Running title

Transverse microtubule array formation

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Cortical microtubule arrays are initiated from a non-random pre-pattern driven by atypical microtubule initiation

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Abstract (247 words)

The ordered arrangement of cortical microtubules in growing plant cells is essential for anisotropic cell expansion and hence for plant morphogenesis. These arrays are dismantled when the microtubule cytoskeleton is rearranged during mitosis and reassembled following completion of cytokinesis. The reassembly of the cortical array has often been considered as initiating from a state of randomness, from which order arises at least partly through self-organizing mechanisms. However, some studies have shown evidence for ordering at early stages of array assembly. To investigate how cortical arrays are initiated in higher plant cells, we performed live cell imaging studies of cortical array assembly in tobacco BY-2 cells after cytokinesis and drug-induced disassembly. We found that cortical arrays in both cases did not initiate randomly, but with a significant over-representation of microtubules at diagonal angles with respect to the cell axis, which coincides with the predominant orientation of the microtubules before their disappearance from the cell cortex in preprophase. In Arabidopsis root cells, recovery from drug-induced disassembly was also non-random, and correlated with the organization of the previous array, although no diagonal bias was observed in these cells. Surprisingly, during initiation only about half of the new microtubules were nucleated from locations marked by GFP-GCP2 tagged gamma-nucleation complexes (γ-TuRC), therefore indicating that a large proportion of early polymers was initiated by a non-canonical mechanism not involving γ-TuRC. Simulation studies indicate that the high rate of non-canonical initiation of new microtubules has the potential to accelerate the rate of array repopulation.
Introduction (5813 characters including spaces)

Higher plant cells feature ordered arrays of microtubules at the cell cortex (Ledbetter and Porter, 1963) that are essential for cell and tissue morphogenesis, as revealed by disruption of cortical arrays by drugs that cause microtubule depolymerization (Green, 1962) or stabilization (Weerdenburg and Seagull, 1988), and by loss of function mutations in a wide variety of microtubule associated proteins (MAPs) (Baskin, 2001; Whittington et al., 2001; Buschmann and Lloyd, 2008; Lucas et al., 2011). The structure of these arrays is thought to control the pattern of cell growth primarily by its role in the deposition of cellulose microfibrils, the load-bearing component of the cell wall (Somerville, 2006). Functional relations between cortical microtubules and cellulose microfibrils have been proposed since the early sixties, even before cortical microtubules had been visualized (Green, 1962). Recent live cell imaging studies have confirmed that cortical microtubules indeed guide the movement of cellulose synthase complexes that produce cellulose microfibrils (Paredez et al., 2006) and have shown further that microtubules position the insertion of most cellulose synthase complexes into the plasma membrane (Gutierrez et al., 2009). These activities of ordered cortical microtubules are proposed to facilitate the organization of cell wall structure, creating material properties that underlie cell growth anisotropy.

While organization of the interphase cortical array appears to be essential for cell morphogenesis, this organization is disrupted during the cell cycle as microtubules are rearranged to create the preprophase band, spindle and phragmoplast during mitosis and cytokinesis (reviewed by Wasteneys, 2002). Upon completion of cytokinesis, an organized interphase cortical array is regenerated, but the pathway for this reassembly is not well understood.

The plant interphase microtubule array is organized and maintained without centrosomes as organizing centers (reviewed in Wasteneys, 2002; Bartolini and Gundersen, 2006; Ehrhardt and Shaw, 2006) and microtubule self-organization is proposed to play an important role in cortical microtubule array ordering (Dixit and Cyr, 2004). In electron micrographs microtubules have been observed to be closely associated with the plasma membrane (Hardham and Gunning, 1978) and live cell imaging provides evidence for attachment of microtubules to the cell cortex (Shaw et al., 2003; Vos et al., 2004). The close association to the plasma membrane restricts the cortical microtubules to a quasi two-dimensional plane where they interact
through polymerization-driven “collisions” (Shaw et al., 2003; Dixit and Cyr, 2004). Microtubule encounters at shallow angles (< 40 degrees) have a high probability of leading to bundling, while microtubule encounters at steeper angles most likely result in induced catastrophes or microtubule crossovers (Dixit and Cyr, 2004). Several computational modeling studies have since shown that these types of interactions between surface-bound dynamical microtubules can indeed explain spontaneous co-alignment of microtubules (Allard et al., 2010; Eren et al., 2010; Hawkins et al., 2010; Tindemans et al., 2010).

The question of how the orientation of the cortical array is established with respect to the cell axis is less well understood. One possibility is that microtubules are selectively destabilized with respect to cellular coordinates (Ehrhardt and Shaw, 2006). Indeed, recent results from biological observations and modeling suggest that catastrophic collisions induced at the edges between cell faces, or heightened catastrophe rates in cell caps could be sufficient to selectively favor microtubules in certain orientation and hence determine the final orientation of the array (Allard et al., 2010; Eren et al., 2010; Ambrose et al., 2011; Dhonukshe et al., 2012).

