^{2+} level, we cultured 0-d cotton seed for 5 d on the same but Ca^{2+} free BT medium. This intervention abolished the green fluorescence, indicating no or little Ca^{2+} left in the fiber cells following the Ca^{2+} starvation treatment, although some residual signals remained visible in the underneath seed coat (Fig. 1, C). Elongating pollen tubes and root hairs are known to have high Ca^{2+} level in the cytosol of their respective apical regions, thus representing ideal positive controls. Loading both cell systems with Fluo-3 resulted in a strong fluorescent signal in their tip regions (Fig. 1,

227 D and E), as expected (e.g. Fan et al., 2011; Suwińska et al., 2017).

228 To provide an unequivocal evidence that the fluorescence signal emitted from the
229 Ca^{2+} -binding Fluo-3 did come from the cytosol of the elongating fibers, plasmolysis
230 was performed on cultured seed and fiber with 0.5 or 1.0 M sorbitol. In comparison
231 with the non-plasmolysed control of 5-d cotton fibers, which exhibited a green
232 fluorescence of Fluo-3 inside the cell wall but outside the vacuole (Fig. 2, A),
233 plasmolysis induced by 0.5 M sorbitol caused the fiber protoplast being pulled away
234 from the cell wall, leaving a gap between the cell wall displaying a blue fluorescence
235 signal from Calcofluor White and the narrow strip exhibiting green fluorescence (Fig.
236 2, B and C). A triple labelling with Fluo-3, Calcofluor White and RH-414, a plasma
237 membrane-specific staining (Zhang et al., 2015) revealed that the Fluo-3 green
238 fluorescence resided within the red fluorescence of RH-414 for plasma membrane
239 (Fig. 2, C). In comparison to that treated with 0.5 M sorbitol (Fig. 2, B and C), the
240 effect of plasmolysis became more evident following incubation with 1.0 M sorbitol,
241 in which the region emitting Ca^{2+} -bound Fluo-3 green fluorescence was pulled further
242 away from the cell wall with the vacuole becoming highly shrunken (Fig. 2, D) due to
243 osmotically driven efflux of water (Ruan et al., 2000). These data established clearly
244 that the Ca^{2+} -bound Fluo-3 green fluorescence was emitted from the cytosol of the
245 elongating cotton fiber cells.

246

247 **Cotton fiber cells exhibited even distribution of the cytosolic Ca^{2+} from tip to**
248 **base throughout the elongation period**

249 Having validated the procedure of using Fluo-3 to visualize cytosolic Ca^{2+} in fiber
250 cells as illustrated above, we systemically imaged Ca^{2+} localization patterns in fibers
251 across the entire elongation phase from 0 to 20 DPA as well as the peak stage of the

secondary cell wall cellulose synthesis at 30 DPA. Representative images from selected stages were shown in Figure 3.

The Ca^{2+} fluorescent signals were observed throughout the cytosols of almost all of the 0~2 d fiber initials (Fig. 3, A and insert). As the fiber cells elongate, the even distribution of cytosolic Ca^{2+} became more evident, reflected by the narrow strip of the fluorescent signals from tip to base with the non-fluorescent vacuole sitting in the centre (Fig. 3, B). Depending on the angle of the confocal imaging, some fiber cells exhibited tip-like fluorescent pattern at the first glance. However, a close examination revealed most of them displayed the fluorescence in the basal region as well (Class II in Fig. 3, B). Fiber cells with a true tip-localization of Ca^{2+} (Class I) was only sporadically observed. The uniform distribution of cytosolic Ca^{2+} pattern persisted to the end of elongation at 20 DPA. However, from 10 DPA onwards, an increased proportion of the fiber cells showed patchy fluorescence for Ca^{2+} , a phenomenon highlighted in 15 DPA fibers where boundaries between vacuole and cytosol became less defined and patchy signals (Class III) were seen across the whole protoplast area (Fig. 3, C). By 30 DPA, the fluorescence became very faint and patchy (Fig. 3, D).

The relative percentages of fibers exhibiting classes I, II and III Ca^{2+} patterns at each stage is shown in Figure 3 (E). As one can see, 0-d fiber initials were all in the class II category, exhibiting uniform Ca^{2+} signals throughout the cytosol. Tip-based location of the fluorescent Ca^{2+} signals was observed only in less than 5% of young fibers at 2-5 DPA and none in any other stages examined (Fig. 3, E). Interestingly, patchy pattern of Ca^{2+} signals (class III) increased from about 20% of 2-5 d fibers to ~48% at 10 DPA and then to ~80% at 15 to 20 DPA when elongation slows down and terminates (Ruan, 2007). By 30 DPA, all fibers displayed a patchy pattern of Ca^{2+} , although the fluorescent signals became very weak at this stage (Fig. 3, D and E).

