A Specific Class of Short Treadmilling Microtubules Enhances Cortical Microtubule Alignment

Dear Editor,

Anisotropic cell expansion is a property of plant cells that is dependent upon the formation of a highly ordered cortical microtubule (MT) array. The orientation of cortical microtubules is critical for guiding cellulose synthase complexes in the plasma membrane and thereby determining the direction of the cell expansion (Paredez et al., 2006). Recent work has demonstrated a link between MT organization and stresses experienced by the cell during plant cell development (Uyttewaal et al., 2012; Sampathkumar et al., 2014), but how order is generated within the cortical microtubule array has been much debated (Dixit and Cyr, 2004; Wightman and Turner, 2007).

The crucial role of the microtubule-severing enzyme katanin in plant microtubule alignment is increasingly recognized (Wightman and Turner, 2007; Lin et al., 2013; Wightman et al., 2013; Zhang et al., 2013). Severing at sites where microtubules cross one another appears to be the predominant site of severing in the cortical MT arrays of cotyledon epidermis (Wightman and Turner, 2007; Wightman et al., 2013). In cotyledons, petiole cells undergo anisotropic expansion and exhibit a highly organized microtubule array in which many of the microtubules are orientated in a similar direction to one another (Supplemental Figure 1). This organized array is dependent upon high rates of severing at microtubule crossover sites for the removal of unaligned microtubules. In contrast, adjacent pavement cells exhibit isotropic expansion, net-like MT arrays, and only very low rates of microtubule severing (Wightman and Turner, 2007; Supplemental Figure 1). In pavement cells, SPIRAL2 (SPR2) protein accumulates at crossover sites, preventing severing by katanin, while constant movement of SPR2 in petiole cells exposes crossovers that become substrates for the enzyme katanin (Wightman et al., 2013).

We have noticed an abundance of short treadmilling microtubules (stMTs) during acquisition of time-lapse data, identifiable by fluorescence decline at both ends. These stMTs appear to form a discrete class of microtubules that can be distinguished on the basis of their size (0.5–2 μm). They exhibit a normal distribution with a median length of 1.3 μm (Figure 1A and 1B). Treadmilling MTs greater than 2 μm in length during time-lapse acquisitions are termed long-tMTs. Long-tMTs represent less about a 10% of the treadmilling MTs and are therefore unlikely to have a major influence on microtubule alignment. We also used the term long MTs to indicate a class of MTs greater than 10 μm, where we were not able to observed any treadmilling either because neither end was visible or where an end was visible we saw no evidence of treadmilling. To seek potential differences of MT behavior in distinct array types, we focused on treadmilling and bundling in pavement and petiole cells of wild-type (WT), spr2-1 and bot1-7 mutants that exhibit different levels of MT ordering (Wightman et al., 2013). The frequency of stMTs was low in the net-like arrays of WT pavement cells, but significantly higher in the ordered arrays found in WT petiole cells and in both pavement and petiole cells of spr2-1 (Figure 1C t-test, $p < 0.001$). This correlates with observed severing frequencies (Wightman et al., 2013), suggesting that severing generates stMTs. This idea is strengthened by direct observations of stMTs resulting from severing at crossover sites (Figure 1A and Supplemental Movie 1). Absence of stMTs in the disordered arrays of the katanin mutant bot1-7 is consistent with a direct role of katanin-mediated severing in generating these short MTs (Figure 1C). This implies that cell types with aligned arrays have more stMTs that may contribute to the alignment of the MT array. To ensure that these stMTs were not an artifact of the YFP-MBD reporter, we also examined GFP-TUA6 fusion lines. We found the same trend of increased stMTs in petiole cells as compared to pavement cells (0.68 vs 0.06 MTs/$10^3 \mu m^2$/min), frequencies similar to those observed in WT with the YFP-MBD.