To date, all models of cortical array assembly assume random initial conditions. However, experimental work by Wasteneys and Williamson (1989a, b) in Nitella tasmanica showed that, during array reassembly after drug-induced disruption, microtubules were initially transverse. This was followed by a less ordered phase and later by the acquisition of the final transverse order. A non-random initial ordering was also observed in tobacco BY-2 cells by Kumagai et al. (2001), who concluded that the process of transverse array establishment starts with longitudinal order, but did not provide quantitative data for the process of array assembly. The initial conditions for the cortical microtubule array formation are important to consider, as they may strongly influence the speed at which order is established, and could even prevent it from being established over a biologically relevant time scale.

In the present study, we used live cell imaging to follow and record the whole transition from the cortical microtubule-free state to the final transverse array and used digital tracking algorithms to quantify the microtubule order. Nucleation stands out as a central process to characterize during array initiation. Lacking a central body to organize microtubule nucleations, the higher plant cell has dispersed nucleation complexes (Wasteneys and Williamson, 1989, 1989; Chan et al., 2003; Shaw et al., 2003; Murata et al., 2005; Pastuglia et al., 2006; Nakamura et al., 2010). Therefore we
performed high time resolution observations to quantify nucleation complex
recruitment, nucleation rates and microtubule nucleation angles. We found evidence
for a highly non-random initial ordering state that features diagonal microtubule
orientation and an atypical microtubule initiation mechanism. Simulation analysis
indicates that these atypical nucleations have the potential to accelerate the recovery
of cortical array density.
Results

After cytokinesis, microtubules in BY2 cells reappear with a transient diagonal order

To investigate array initiation, we used tobacco Bright Yellow 2 (BY-2) suspension cells expressing GFP fused to tobacco α-Tubulin (GFP-TUA). These cells feature highly ordered arrays oriented transversely to the axis of growth, have a relatively high mitotic index, and are ideal for drug treatment in flow cell experiments. Furthermore, the potential crosstalk with neighbors is limited because BY-2 cells generally grow in cell files that break up into individual cells (Chan et al., 2011; Crowell et al., 2011; Fujita et al., 2011).

Using point-scanning confocal microscopy, we acquired images from the plane of the cell cortex every 3-5 minutes and measured microtubule length density and ordering after cytokinesis. The first visible microtubules appeared in the cortex after the phragmoplast reached the optical plane of the cell cortex (Figure 1a, Figure S1 and Movie S1) and within ~45 minutes the length density, defined as microtubule length per square micrometer, leveled at around 0.5 µm/µm² (= µm⁻¹, mean of 6 cells; Figure 1b). With the increase in length density, the microtubules also became increasingly bundled, as indicated by increases in the fluorescence intensities of individual microtubule structures. As our focus was on microtubule orientation, we gave bundles the same weight as individual microtubules.

The angles of microtubules with respect to the cell elongation axis were measured and visualized in a contour plot (Figure 1c). Time is presented along the x-axis and the angular distribution over the interval from 0° to 180° along the y-axis (20 bins). The color range represents the fraction of the total microtubule length, so that orientation patterns at both low and high microtubule densities can be compared. Surprisingly, the majority of the microtubule length was diagonally oriented at 45° and 135° angles to the elongation axis in the early stages of array reformation, forming two clear peaks in the angular frequency histogram.

To quantify the transition from the diagonal to the transverse cortical microtubule order, the angular distribution data were filtered to produce the weighted diagonal order parameter $D$ and the weighted transverse order parameter $T$ (see Supplementary Information). From the means of the $D$ and $T$ order parameters over
time, we infer that the diagonal ordering was dominant for the first ~25 minutes after which it was replaced by transverse ordering (Figure 1d).

**Transient diagonal ordering during recovery from oryzalin treatment in BY2 cells**

To establish if the mechanism of transverse microtubule ordering via a transient diagonal phase is generic or cell cycle dependent, we immobilized BY-2 cells expressing GFP-TUA in flow cells and treated them for 1 hour with 20 µM oryzalin to reversibly depolymerize the cortical microtubule array (Morejohn et al., 1987)(Figure 2a and Movie S2). This concentration and duration of oryzalin treatment was sufficient to eliminate all detectible GFP-TUA labeled microtubules. Both the microtubule length density increase and the development of ordering after oryzalin wash out were similar as observed after cell division (Figure 2b). The average plateau density was reached ~25 minutes after appearance of the first cortical microtubules, which is ~45 minutes after the start of the oryzalin wash out (mean of 8 cells). The first microtubules reappeared at diagonal angles to the elongation axis (45° and 135°; Figure 2c). On average, the transient diagonal ordering was replaced by the final transverse ordering after ~40 minutes (Figure 2d). Thus, it appears that both the pattern and kinetics of assembly and ordering are similar whether the array is disassembled by native mechanisms during the cell cycle, or by drug treatment.