277

278 **Steady net Ca^{2+} fluxes were similar between tip and basal regions of fiber cells**

279 Net Ca^{2+} fluxes were measured from apical and basal parts of the cotton fiber cells
 280 (illustrated in Supplemental Figure 1) using MIFE technique. No significant (at $P <$
 281 0.05) difference in the steady-state Ca^{2+} flux was found between the two regions, with
 282 net Ca^{2+} uptake of 25 to 40 $\text{nmol m}^{-2} \text{s}^{-1}$ measured (Fig. 4, A). These steady-state Ca^{2+}
 283 fluxes were largely independent of the cell age and not statistically different ($P < 0.05$)
 284 between growing (5- and 10-d old) and non-growing (20 d old) fibers (Fig. 4, A). Also
 285 similar and not statistically different (except one value for 20 d old base) were steady
 286 net K^{+} fluxes (Fig. 4, C). At the same time, net H^{+} fluxes showed a clear
 287 age-dependent trend (Fig. 4, B), showing a maximal net H^{+} efflux at 10 d. These
 288 results are consistent with the previous observations that 10 d is the turning point of
 289 switching from symplastic to apoplastic pathway when cells enter the rapid phase of
 290 elongation and probably possess strongest ATPase activity (Ruan et al., 2001). In
 291 non-growing 20-d fibers, this H^{+} efflux is ceased, with net H^{+} uptake detected (Fig. 4,
 292 B). At any age, the magnitude of net H^{+} fluxes was higher in the tip compared with
 293 the base of the fiber (Fig. 4, B) suggesting higher metabolic activity in this region.

294 **Elongating fibers displayed ion flux oscillations in both tip and basal regions**

295 One of the hallmarks of growing pollen tubes and root hairs is a presence of clearly
 296 pronounced ultradian (a minutes' range of periods) oscillations that are observed in
 297 the cell tip but absent in the basal region (Monshausen et al., 2008; Zhou et al., 2014;
 298 Mangano et al., 2018; Hoffmann et al., 2020). Here, net Ca^{2+} ion flux oscillations
 299 were observed in both tip and base regions of the 10-d cotton fiber (Fig. 4, D), further
 300 supporting the concept of the diffusive fiber growth. The frequency of these
 301 oscillations were similar to those reported in the literature for root hairs and pollen

tubes and ranged between 2 and 8 min in period. These oscillations were absent in non-growing (20 d old) fibers (as illustrated in Fig. 4, E for H^+ flux data). Given a well-established role of Ca^{2+} oscillations in a broad range of plant developmental and adaptive responses (Tian et al., 2020), it is, therefore, plausible to suggest that the difference between growing and non-growing fiber cells may be potentially related to their ability to encode some vital information by means of Ca^{2+} flux oscillations.

H_2O_2 stimulate Ca^{2+} and H^+ efflux from elongating but not elongated fiber cells

Previous studies on both root hairs (Foreman et al., 2003) and pollen tubes (Lee and Yang, 2008) have revealed an important role of H_2O_2 as a component of the cell growth mechanism. H_2O_2 was also shown to be involved in cotton fiber growth (Tang et al., 2014). Maintaining cytosolic ROS homeostasis has been implicated in regulating cotton fiber elongation, with low H_2O_2 levels likely promoting elongation (Li et al., 2007). To check how ROS may impact on ion flux dynamics, we studied net ion flux responses to physiologically relevant concentration of H_2O_2 in two zones of growing (10 d) and 20-d non-growing fiber cells (Fig. 5).

In the growing fibers, H_2O_2 treatment stimulated massive net efflux of Ca^{2+} (Fig. 5, A) and H^+ (Fig. 5, B), indicative of stimulation of respected ion pumps and resulted in a significant shift towards net K^+ uptake (less efflux; Fig. 5, C) that could be essential for both turgor-driven growth and a charge balance. No significant effects of H_2O_2 on Ca^{2+} and H^+ fluxes were observed in the non-growing cells, and H_2O_2 treatment here resulted in increased net K^+ efflux from the cell. Thus, it appears that desensitization of the cell and a loss of its ability to respond to H_2O_2 may be causally related to the fiber growth mechanism. The magnitude of H_2O_2 -induced Ca^{2+} flux response was ~2 fold higher in the fiber tip than that in the base (Fig. 5, A and D) in

growing cells. Similar patterns were also observed in the magnitude of H₂O₂ -induced changes in net H⁺ flux (Fig. 5, B and E). Here, growing cells showed intrinsically more negative H⁺ flux values (net H⁺ efflux) compared with non-growing cells (net H⁺ uptake). Application of H₂O₂ has further increased net H⁺ efflux in growing cells, with much stronger effects reported for the tip (Fig. 5). These findings are consistent with the above observations of higher H⁺-ATPase pumping activity in the tip region, once again pointing at likely higher metabolic activity in this zone. Net K⁺ responses to H₂O₂ in the base were qualitatively similar to those in the tip, although several fold lower in magnitude (Fig. 5, C and F).