It has been suggested that treadmilling is an essential feature of plant cells that may help array reorganization (Shaw et al., 2003). In order to examine the behavior of stMTs and determine their contribution to MT ordering, we followed the fate of individual stMTs after time-lapse acquisition. By tracking their path, we found that treadmilling MTs change their original path by moving in a curved trajectory (Figure 1A and Supplemental Movie 1). Examination of stMTs reveals that 91% bundle onto other microtubules, while 9% depolymerize completely (N = 192). This contrasts dramatically with the collision outcomes of long MTs, which bundle only if a shallow angle collision occurs, but typically form crossovers if the angle is higher than 45° (Wightman and Turner, 2007). We thus compared bundling in pavement and petiole cells of WT, spr2-1, and bot-1. We found that bundling of long MTs, as a result of plus-end growth, was relatively constant in pavement and petiole cells across backgrounds (Supplemental Figure 2). However, bundling of stMTs was significantly higher in both WT and spr2 petiole cells compared with the corresponding pavement cells and increased in both pavement (t-test, $p < 0.01$) and petiole cells (t-test, $p < 0.001$) for spr2-1 relative to the WT (Supplemental Figure 2). Since most stMTs bundle onto other MTs, this reflects differences in stMT densities across cell types.
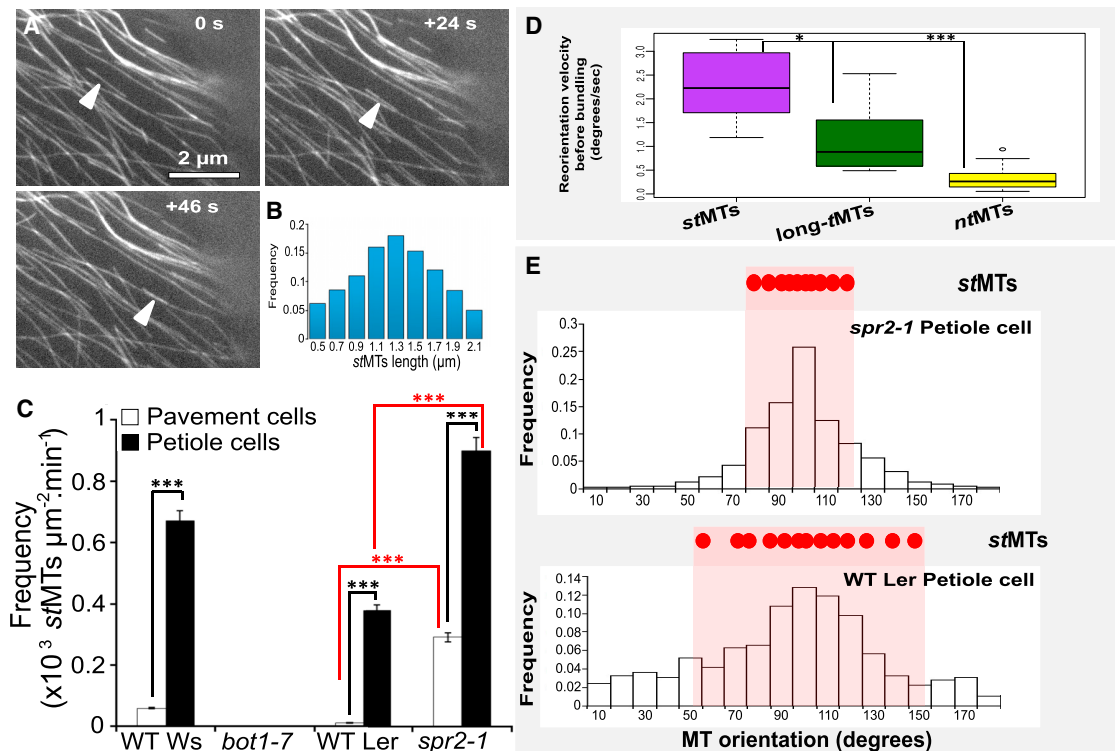
Figure 1. Microtubule Severing Generates Treadmilling Microtubules that Increase Microtubule Alignment.
(A) Example of a short *st*MT microtubule that bundle onto an aligned MT in a WT petiole cell. Arrowhead indicates an position of a *st*MT.
(B) *st*MTs form a discrete class of microtubules based on their size distribution.
(C) Frequency of short treadmilling MTs (*st*MTs) in pavement and petiole cells of different backgrounds, normalized for both area and time. WT WS, 12 cells, 29 min, pavement N = 13, petiole N = 126; WT Ler, 42 cells, 68 min, pavement N = 5, petiole N = 145; botero1-7, 12 cells, 68 min, pavement 0, petiole 0; spiral2-1, 49 cells, 88 min, pavement 243; petiole 1133. Asterisks indicate significance of t-tests, ***$p < 0.001$.
(D) Reorientation velocity of *st*TMTs long-*t*MTs, and long MTs. For each category, n = 20 MTs. *$p < 0.05$, ***$p < 0.001$.
(E) Distinct pattern of bundling location of *st*MTs and long MTs. Upper graph shows a hyper aligned (*spr2-1* petiole cell), lower graph shows a WT Ler petiole cell with an aligned array. In each case, the histogram shows the distribution of angles, with 90° representing the alignment axis, following Wightman et al. (2013). Red dots show the location of bundling on the array.