**Diagonal ordering also occurs during array disassembly in BY2 cells**

Interestingly, a diagonal bias for microtubule orientation was also observed during late stages of array disassembly as cells exit interphase and form prophase bands (observations from 5 cells, Figure S2). Likewise, the same bias was observed in late stages of microtubule depolymerization caused by oryzalin application (Figure 2c and d). The microtubule length density started to decrease less than a minute after drug application and reached zero microtubules after ~16 minutes. Within 2 minutes after oryzalin addition, a diagonal microtubule order took over the dominant transverse order and lasted until the last microtubules were depolymerized (Figure 2d). Thus diagonal biasing of microtubule orientation appears to be a feature both of the last stages of array disassembly and the first stages of array re-assembly, irrespective of whether arrays are taken apart by cellular mechanisms or by drug treatment.
Microtubule nucleation has a diagonal bias during array initiation in BY2 cells

A bias in microtubule orientation might occur because microtubules are preferentially created in specific orientations, or because they are selectively destabilized, or if they are reoriented once initiated. To assess the origin of the diagonal microtubule ordering, we made movies at high time resolution (2s intervals) of BY2 cells expressing GFP-TUA cytokinesis and oryzalin wash out (Movie S3). We observed that in the first 30 minutes the majority of new microtubules were nucleated at the cell cortex at locations free of other detectable microtubules. The majority of nucleations during this period were free nucleations (274 out of 352, 77%, in 6 cells after cytokinesis, and 73 out of 117, 62%, in 5 cells after oryzalin wash out). These observations are in contrast to those of interphase nucleation, where microtubule-associated microtubule nucleations have been observed to comprise greater than 99% of nucleations in wild type Arabidopsis cells (Murata et al., 2005; Nakamura et al., 2010; Kirik et al., 2012). We measured the angles of these free nucleations with respect to the cell axis after both cytokinesis (Figure 3a) and oryzalin wash out (Figure 3b). We did not analyze microtubule nucleations in the same orientation as the microtubule they nucleated on, as they do not give rise to new microtubule orientations. A Bayesian statistical analysis of these data (see Materials and Methods) revealed a significant bias for nucleations to occur along the diagonal directions both after cytokinesis and oryzalin wash out.

In Arabidopsis root cells, a large fraction of nucleations during array initiation are free of labeled γ-tubulin complexes

We found it remarkable that the nucleation bias had the same orientation as the cortical microtubule order just before disappearance. This suggested that a ‘memory’ of the previous array organization might be maintained at the cell cortex. We could imagine three alternative models. First, nucleation complexes recruited to the previous array might persist at the cell cortex, retaining orientational information. Second, there might be other orientational information at the cell cortex that acts to orient newly recruited nucleation complexes as they initiate the next array. Finally, a subset of the previous array might be resistant to disassembly either by native mechanisms or by drugs, and they may be either small enough to evade detection by GFP-TUA5 labeling (or that the specific isoform of alpha tubulin we labeled is not or...
less incorporated into these structures). These disassembly-resistant remnants might act as orientated seeds for initiating new polymerization during array reassembly.

To distinguish among these hypotheses, we assayed the localization and dynamic behavior of γ-tubulin complexes and their relationship to new nucleations using Arabidopsis plants expressing both a γ-tubulin complex marker (GCP2-3xGFP, Nakamura et al., 2010) and a compatible tubulin marker (mCherry-TUA5, Gutierrez et al., 2009, see Movie S4). To facilitate our analysis, we used a 1 hr treatment of 1 µM oryzalin to dissemble existing cortical arrays in Arabidopsis root epidermal cells. After drug washout, we acquired images of the cell cortex at high time resolution (2s intervals). We observed no evidence for persistent GFP-labeled nucleation complexes at the cell cortex, thus refuting the first hypothesis; that nucleation complexes recruited to the previous array might persist at the cell cortex to initiate the new array.

We then scored all observed nucleation events in the field of view, asking if GCP2-3xGFP was present at the position of microtubule nucleation. As labeled complexes are present and motile in the streaming cytosol (Nakamura et al., 2010), we required that punctae GFP signal be present at the position of nucleation for at least two consecutive image frames to be scored positively. In control cells that were not pretreated with oryzalin, we found that 68 out of 70 nucleations (97%) colocalized with the GCP2-3xGFP label (Figure 4a and Movie S5, data acquired from 6 cells on 6 plants), a frequency in good agreement with the ~98% found by Nakamura et al. (2010) in hypocotyl cells. By contrast, in oryzalin treated cells, only a little over half of the observed nucleations (45 out of 81, 56%) colocalized with the GCP2-3xGFP label in the first 20 minutes after the start of oryzalin wash out, a dramatically lower proportion (p << 0.0001, one-tailed binomial test, 8 cells). Thus, while only ~3% of nucleations was not observed to be accompanied by GCP2-3xGFP in mature arrays, this frequency raised to ~44% during early stages of array assembly (Figure 4b). The lack of detectable γ-TuRC label at nearly half of the early nucleations argues strongly against the second hypothesis for diagonal nucleation orientation; that orientational information at the cell cortex directs the orientation of new nucleation complexes recruited to the cell cortex during early array assembly. We also found no evidence for involvement of two candidates for such orientational information, the cortical actin cytoskeleton and cellulose microfibrils, by disruption experiments with latrunculin B (Figure S3) or isoxaben (Figure S4), respectively.
On the other hand, the marked reduction in GCP2-3xGFP co-localization was consistent with the third hypothesis; that a large and significant proportion of nucleations during early array recovery arise from seeds not associated with γ-tubulin complexes. We term these nucleation events non-canonical nucleations because they lack association with detectible γ-TuRCs as determined by GCP2-3xGFP labeling, an essential subunit of the core γ-TuRC.

In the *Arabidopsis* root epidermal cells, we also determined the orientation the orientation of the cortical microtubule array during oryzalin treatment and the recovery from oryzalin wash out. We found that in these cells, the orientational bias of the initial array after oryzalin wash out was the same as the bias that we found during the depolymerization (Figure 4c and d, Figure S5, Movie S6). This was the case not only for transversely oriented cortical microtubule arrays, but also for oblique and longitudinal arrays (Figure 4c and d, Figure S5, Movie S6).