Cotton fiber elongation is dependent on Ca²⁺ channel activities

Ca²⁺ flux and its cytosolic homeostasis are largely dependent on the orchestrated activities of numerous plasma membrane Ca²⁺ channels and efflux systems (Ca²⁺-ATases and CAX Ca²⁺/H⁺ exchangers; Bose et al., 2011; Demidchik et al., 2018). To assess a possible role of these transporters in cotton fiber elongation, cotton seeds at 0 or 6 DPA were transferred into the BT medium containing one of the following three blockers-ruthenium red (RR; a known blocker of the tonoplast Ca²⁺-permeable slow vacuolar channels; Pottosin et al., 1999), verapamil (VP; a blocker of the plasma membrane-based voltage-dependent Ca²⁺ channel; De Vriese et al., 2018) and gadolinium (Gd³⁺; a blocker of non-selective cation permeable NSCC channels; Demidchik and Maathuis, 2007; Zepeda-Jazo et al., 2011). The first batch of seeds were cultured from 0 to 6 DPA to examine the impact of the blockers on early fiber elongation. As shown in Figure 6 (A), application of RR and VP reduced fiber elongation by about 20% in comparison with the control with no effect on seed fresh weight, whereas inclusion of Gd³⁺ blocked fiber growth completely and severely

352 inhibited seed growth (Supplemental Figure 1). We further tested the effect of these
 353 Ca^{2+} channel blockers on mid stage fiber elongation, namely from 6 DPA onwards. To
 354 this end, application RR, VP and Gd^{3+} reduced fiber elongation by about 40%, 75%
 355 and 60%, respectively with moderate inhibitory effect on seed weight by RR or VP,
 356 but not by Gd^{3+} treatment (Fig. 6, B; Supplemental Figure 1). These findings indicate
 357 the essential roles of Ca^{2+} channels in the cotton fiber elongation.

358

359

Discussion

Plant cells grow diffusively across the whole cell surface (Geitmann and Ortega, 2009; Tian et al., 2015) or specifically at the tip area only (Rounds and Bezanilla, 2013; De Jong et al., 2019). The former is characterized with incorporation of a new wall material uniformly distributed across the cell surface leading to even growth over the cell axis as observed in most cell types including, for example, leaf mesophyll cells, epidermal trichomes and pavement cells (Smith and Oppenheimer, 2005; Yanagisawa et al., 2015). Some plant cells concentrate their wall extension through incorporating new wall material only to the apical site, hence becoming tip-growth such as that in the pollen tubes (Qu et al., 2012; Gu et al., 2015) and root hairs (Monshausen et al., 2008; Fan et al., 2011). As outlined in the Introduction, it remains unresolved as whether cotton fiber cells, the most important cell types producing cellulose for the gloable textile industry, follow a tip- or diffusive-based growth pattern. Here, we provided compelling cell biology and eletrophysiology evidence that elongating cotton fiber employs a diffusive but not tip growth mechanism as schematically illustrated (Fig 7).

Cotton fibers follow a diffusive growth as evidenced by a uniform distribtion of the intracellular free cytosolic Ca^{2+} and similar Ca^{2+} flux patterns in both tip and basal zones

By confocal imaging of the Ca^{2+} -bindig fluorescent dye, Fluo-3, we showed that Ca^{2+} was uniformly distributed in the cytosol of the fiber cells throughout its elongation period from 0 to 20 DPA (Figs. 1 to 3). Occusionally, less than 5% of young fiber cells appeared to show tip-localized Ca^{2+} at 2-5 DPA, which probably was an artifact derived from the the cytosolic Ca^{2+} being pushed to the tip area during sectioning and

imaging process. It is interesting to note from 10 DPA onwards, an increased proportion of the fiber cells exhibited patchy fluorescence of Ca^{2+} -binding Fluo-3, which reached ~ 80% by 15 to 20 DPA (Fig. 3, E), when fiber cells enter the transition from elongation to cell wall cellulose synthesis (Ruan, 2007). The fluorescence of Fluo-3 is dependent on esterase activity which catalyzes the removal of the ester group of the non-fluorescent dye Fluo-3 ester once it has diffused from the cell wall matrix to the cytoplasm, thereby allowing Fluo-3 to bind Ca^{2+} leading to emission of fluorescence (Zhang et al., 1998). Previous studies indicate that as cotton fiber cells approach to the end of the elongation at ~ 15 DPA, the tonoplasts become increasingly leaky, which may generate highly localized pH change and even release additional esterase from the vacuole (Ruan et al., 2001; Ruan et al., 2004), resulting in concentrated Fluo-3 in those areas, hence patchy fluorescence (Fig. 3).