Of the three classes of MTs defined above, long MTs exhibited significantly slower reorientation (Figure 1D). We found that *st*MTs reoriented significantly faster than long-*t*MTs (t-test, $p < 0.05$), and even more significantly than long MTs, which are restricted in their amount of reorientation due to their length and lack of treadmilling, and can thus only bundle onto a neighboring MT. Reorientation was significantly correlated with treadmilling MT length (Supplemental Figure 3). Together with the finding that long MTs have very limited reorientation potential, this suggests that both MT length and treadmilling are important determinants of MT reorientation capacity. Long MTs are constrained by their length and can only reorientate by swiveling of the plus end. Their reorientation thus depends on their initial location and the angle at which they intercept another MT (Figure 1; Wightman and Turner, 2007). By contrast, *st*MTs are not limited by their length or fixed position (part of a bundle) in the array and can thus move diffusely through the array.

To assess whether the increased treadmilling and the resulting bundling played a role in MT organization, we recorded the localization of *st*MTs bundling events in petiole cells. We selected 11 WT petiole cells that varied in their level of array alignment and

determined the angle between the MT that the *st*MT bundled onto and the mean array alignment (see Supplemental Figure 4 for graphic explanation). Compared with the well-aligned arrays, we found that *st*MTs bundling in more disorganized arrays resulted in a wider range of array bundling angles (Supplemental Figure 4, Supplemental Materials and Methods). This indicates that *st*MTs bundling follows the existing topology of the array. This is consistent with a diffuse pattern of bundling of stMTs, likely resulting from their high reorientation capabilities. Hence, highly aligned arrays, where most microtubules are in the same orientation, predetermine the bundling location of *st*MTs and therefore promote the self-reinforcement of the aligned array. To further illustrate that treadmilling MTs bundle on MTs oriented similarly, we selected a WT and a *spr2-1* petiole cell with aligned and hyper-aligned MT organization, respectively, and noted the angle between the MT targeted by bundling *st*MTs and the mean array angle (Figure 1E). Consistent with our 11-cells analysis (Supplemental Figure 4), we found that the most highly aligned arrays in *spr2-1* petiole cells resulted in bundling occurring over a significantly narrower range than for WT petiole cells (Figure 1E; F-test, $p < 0.05$). This implies that the bundling of *st*MTs reinforces the topological properties already existing in

the array by increasing MT density of the pre-existing MT alignment. Since aligned arrays have by definition a large proportion of MTs in the same orientation, the chance that *st*MTs bundle onto an aligned MT will be much greater. We thus demonstrate a feedback mechanism that enhances alignment because *st*MT bundling depends upon the pre-existing MT order, and is most proficient in those cells where MTs are highly aligned (Figure 1E and Supplemental Figure 4). The feedback results from the fundamentally distinct behavior of *st*MTs; these short-lived MTs bundle continuously, reinforcing the existing topological properties of the array.