**Simulations**

We performed mechanistic simulations to ask if the observed prevalence of diagonal microtubule nucleation was sufficient to explain the degree of observed diagonal ordering during the initial stages of array assembly and to ask what affect these non-canonical nucleations might have on the evolution of array density and ordering. In the simulations, cortical microtubules interact on a cylindrical cell-shaped surface of dimensions similar to that of the tobacco BY-2 cells used in our *in vivo* experiments (Figure 5a)(Tindemans et al., 2010; Deinum et al., 2011). To test for the influence of the non-canonical nucleation events, these nucleations were treated as a separate class, their density and orientations was chosen to match the distributions determined from the live cell experiments as described above in BY2 cells. At the start of the simulations we add a finite pool of microtubule fragments with the density of $d_0 0.1/\mu m^2$ and an activation rate of $r_s 0.003/s$ (see the Materials and Methods section for further details on the simulation technique and the parameters employed). In simulations that include all nucleation events the length density initially rises steeply, reaching ~80% of the final density in just 10 minutes, then transiently leveling off (Figure 5b and illustrated in figure 4a at 15 min.). By contrast, in simulations without the non-canonical nucleation class, the length density rises more gradually and steadily, reaching 80% of the final density only after about 40
minutes (Figure 5b). Thus, the non-canonical nucleations appear to have the potential to significantly accelerate the recovery of array density during array re-assembly.

When simulations were run without the non-canonical nucleation class there was no initial bias of the angular distribution (Figure 5c). As expected, when these nucleations were added to the simulation, a clear initial bias at 45 and 135 degrees is created (Figure 5d), markedly similar to our experimental observations (Figure 1c). The diagonal and transverse order parameters $D$ and $T$ as a function of time for the simulations with the non-canonical nucleation class (Figure 5e) both qualitatively and quantitatively match the values we found experimentally (Figure 5d). Thus, the inclusion of the diagonally biased nucleation events observed in living cells have the potential to explain both the initial diagonal ordering and the observed evolution of array ordering in these cells. While non-canonical nucleations had the effect of lowering the initial transverse ordering state of the simulated arrays, the difference is not significant and the order parameters level off to the same value (Figure 5f), indicating that biased non-canonical nucleations did not present a barrier to achieving proper array ordering.
Discussion

The transverse arrangement of the cortical microtubule array is essential for anisotropic growth, yet little was known about how it arises from a disassembled state, the situation that recurs after each cell division during the life of the cell. The currently accepted self-organization models for transverse cortical microtubule array establishment, based on microtubule interactions (Allard et al., 2010; Eren et al., 2010; Hawkins et al., 2010; Tindemans et al., 2010), assume that new microtubules appear with random initial orientations. We found that the first microtubules in new arrays of tobacco BY-2 cells were in fact not randomly oriented, but showed significant ordering at orientations of both 45° and 135°. This was true both for array assembly during the cell cycle as well as reassembly of arrays after oryzalin washout. Organization in these arrays did not evolve by gradual ordering from a disorganized, random state, but by a transition from one ordered state to another.

Exploration of the cause for the non-random initiation of array establishment revealed that there was a significant bias in the orientation of early microtubule nucleations, sharing the same 45° and 135° bias relative to the cell axis that was observed for array ordering. Results from simulation studies incorporating these oriented nucleations matched experimental observations very closely, indicating that this population of directionally biased nucleations is sufficient to explain the fast-forming initial diagonal ordering state of new arrays, and together with self-ordering based on microtubule interactions, is sufficient to explain the transition from this transiently ordered array into the final transverse array.

Analysis of oryzalin treatment in Arabidopsis root epidermal cells showed that also in these cells the initial bias of microtubule orientation correlates with the last orientation before disassembly. This was true for not only the transverse cortical microtubule array, but also for oblique and longitudinal orientation, which strongly suggests that the bias depends on elements of the microtubule cytoskeleton before depolymerization.

We considered several alternative ideas for the mechanism of nucleation orientation. In interphase cells, the majority of nucleations at the cell cortex occurs from the sides of existing microtubules with a major peak at about 40° (Murata et al., 2005) and a secondary peak at 0° (i.e. parallel to the parent polymer) (Chan et al., 2009; Nakamura et al., 2010; Kirik et al., 2012). Thus, in the interphase arrays studied to date, existing microtubules largely determine the orientation of new microtubule
nucleation. The vast majority of the nucleation events in the cortex of Arabidopsis interphase cells have been observed to be marked by tagged components of the γ-TuRC complex (>98%, Nakamura et al., 2010; Kirik et al., 2012). However, at the start of array assembly there are no obvious existing microtubules (this study, Wasteneys and Williamson, 1989, 1989) to recruit and position nucleation complexes (Nakamura et al., 2010), and therefore it was necessary to consider other mechanisms for nucleation orientation. One possibility was that oriented γ-TuRC complexes are simply retained at the cell membrane from the previous cortical array, an idea that was directly contradicted by our observations in Arabidopsis root cells. A second possibility was that newly recruited γ-TuRCs are positioned by cryptic orientational information at the cortex. Surprisingly, however, we found that labeled γ-TuRC complexes failed to be detected at ~44% of the nucleation events observed during array initialization compared to ~3% at steady state microtubule density. This result both effectively eliminated a mechanism based solely on oriented γ-TuRCs and revealed that many early nucleations apparently arise from non-canonical nucleation sites that lack a γ-TuRC. The GCP2 protein is a core component of the γ-TuRC complex and localizes to both mitotic and interphase microtubule arrays. The possibility that less of the GCP2 protein, which we use as the γ-TuRC complex marker, is incorporated in the γ-TuRC complexes therefore appears to be small, ruling out a bias due to insufficient labeling.