In contrast to tip-based growing cells where Ca^{2+} oscillations are restricted to the apical region, the growing cotton fiber cells displayed Ca^{2+} and H^+ oscillation in both tip and basal regions at 5 and 10 DPA but not at 20 DPA when elongation has completed (Fig. 4, Fig. 7). Ion flux oscillations are firmly associated with a broad range of developmental and adaptive processes, and rapid (ultradian) oscillations are considered as a hallmark of each elongating cells. Indeed, a strong association was found between oscillations in H^+ and Ca^{2+} fluxes into epidermal cells and root growth rate (Shabala et al., 1997; Shabala and Newman, 1997), with no oscillations found in roots growing slower than $2 \mu\text{m min}^{-1}$. These oscillations were always observed in the elongation and meristematic regions of plant roots, but only occasionally in the mature root zone (Shabala and Knowles, 2002). The same is true for the polarized cell growth of pollen tube and root hair in which tip-focused Ca^{2+} oscillations specify the signalling events for rapid cell elongation (Tian et al., 2020).

A critical component of ion flux oscillations are H^+ -ATPase pumps that energize membranes and form a feedback loop(s) with various voltage-dependent cation and anion channels (Hansen, 1978; Gradmann, 2001; Shabala et al., 2006). By controlling voltage-gated Ca^{2+} channels (Demidchik et al., 2018), such oscillations in the H^+ -pump activity may rapidly modulate cytosolic free Ca^{2+} concentrations in the cell creating specific Ca^{2+} “signatures” and activating an array of signalling pathways (Tian et al., 2020). Here we showed that, contrary to reports for pollen tubes or root hairs, such Ca^{2+} and H^+ flux oscillations were observed in both tip and basal regions of the growing cotton fiber cell (Fig. 4, D) but only in growing cells (Fig. 4, E). The disturbance to cell’s ability to transport Ca^{2+} across the plasma membrane (by pharmacological agents) resulted in a significant (up to 70%) reduction in the fiber growth (Fig. 6). Taken together, this data indicates that elongating cotton fiber employs a diffusive, but not tip growth, mechanism that is strongly dependent on external Ca^{2+} transport from the apoplast across the plasma membrane. It also appears that oscillations in the fibre tip are faster than in the base, suggesting a possibility of the frequency encoding of growth-related signals. The details of this process warrants a separate investigation.

Growing and non-growing cotton fibers exhibit different flux dynamics of Ca^{2+} , H^+ and K^+ and their response to H_2O_2

H_2O_2 has been shown to function as a signalling molecule to promote cotton fiber elongation, likely through enhancing the activity of Ca^{2+} -binding protein, Calmodulin (Tang et al., 2014). H_2O_2 is also known as a potent activator of several types of Ca^{2+} -permeable ion channels, forming so called ‘ Ca^{2+} -ROS hub’ and amplifying cytosolic Ca^{2+} signals (Demidchik et al., 2018) in root epidermis and leaf mesophyll

cells. Here, however, application of H₂O₂ resulted in a massive net Ca²⁺ efflux (Fig. 5, A and D) that was observed in both apical and basal region but only in growing fibers. As electrochemical [Ca²⁺] gradient across the plasma membrane favours thermodynamically passive Ca²⁺ uptake, the observed net Ca²⁺ efflux can be only a result of operation of some active Ca²⁺ transport system (such as Ca²⁺-ATPase or Ca²⁺/H⁺ exchanger) activated by H₂O₂. Zepeda-Jaso et al. (2011) reported that hydroxyl radicals (another type of ROS) caused activation of eosin yellow-sensitive (a specific Ca²⁺ pump inhibitor) Ca²⁺ efflux from pea root epidermis, and that these effects were potentiated by polyamines (Velarde-Buendía et al., 2012). Thus, we speculate that a similar scenario may be applicable here.

The physiological rationale behind activation of Ca²⁺ efflux remains a subject of a separate study. ACA-type (plasma membrane-based) Ca²⁺-ATPases are essential component of the polarized cell growth. Four ACAs (ACA2, 7, 9, 11) are highly expressed during most of the pollen developmental stages (García Bossi et al., 2020), and insertional mutants of ACA9 show reduced pollen tube growth (Schjøtt et al., 2004). Ca²⁺-ATPases are also essential for a root hair growth. In our case, net Ca²⁺ efflux was activated by ROS treatment (Fig. 5). The most likely explanation on the stimulation of Ca²⁺ efflux by H₂O₂ is that such stimulation of Ca²⁺ ATPase is required to return cytosolic [Ca²⁺] levels down to the basal levels, once the ROS signalling is over (Bose et al., 2011). Indeed, the above “Ca²⁺-ROS hub”, that is formed by the H₂O₂-inducible Ca²⁺ permeable non-selective cation channel and NADPH oxidase (Demidchik et al., 2018), operates in a positive feedback manner and can result in an uncontrollable increase in ROS accumulation in the growing cell, with a danger of a possible damage to key macromolecules and cellular structures. Hence, once the ROS signalling is over, operation of this self-amplifying system should be ceased. The