Overexpression of katanin leads to increased bundling (Stoppin-Mellet et al., 2006), but cannot induce MT alignment in pavement cells where the presence of immobile SPR2 protein protects crossovers (Wightman et al., 2013). Although Shaw et al. (2003) concluded that treadmilling MTs change MT organization through MT reorientation and bundling, they did not provide an alignment mechanism. Feedback between treadmilling, bundling and array alignment plays a key role in the maintenance of MT alignment (Figure 1E and Supplemental Figure 4). Feedback of topology on itself is an emerging theme in developmental biology (Jaeger et al., 2008). For example, in cotyledons, the lobed shapes of pavement cells are predicted to generate a stress pattern that controls microtubule orientation, which itself feedbacks on cell shape (Sampathkumar et al., 2014). Feedback of a topological feature on itself implies that stochastic events play an important role in development. In this case, the diffuse distribution of *st*MT bundling across the array, fostered by the high reorientation potential of these types of MTs, plays an important role. However, this feedback cannot drive alignment *ad hoc* and is dependent upon microtubule severing at crossover sites to (i) generate the *st*MTs and (ii) eliminate unaligned MTs (Wightman et al., 2013).

Our study shows cell-type specific and array-dependent rates of short MT treadmilling. In severing-active cells with ordered arrays, large pools of *st*MTs with a high reorientation potential bundle onto the aligned array. Feedback between MT order and bundling of these species of microtubule explains how they contribute to MT alignment.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

ACKNOWLEDGMENTS

Guillaume Chomicki[1,2],
Raymond Wightman[1,3] and Simon R. Turner[1,*]

[1]Faculty of Life Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, UK
[2]Present address: Department of Biology, Systematic Botany and Mycology, University of Munich (LMU), 67 Menzinger Street, 80638 Munich, Germany
[3]Present address: The Sainsbury Laboratory, University of Cambridge, Bateman Street, Cambridge CB2 1LR, UK
*Correspondence: Simon R. Turner (simon.turner@manchester.ac.uk)
http://dx.doi.org/10.1016/j.molp.2016.05.008

REFERENCES

Dixit, R., and Cyr, R. (2004). The cortical microtubule array: from dynamics to organization. Plant Cell **16**:2546–2552.

Jaeger, J., Irons, D., and Monk, N. (2008). Regulative feedback in pattern formation: towards a general relativistic theory of positional information. Development **135**:3175–3183.

Lin, D., Cao, L., Zhou, Z., Zhu, L., Ehrhardt, D., Yang, Z., and Fu, Y. (2013). Rho GTPase signaling activates microtubule severing to promote microtubule ordering in *Arabidopsis*. Curr. Biol. **23**:290–297.

Paredez, A.R., Somerville, C.R., and Ehrhardt, D.W. (2006). Visualization of cellulose synthase demonstrates functional association with microtubules. Science **312**:1491–1495.

Sampathkumar, A., Krupinski, P., Wightman, R., Milani, P., Berquand, A., Boudaoud, A., Hamant, O., Jönsson, H., and Meyerowitz, E.M. (2014). Subcellular and supracellular mechanical stress prescribes cytoskeleton behavior in *Arabidopsis* cotyledon pavement cells. Elife **3**:e01967.

Shaw, S.L., Kamyar, R., and Ehrhardt, D.W. (2003). Sustained microtubule treadmilling in *Arabidopsis* cortical arrays. Science **300**:1715–1718.

Stoppin-Mellet, V., Gaillard, J., and Vantard, M. (2006). Katanin's severing activity favors bundling of cortical microtubules in plants. Plant J. **46**:1009–1017.

Uyttewaal, M., Burian, A., Alim, K., Landrein, B., Borowska-Wykręt, D., Dedieu, A., Peaucelle, A., Ludynia, M., Traas, J., Boudaoud, A., et al. (2012). Mechanical stress acts via katanin to amplify differences in growth rate between adjacent cells in *Arabidopsis*. Cell **149**:439–451.

Wightman, R., and Turner, S.R. (2007). Severing at sites of microtubule crossover contributes to microtubule alignment in cortical arrays. Plant J. **52**:742–751.

Wightman, R., Chomicki, G., Kumar, M., Carr, P., and Turner, S.R. (2013). SPIRAL2 determines plant microtubule organization by modulating microtubule severing. Curr. Biol. **23**:1–6.

Zhang, Q., Fishel, E., Bertroche, T., and Dixit, R. (2013). Microtubule severing at crossover sites by katanin generates ordered cortical microtubule arrays in *Arabidopsis*. Curr. Biol. **23**:2191–2195.