We do not know the molecular identity of the microtubule cytoskeleton elements that provide the seeding for the initial bias in microtubule initiation and ordering. While most microtubules nucleated by γ-TuRCs (Teixidó-Travesa et al., 2012), nucleation has also been found to be facilitated by several microtubule plus-end-binding proteins (Rogers et al., 2008; Rusan and Rogers, 2009), transforming acidic coiled coil (TACC) family proteins, and RanGTP-activated factors (Gruss and Vernos, 2004; Clarke and Zhang, 2008). It is not clear whether all these factor act as true nucleators or if they might stabilize short microtubule fragments that could act to restart polymer growth (Teixidó-Travesa et al., 2012). When we washed out oryzalin in Arabidopsis epidermal root cells after 30 minutes short but still visible fragments of microtubules started to reinitiate growth (Movie S7). The non-canonical microtubule initiations we describe in this paper are most likely not derived from γ-TuRC complexes, because of the absence of GCP2-3xGFP marker at the cell cortex during
the disassembly phase, suggesting that another factor may serve as the nucleator or that initiations may arise from stabilized short fragments of microtubules.

We therefore speculate that short fragments of microtubules may act as nucleators during early cortical array recovery. This would provide a mechanism by which the orientation of new initiations is tied to that of the previous array, in the absence of a microtubule scaffold that positions and orients nucleation complexes. Previous studies by Wasteneys and Williamson (1989b) in Nitella are also consistent with this possibility. These investigators observed that while Nitella microtubules returned in their original transverse orientation during recovery from oryzalin, orientation was random after longer, and presumably more complete, oryzalin treatment (Wasteneys and Williamson, 1989, 1989). In our studies, incomplete drug action cannot explain observations of array re-assembly following cytokinesis, since there was no drug treatment in these cells and the extremely similar mode and kinetics of array reassembly we observed between these cells and those recovering from oryzalin treatment suggest that a similar mechanism is responsible in both situations.

It is as yet unclear how such sort pieces of microtubules persist at the cell cortex during drug treatments for over an hour and even longer during cytokinesis. We did observe that the rate of disassembly slows down over time. It is well possible that microtubule disassembly is in part dependent on how much the microtubule is physically loaded with microtubule associated proteins. Indeed, disassembly of microtubules could elevate the cytosolic concentration of microtubule associated proteins by decreasing the microtubule binding surface. An increased concentration of free MAPs in turn could drive association with any polymer remaining, potentially acting to stabilize short microtubule fragments.

A number of questions remain. First, microtubule seeds from the previous cell cycle were not readily detected by imaging of GFP-TUA. However, this might be easily explained if the seeds are small enough to contain only a few labeled subunits (only a fraction of alpha subunits in the cell are tagged), a degree of labeling that may well lie below the high background of unpolymerized subunits in the cytosol. It is also formally possible that the seeds may have a composition that does not include the labeled tubulin isoform used for imaging. Such small fragments would be difficult to detect in electron micrographs because of their small size and their relatively low estimated density (0.1/µm²).
A second puzzle is why the presumed source of the oriented seeds – the last cohort of microtubules at the end of array disassembly – has a diagonal bias to the cell axis in the BY2 cells. One possibility is that the bias arises from the normal formation of the newest microtubules by branching nucleation at about 40 degrees to their mother polymers (Wasteneys and Williamson, 1989, 1989; Murata et al., 2005; Chan et al., 2009; Nakamura et al., 2010). In a transversely oriented array, these nucleations would lie approximately at 45° and 135° to the cell axis, and would have a high likelihood of interacting with the dominant population of transverse microtubules. These interactions can lead to incorporation into bundles by treadmilling motility (Shaw et al., 2003; Dixit and Cyr, 2004), or catastrophe (Dixit and Cyr, 2004), both of which would tend to diminish the population of diagonally oriented polymers. However, as the microtubule array is broken down and microtubule density drops, encounters would be predicted to be less frequent and therefore the likelihood of aligning or eliminating branching microtubules will be reduced.

It is also not clear why there is a transient diagonally ordered phase during disassembly in BY2 cells and not in our experiments in *Arabidopsis* root epidermal cells. The disassembly occurred ~ 5 times faster in the *Arabidopsis* root epidermal cells. It is possible the diagonal bias found in BY2 cells is dependent on growth and nucleation during the drug treatment. This effect could be much smaller in the *Arabidopsis* root cells because of the shorter time of microtubule depolymerization.