460 H₂O₂-induced activation of Ca²⁺-ATPase may serve just this purpose.

461 The elongating (5 and 10 DPA), but not elongated (20 DPA), cotton fibers
462 exhibited net H⁺ efflux, with the strongest efflux observed at 10 DPA (Fig. 4). This
463 efflux was vanadate-sensitive (data not shown), implying involvement of H⁺-ATPase.
464 The magnitude of H⁺ efflux was further stimulated by H₂O₂ treatment (Fig. 5)
465 implying activation of H⁺-ATPase (in addition to that of Ca²⁺ ATPase). This
466 activation would hyperpolarize the plasma membrane and positively impact on
467 acquisition and transport of essential nutrients and metabolites (Ruan, 2007).

468 Cotton fiber growth relies on the import of nutrient resources from the basal ends
469 connecting the underlying seed coat. During fiber elongation, the cellular pathway for
470 nutrient import switches from symplasmic pathway via plasmodesmata early in
471 elongation to apoplasmic route at ~10 DPA at the onset of rapid fiber elongation
472 (Ruan et al., 2001; Ruan et al., 2004). This switch coincides with strong expression of
473 a group of plasma membrane sugar and K⁺ transporters to uptake sugars and K⁺,
474 major osmotical solutes in the fiber cells (Ruan et al., 2001; Zhang et al., 2017b).
475 Here, we show that H₂O₂ treatment resulted in a shift towards net K⁺ uptake in a
476 growing cotton fiber (Fig. 5, C and F) that could be explained by both H⁺-ATPase
477 pump-mediated membrane hyperpolarization (hence, opening inward-rectifying K⁺
478 channels) and increased pH gradient across the plasma membrane to provide a driving
479 force for operation of the high affinity K⁺ uptake systems (e.g. K⁺/H⁺ exchangers;
480 Rubio et al. 2020). The highest H⁺-ATPase activity and its increased sensitivity to
481 H₂O₂ at 10 DPA may be also essential to transport sucrose (via H⁺/sucrose symporter
482 (Ruan et al., 2001; Zhang et al., 2017b). Together with K⁺, sucrose import may also
483 osmotically drive fiber elongation as K⁺ and malate account for ~ 50% of the total
484 osmolality with soluble sugars making up the remaining 50% and they collectively

play essential role in generating osmotic potential, hence turgor (Dhindsa et al., 1975; Ruan et al., 2001; Wang et al., 2010). In this context, activation of H⁺-ATPase by ROS could be a critical step in generating cell turgor for fiber elongation.

Materials and Methods

Plant Material

Cotton (*Gossypium hirsutum* cv. Coker 315 plants were grown under controlled conditions as previously described (Ruan et al., 1997). Cotton seeds were sown in a potting mixture (Metro-Mix 200 growing medium, Scotts, Columbus, OH). The plants were raised under greenhouse conditions with partial temperature control (25-30 °C during the day for 14 h and 18-22 °C during the night for 10 h. About 100 g per pot of Osmocote (Scotts), a controlled release fertilizer with N:P:K at 1:1:1, was applied once every 20 d. The plants were watered once every 2 d. Cotton bolls were sampled at 9:00~ 10 am on d of anthesis for ovule culture.

Cotton Ovule Culture

The procedure of cotton ovule culture was carried out as described (Li et al., 2010) with some modifications. Cotton bolls (0 DPA) at 1st nodes of middle fruiting branches were collected and surface-sterilized with 70 % (v/v) ethanol for 30-60 s and a quick exposure to methane burner flame, followed with treatment of 6% (v/v) sodium hypochlorite (NaOCl) for 20 min. Thereafter, the bolls were washed with sterile water to remove residual NaOCl. Ovules were removed aseptically from middle part of each locule and floated on 50 ml liquid BT medium (Beasley and Ting, 1974) in a 300 ml- plastic culture bottle with 20 ovules per flask. The bottles were placed in the dark at 30 °C without shaking until observations.

The BT culture medium was prepared according to (Beasley and Ting, 1974). indole-3-acetic acid (IAA) sodium salt and gibberellic acid (GA₃) potassium salt were dissolved in water and filter sterilized to obtain stock solutions of 50 mM and 5 mM, respectively. In practice, 5 μM IAA and 0.5 μM GA₃ were added to the BT medium after autoclaving. All above operations were performed under sterile condition.