Irrespective of whether the source of oriented nucleation in early array assembly is due to seeds from the previous array or another mechanism, our observations reveal the existence of a substantial class of non-canonical nucleations not associated with γ-TuRCs that contribute to the initiation of the cortical array. In simulation studies we explored how these non-canonical nucleations may affect array reassembly and found this class of oriented nucleations to have the potential to significantly accelerate recovery of array density without significantly impeding the acquisition of the ultimate ordering which is driven by microtubule interactions. The existence of this mechanism may address a fundamental dilemma the plant cell faces in rebuilding an array from scratch. In interphase cells, nucleation from γ-TuRC complexes was observed to be approximately 10-fold more likely when they are localized to microtubules than to other locations at the cell cortex (Nakamura et al., 2010). If this reflects a fundamental property of γ-TuRC activation, then the cell may face limitations in how fast it can initiate new arrays when there are no existing
cortical microtubules to recruit γ-TuRCs and contribute to their activation. Our live cell observations and simulation studies reveal a class of nucleations that do not require γ-TuRC recruitment and activation at cortical microtubules may act as a primer to accelerate the assembly of the new array.
Materials and methods

Plant material

Tobacco (Nicotiana tabacum L.) Bright Yellow-2 (BY-2) suspension cultured cells were grown according to standard protocols (Nagata et al., 1992). We stably transformed BY-2 cells using Agrobacterium tumefaciens LBA4404 mediated procedures with a reporter construct consisting of the enhanced green fluorescent protein fused to tobacco α-tubulin (sGFP-TUA) under control of the CaMV 35s promoter, kindly provided by Dr. S. Hasezawa, University of Tokyo, Japan (Kumagai et al., 2001). The BY-2 cell line expressing eGFP-FABD was generously provided by Dr. T. Ketelaar (Wageningen University) (Ketelaar et al., 2004). We used Agrobacterium tumefaciens to transform the pGCP2-GCP2-3xGFP construct, kindly provided by Masayoshi Nakamura and Takashi Hashimoto (Nara Institute of Science and Technology, Ikoma, Japan), into an Arabidopsis thaliana Col-0 expressing 35s-mCherry-TUA5 (Gutierrez et al., 2009).

Specimen mounting

Transformed cells were imaged in thin ~10 to 20 μL gas permeable micro-chambers lined on one side with Biofoil (VivaScience, Hannover, Germany) and a 24 x 24 mm coverslip on the other side as described earlier (Vos et al., 2004). Slides were sealed with VALAP (1:1:1 Vaseline : lanolin : paraffin). For oryzalin treatments, cells were immobilized in plastic flow cells (1 channel of 100 μL with a height of 0.4 mm; Ibidi, Munich, Germany) that were pretreated with 1 mg/mL poly-L-lysine solution in dH2O for 30 min at room temperature. Ten flow cell volumes of 20 μM oryzalin (from 20 mM stock in DMSO) in BY-2 medium were perfused through the channel with cells and after 1 hour, washed out with constant perfusion of BY-2 medium at a flow rate >0.1 mL/min. For latrunculin B and isoxaben experiments, 10 mL of a BY-2 culture was incubated for at least 3 hours in 0.5 or 1.0 μM latrunculin B or at least 24 hours in 10 μM isoxaben before adding 20 μM oryzalin and cell immobilization in a flow cell. Washes with latrunculin B or isoxaben were initiated after 1 hour to allow the microtubule cytoskeleton to recover, but not the actin cytoskeleton or the cellulose microfibril production. The immobilization, the perfusion of medium with 0.1% DMSO, 0.1% ethanol and the confocal imaging did
not influence the cytoarchitecture or microtubule organization of the tobacco BY-2 cells (data not shown).

The Arabidopsis plants were grown on standard Hoagland's medium and gently mounted between an objective slide and coverslip spaced by two strips of double sided Scotch tape. For the oryzalin treatment, the plants were transferred to a six well plate containing 1.0 µM oryzalin for an hour to depolymize the microtubules. Oryzalin was washed out at a flow rate of ~0.5 mL dH₂O/min.

Microscopy

For the long-term microtubule analysis we used confocal laser scanning microscopy (CLSM). Images and time-lapse movies were produced with a 63x / 1.4 NA oil immersion DIC lens on an Axiovert 200M microscope equipped with a Pascal CLSM unit (Zeiss, Jena, Germany). The GFP was excited with the 488 nm argon laser and a 505 nm long-pass emission filter. To see all microtubules in the cortex, a pinhole of 1.5 to 2 airy disc units (1.0 to 1.4 µm in the Z-axis) was used. The scan time was 4 to 8 µsec/pixel and the temporal resolution was 3 to 5 minutes. Alternatively, time-lapse Z-series of 2.5 µm thickness were made on a Leica DM IRB microscope equipped with the perfect focusing system, a CSU22 spinning disk set up (Yokogawa, Tokyo, Japan) and a C9100 EM-CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). We used a 100x / 1.4 NA objective lens and excited the GFP with a 488 nm argon laser. Five 0.5 µm optical sections, each taking 250 ms, were typically obtained at 3-minute intervals. The visible area with cortical microtubules varied from about 200 to 600 µm².