515

516 **Confocal imaging of Ca²⁺ patterning**

Cotton ovules with fibers attached were pre-loaded with the intracellular Ca²⁺ sensitive fluorescent probe, fluo-3 acetoxymethyl (Fluo-3 thereafter) ester (Biotium, US) following a protocol adapted from (Zhang et al., 1998). For loading, cotton ovules were incubated in the BT medium contains 20 μM Fluo-3 ester (stock in DMSO), 200 μM CaCl₂, and 50 mM sorbitol in a 12-well cell culture plate (2 ml per well) for 3 h at 4 °C in the dark to minimize the hydrolysis of AM ester by potential extracellular esterases. Cotton ovules were then transferred to a new BT medium incubated for 2 h at 26 °C to allow the cleavage of the loaded Fluo-3 ester to Fluo-3 by cytosolic esterases, thereby trapping the impermeable Fluo-3 in the cytosol of fiber cells. Afterwards, hand-cut sections of cotton ovule with fiber attached were washed 3 times by phosphate-buffered saline (PBS) plus 100 mM sucrose. In specified instances, sections were counterstained with 0.1 % (w/v) Calcofluor White for 30 s to label the cell wall and the (Zhang et al., 2015). Thereafter, sections were transferred to 1 ml of PBS containing 100 mM sucrose for preparation of confocal imaging. Sections of cotton ovules were carefully placed on slides to ensure most fiber cells were placed on the same focal plane.

Fluorescent imaging of cotton ovule and fiber cells was performed using an Olympus FV1000 confocal laser scanning microscopy (Olympus, Japan). Fluo-3 was

excited with a 488 nm diode laser (15 mW, laser power set to 50 %) and emitted fluorescence captured at 515 nm, while Calcofluor White was excited with a 405 nm UV laser (50 mW, laser power set to 15 %) and emitted fluorescence captured at 460 nm. Gain of the photomultiplier tube was set to 600 V for Fluo-3, and 400 V for Calcofluor White.

To imaging Fluo-3 fluorescence in cotton root hairs, 14 d old seedlings were chosen for root sampling, and loading of Fluo-3 as that for cotton ovules. For imaging of Fluo-3 in pollen tubes, pollen grains were harvested from blooming flowers and suspended in the culture medium (1.0 mM KCl, 0.06 mM CaCl₂, 1.0 mM H₃BO₃, and 1.0 mM MES, pH 6.0 (Tris)) at room temperature (26 °C) for 30 min. Then Fluo-3 ester (20 μM final concentration) was added to the culture medium and incubated for 2 h at 4 °C in darkness. Pollen grains were then washed three times with dye-free culture medium and then incubated for 2 h at 26 °C before fluorescent imaging.

Plasmolysis of fiber cell

To better identify the subcellular localization of the Ca²⁺ signal, Fluo-3 loaded cotton ovules (5 DPA) were counterstained with 10 μM RH-414, a cell membrane tracker (Molecular Probes, US), during the last 30 min of cleaving Fluo-3 ester, before hand-cutting. Thereafter, sections of cotton ovule were soaked in the PBS solution containing 500 mM or 1 M sorbitol for 20 min to achieve moderate or severe plasmolysis of the fiber cells, respectively. RH-414 was excited with a 559nm diode laser (15 mW, laser power set to 25%) and emitted fluorescence captured at 625 nm. Gain of the photomultiplier tube was set to 500 V.

Non-invasive H^+ , Ca^{2+} and K^+ flux measurements using MIFE™ technique

The H^+ , Ca^{2+} and K^+ -selective microelectrodes were prepared and calibrated as described elsewhere (Shabala et al., 2013). A lock from the bolls of 5 DPA or 10 DPA or 20 DPA was gently removed and secured on a glass slide using a thin paraffin strip and conditioned in basal salt medium (BSM; 120 mM Glucose, 1 mM KCl, 0.1 mM $CaCl_2$ pH 5.7 ± 0.2) for 40 minutes at room temperature for recovery of growth (Supplemental Figure S1). The net H^+ , Ca^{2+} and K^+ fluxes were measured from apical and basal regions of the cotton fiber at 40 μm away from individual fiber cells using MIFE technique for up to 60 min. To study the H_2O_2 -induced flux changes, steady-state ion fluxes were measured for 10 minutes at respective regions of the cotton fiber. Thereafter, 5 mM H_2O_2 was added to the BSM, and transient ion fluxes were monitored for further 30-40 min.