For the nucleation analysis we used a confocal spinning disk microscope described earlier (Gutierrez et al., 2009), except that a Nikon Eclipse Ti microscope with the perfect focusing system and a 100x 1.45 NA oil objective replaced the Zeiss Axiovert 200. Alternatively, we used a total internal reflection fluorescence (TIRF) microscopy on a Nikon Eclipse Ti microscope with the perfect focusing system. We used a 100x 1.49 NA TIRF oil objective and excited with a solid-state 478nm laser (Cobolt AB) and using a Semrock 535/39 emission filter. The microscope was equipped with a manual Nikon TIRF arm and a QuantEM EM-CCD camera (Photometrics). We used 800 ms exposure time and a 2 or 2.14 s time interval for the spinning disk and TIRF microscope respectively.
**Data analysis**

Time-lapse images were converted into 8-bit tiff file stacks with ImageJ (W. S. Rasband, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007). Z-stacks were converted to average or maximum projections of 3 to 5 sections. The ImageJ StackReg plug-in was used to align the images of a stack (Thevenaz et al., 1998). All visible microtubules in the images were traced using the semi-automatic ImageJ plug-in NeuronJ (v1.01) (Meijering et al., 2004). The microtubule tracings were stored as a series of x and y pixel coordinates with a maximum distance of 5 pixels in both the x and y direction between subsequent points. A Perl script was used to extract the line segments and distribute their lengths over 20 evenly spaced bins according to their angle with the x-axis. The script corrects for the uneven distribution due to discrete pixel values of possible segment angles produced by NeuronJ (see Supplementary Information and Figure S6 for the verification procedure). As we could not distinguish between the plus and minus ends of microtubules, every line segment was assigned an angle in the interval from 0° to 180°. The bins had a width of 9° and were centered on 0°, 9°, etc., up to 171°.

For each image, the microtubule length density was obtained by dividing the total microtubule length by the area of the cortical section in the images. The angular distribution data are presented in contour plots produced with Origin (OriginLab, Northampton, MA, USA) as the fraction of microtubule length at each time point. For clarity, an extra 180° bin is depicted as a copy of the 0° bin in each graph. The angle bins are along the y-axis and time along the x-axis, and a rainbow color gradient indicates the cumulative microtubule length or fraction in 20 equal sized steps, ranging from blue (few microtubules) to red (maximum length or fraction). Plots of mean distributions of several experiments were produced by aligning the timing of individual cells to the moments of zero microtubules after breakdown or before re-polymerization, and averaging the fractions.

To calculate the increase in microtubule density after cytokinesis and oryzalin wash out, and the final plateau value, data from individual experiments were fitted with the linear function: if t > Tp, then density = P1+P2·Tp, else density = P1+P2·t, with Tp as the time to reach the plateau density. The time of emergence of diagonal (45° and 135°) and transverse (90°) microtubule ordering was analyzed by filtering the angle bins with two test functions: T for transverse ordering and D for diagonal ordering.
ordering (see Supplementary Information and Figure S7). Both functions have the property that a randomized system yields a value of zero. A system that is perfectly ordered (in the transverse direction for $T$ and the diagonal direction for $D$) produces a value of 1.

For the nucleation analysis we determined the position in the cell, the time point, the angle with respect to the cell axis, whether the nucleation was free or microtubule bound and the angle of the seed microtubule in case of branching nucleation. For further analysis we only used the microtubule nucleations that were unbound. To assess whether a bias exists for nucleation along diagonal directions, we defined 15° degree bins around the 45°, 135°, 225° and 315° degree directions, and scored microtubules in these bins as being diagonal. We introduced a diagonal biasing parameter $\delta$ by equating the probability of a diagonal nucleation to a non-diagonal nucleation

$$P_{\text{diag}}(\delta) = \frac{1}{6}\delta \frac{\delta}{6}(1-\delta)$$

This parameter is normalized such that $\delta=0$ implies there are no diagonal nucleations, $\delta=1$ implies all nucleations are diagonal, while $\delta=\frac{1}{2}$ is the neutral case in which there is no bias, in which case the proper unbiased weight $\frac{1}{6}=60^\circ/360^\circ$ is accorded to the diagonal bins. We then performed a maximum likelihood estimate of $\delta$ by evaluating the likelihood ratio

$$\frac{L(\delta)}{L(1/6)} = \frac{P_{\text{diag}}(\delta)^M (1-P_{\text{diag}}(\delta))^N}{(1/6)^M (1/6)^{N-M}}$$

where $M$ is the number of diagonal microtubules observed out of a total of $N$. For the nucleations after cytokinesis we have $M=66$ and $N=274$, yielding $\delta=0.61$. For the nucleations after oryzalin washout we have $M=26$ and $N=73$, yielding $\delta=0.73$ (see Figure S8 a and b). Note that a standard one-tailed binomial analysis also rejects the null hypothesis of no bias with $p < 0.001$ for the post-cytokinesis case and $p < 0.0001$ for the oryzalin washout case.

**Simulation Methods**

We performed simulations of interacting cortical microtubules using the event-based algorithm (Tindemans et al., 2010). The simulations are implemented on a cylindrical cell geometry with a length of 80 µm and a radius of 40 µm.
Microtubules that impinge on the edges of the cylinder undergo catastrophes, a boundary condition that was shown to robustly select a transverse orientation of the steady-state array (Allard et al., 2010; Eren et al., 2010).