Pharmacological experiments

To examine the role of Ca^{2+} channels in cotton fiber growth, the pharmacological approach was undertaken. Ruthenium red (RR; known blocker of the tonoplast Ca^{2+} -permeable slow vacuolar channels), verapamil (VP; a blocker of the plasma membrane-based voltage-dependent Ca^{2+} channel) and gadolinium (Gd^{3+} ; a blocker of non-selective cation permeable NSCC channels) were added to the culture medium individually at specified DPA. RR and Gd^{3+} were added from aqueous stocks of 5 mM and 50 mM, respectively; VP was added from a 100 mM stock in ethanol. The pharmaceuticals were diluted prior to use to working concentrations of 50 μM , 0.2 mM, and 1 mM for RR, VP, and Gd^{3+} , respectively.

Measurement of the fiber length

Cotton ovules with fibers attached were boiled in 100 ml of distilled water with 2-3 drops of 1 M HCl inside for 1 min. Afterwards, each ovule was carefully placed on a convex dish, and fibers grown on the ovule were streamed out with a jet of water. Then, fiber length was determined from the attachment point of fiber and ovule to the edge where most fibers terminate at. A vernier caliper together with a dissecting microscope were employed for the measurement.

Data analysis

Fluorescent images captured by confocal microscope were edited in software FV10-ASW 4.0 viewer. Schematic diagram was drawn by software PowerPoint 2013 (Microsoft). Graphs were plotted by Excel 2013 (Microsoft). Student's T test and ANOVA analyses were performed in SPSS 20 (IBM).

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621 **Figure legends**

622 **Figure 1.** Validation of Ca²⁺ indicator Fluo-3 in cotton fibers, pollen tubes and root
623 hairs.

624 Green fluorescence came from the membrane-impermeable intracellular Ca²⁺
625 indicator Fluo-3, whereas blue fluorescence reflected the cell wall indicator
626 Calcofluor White (CW) binding to cellulose. Cotton seeds (5 days post anthesis, DPA)
627 with fibers attached were co-stained with Fluo-3 and CW (A), or with CW only (B).
628 (C) Cotton fibers derived from seeds cultured in a Ca²⁺-free BT medium were
629 co-stained with Fluo-3 and CW at 5 DPA. Note, the presence of Ca²⁺ fluorescence
630 signals (arrowheads) in (A) outside the vacuoles (asterisks) but its absence in the
631 negative controls of (B) and (C). (D and E) Representative images of cotton pollen
632 tube and cotton root hair, respectively, stained with Fluo-3, showing Ca²⁺ fluorescence
633 at the tip regions. Scale bars in (A, B and C) = 100 μm, in (D and E) = 50 μm.

634

635 **Figure 2.** Localization of Ca^{2+} in the cytosol of elongating fiber cells. Ca^{2+} was
636 indicated by green fluorescence emitted from Fluo-3.

637 Cell wall and plasma membrane were labelled with Calcofluor White (CW, blue
638 fluorescence) and with RH-414 (red fluorescence), respectively. 5 d cotton seeds with
639 fibers attached were incubated for 30 min in PBS medium (A), or the PBS with
640 sorbitol at 0.5 M (B and C) or 1 M (D) before confocal imaging. Note, in comparison
641 with the control (non-plasmolysed in (A)), plasmolysis resulted in protoplast being
642 pulled away from the cell wall (B, C), a phenomenon becoming more evident under
643 1.0 M sorbitol treatment (D). In all cases, Ca^{2+} was localized in the cytosol between
644 the cell wall and vacuole. Scale bars = 15 μm .

645 **Figure 3.** Dynamics of Ca^{2+} patterning in cotton fibers from elongation to rapid cell
646 wall cellulose synthesis.

647 Ca^{2+} was indicated by green fluorescence emitted from Fluo-3 labelling. (A) Young
648 fiber cells elongated from seed epidermis (arrows) at 2 DPA, showing Ca^{2+} signals in
649 the cytosol (insert). (B) Elongating fiber cells at 5 DPA, exhibiting Ca^{2+} signals
650 occasionally concentrated to the tip area but mostly in the peripheral regions of the
651 fibers, termed as pattern class I and II, respectively. Asterisks in (A and B) indicate
652 vacuoles. (C) Cotton fibers at 15 DPA, a transition phase from elongation to
653 secondary cell wall cellulose synthesis. The fluorescent Ca^{2+} signals became
654 aggregated or patchy, categorized as pattern class III. (D) Cotton fibers at 30 DPA,
655 undergoing intensive cellulose synthesis, displayed much reduced Ca^{2+} signals, in
656 comparison with that in the early stages (A, B, and C). Scale bars = 100 μm . I The
657 relative percentages of classes I, II and III Ca^{2+} patterns across fiber development,
658 which were calculated by counting at least 30 individual fiber cells randomly selected
659 from 10 seeds at each stage.

660

661 **Figure 4.** Net ion flux profiles across the plasma membrane of the cotton fiber cells

662 measured by the non-invasive microelectrode MIFE technique.

663 Panel A to C show mean values of steady-state net Ca^{2+} (A), H^+ (B), and K^+ (C) fluxes

664 measured from the tip and basal regions of growing (5 and 10 DPA) and non-growing

665 (20 DPA) cotton fiber cell. Mean $\pm$ SE ($n = 5$ to 8). Data labelled with different

666 low-case letters is significant at $P < 0.05$. *ns* = not significant. (D) oscillations in net

667 Ca^{2+} measured from the tip and basal regions of growing cotton fiber (10 DPA). (E)

668 dynamics of net H^+ fluxes measured from the tip region of the cotton fiber cells as a

669 function of its age. The ultradian H^+ oscillations were observed from growing (5 and

670 10 DPA) but not in non-growing (20 DPA) cells. One (of 6) typical example is shown

671 for each treatment. The sign convention for all MIFE data is “influx positive”.

672

673 **Figure 5.** Transient responses of net Ca^{2+} (A and D), H^+ (B and E) and K^+ (C and F)

674 fluxes to 5 mM H_2O_2 treatment measured from growing (10 DPA) and non-growing

675 (20 DPA) fiber cells in tip and base regions. Error bars indicate SE ($n = 5$ to 8). The

676 sign convention is “influx positive”.

677

678 **Figure 6.** Effects of Ca^{2+} channel blockers on *in vitro* cotton fiber growth.

679 Cotton seeds at 0 or 6 DPA were cultured in the BT medium contains either 50 μM

680 Ruthenium Red (RR), or 200 μM Verapamil (VP), or 1 mM Gadolinium (Gd^{3+}) for 6

681 d. Data labelled with different low-case letters is significant at $P < 0.05$ (One-way

682 ANOVA, Duncan’s test). Error bars indicate SE ($n=10-20$).

683

684 **Figure 7.** A schematic model on diffusive growth of cotton fiber cells characterized

by Ca^{2+} patterning and ion fluxes.

Cytosolic Ca^{2+} was found to be evenly distributed from tip to basal regions with Ca^{2+} and H^+ oscillations detected in both areas in the elongating fiber cells, which also responded to H_2O_2 treatment by a massive increase in the net Ca^{2+} and H^+ efflux in both tip and basal regions. By contrast, non-growing cells (20 DPA) did not respond to H_2O_2 treatment and lacked Ca^{2+} and H^+ oscillations (see text for more details). The findings indicate that the desensitization of the fiber cell and a loss of its ability to respond to H_2O_2 may be causally related to the termination of the cotton fiber elongation. Basal location of sugar and k^+ transporters was based on Ruan et al (2001).

Supplemental Data

Supplemental Figure S1. The effect of Ca^{2+} blockers on cotton ovule growth.

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Figure 1. Validation of Ca^{2+} indicator Fluo-3 in cotton fibers, pollen tubes and root hairs.

Green fluorescence came from the membrane-impermeable intracellular Ca^{2+} indicator Fluo-3, whereas blue fluorescence reflected the cell wall indicator Calcofluor White (CW) binding to cellulose. Cotton seeds (5 days post anthesis, DPA) with fibers attached were co-stained with Fluo-3 and CW (A), or with CW only (B). (C) Cotton fibers derived from seeds cultured in a Ca^{2+} -free BT medium were co-stained with Fluo-3 and CW at 5 DPA. Note, the presence of Ca^{2+} fluorescence signals (arrowheads) in (A) outside the vacuoles (asterisks) but its absence in the negative controls of (B) and (C). (D and E) Representative images of cotton pollen tube and cotton root hair, respectively, stained with Fluo-3, showing Ca^{2+} fluorescence at the tip regions. Scale bars in (A, B and C) = 100 μm , in (D and E) = 50 μm .

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A **B** **C** **D** **E**

Figure 4. Net ion flux profiles across the plasma membrane of the cotton fiber cells measured by the non-invasive microelectrode MIFE technique.

Panel A to C show mean values of steady-state net Ca^{2+} (A), H^+ (B), and K^+ (C) fluxes measured from the tip and basal regions of growing (5 and 10 DPA) and non-growing (20 DPA) cotton fiber cell. Mean $\pm$ SE ($n = 5$ to 8). Data labelled with different low-case letters is significant at $P < 0.05$. *ns* = not significant. (D) oscillations in net Ca^{2+} measured from the tip and basal regions of growing cotton fiber (10 DPA). (E) dynamics of net H^+ fluxes measured from the tip region of the cotton fiber cells as a function of its age. The ultradian H^+ oscillations were observed from growing (5 and 10 DPA) but not in non-growing (20 DPA) cells. One (of 6) typical example is shown for each treatment. The sign convention for all MIFE data is “influx positive”.

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