The kinetic parameters for the dynamics of the microtubule plus-ends are based on Vos et al. (2004): growth speed $v^+ = 0.08 \mu m/s$, shrinkage speed $v^- = 0.16 \mu m/s$, spontaneous catastrophe rate (switch from growing to shrinking state) $r_c = 0.003 /s$ (a value slightly lower than that of Vos et al. 2004, consistent with a likely overestimation of this quantity in that work due to undetected collision-induced catastrophes), and rescue rate (switch from shrinking to growing state) $r_r = 0.007 /s$. The minus-ends of microtubules shrink with a constant treadmilling speed of $v_t = 0.01 \mu m/s$, following Shaw et al. (2003) and identical to Deinum et al. (2011).

The results of angle-dependent collisions between growing microtubules and obstructing ones, follow the simplified scheme also employed by Allard et al. (2010), Eren et al. (2010) and Deinum et al. (2011). All collisions with an incidence angle below 40° result in collision induced bundling, where the incoming microtubule changes direction and continues to grow along the obstructing one. The outcomes of steep angle encounters vary greatly from cell type and stage (Wasteneys and Ambrose, 2009), therefore we measured these outcomes in our 2s dataset after cytokinesis in BY-2 cells. We found that of 70 encounters > 40° in 4 cells, 14 (20%) encounters induced a catastrophe and 56 (80%) resulted in a crossover. Therefore in our simulations collisions with an incidence angle larger than 40° have a 20% probability of undergoing an induced catastrophe, where they switch to a shrinking state, and a 80% probability of simply crossing over the obstructing microtubule.

New microtubules are nucleated at a constant overall rate of $r_n = 0.0002 /s/(\mu m)^2$, which we estimated from our observations of the nucleations after cytokinesis (Deinum et al., 2011). Nucleations occur either at an arbitrary location in the model cortex or from a microtubule. We modeled the portioning of nucleation events between microtubule-free and microtubule-bound by a density-dependent chemical equilibrium, which accounts for the affinity of nucleation complexes for the microtubules. The fraction of microtubule-bound nucleations is given by

$$f_{\text{bound}} = \frac{\rho}{\rho_s + \rho_s}$$

where $\rho$ is the (time-dependent) length density ($\mu m/\mu m^2$) of the microtubules, and the cross-over density $\rho_s = 0.1 \mu m/\mu m^2$, determines the location of the equilibrium,
which we chose in order to match the observed timescale of the crossover towards the final transversely ordered state. The microtubule-bound nucleations have an orientational distribution with respect to the parent microtubule, which is a coarse-grained representation (Deinum et al., 2011) of the experimentally observed patterns (Chan et al., 2009). We have reduced the rate of unbound nucleations by the a factor of 10 to \( r_{n,\text{free}} = 0.00002 \, /s/(\mu m)^2 \), following the data presented by Nakamura et al. (2010) for a steady state microtubule array.

At the start of the simulations we add a finite pool of microtubule fragments with the density of \( d, \ 0.1/\mu m^2 \) and an activation rate of \( r, \ 0.003 /s \). These values were based on the free nucleation rate of BY-2 cells after cytokinesis. Only these reactivating microtubule fragments have a bias towards the diagonal directions of 45° and 135°. This bias was implemented by drawing the direction of nucleation with respect to the parent microtubule from the following distribution

\[
\psi(\theta) = \frac{1}{2\pi I_0(\alpha)} \exp \left\{ \alpha \cos \alpha(\theta - \frac{\pi}{4}) \right\}
\]

where the angle \( \theta \) is expressed in radians, \( \alpha \) is a parameter that sets the degree of bias, and \( I_0 \) is a modified Bessel function of the first kind (see e.g. Abramowitz and Stegun, 1970). We chose \( \alpha = 1.5 \), which reproduces the experimentally determined ratio between the nucleations in 15° bins around the diagonal directions and those in the remaining directions, for the case after cytokinesis. In the control simulations, this pool of microtubule nucleations was not present.

All simulations were started from an initially empty cortex. The time evolution of the angular distributions of microtubules was analyzed using the same filters also used for the experiments (see Supplementary Methods). The microtubule density is reported in terms of an ‘optical density’ in which overlapping microtubules in bundles do not separately contribute to the density, but only the bundle as a whole, mimicking the values measured in the experiments. All simulation results are the average of 500 independent simulations performed with the same parameters. The results were also used for subsequent calculations of \( D \) and \( T \) and Figure 5c.

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Figure legends

**Figure 1** Return of cortical microtubules after cell division in BY-2 cells. (a) Cortical microtubules in two daughter cells after cytokinesis. The first frame (T-10 minutes) shows the late phragmoplast in the cortex. Time is indicated in minutes, scale bar is 10 µm. (b) Microtubule length density increase over time after cytokinesis (mean of 6 cells). The mean density plateaus at ~0.54 µm⁻¹ and is reached after ~46 minutes, based on linear curve fitting of the individual data points of 6 cells (red line). Bars represent standard error (SE). (c) Angular distribution over time presented as the fraction of the total microtubule length at each measurement (mean of 6 cells). The first microtubules are ordered along the diagonal cell axes of 45° and 135°. (d) Diagonal (green circles) and transverse (red squares) microtubule order parameters, $D$ (green error bars) and $T$ (red error bars), after cell division (means of 6 cells ± SE) and the exponential curve fittings (black lines, based on all individual data points). At ~25 minutes after $t_0$ (last measurement before microtubules became visible) the transverse microtubule ordering became dominant over the diagonal microtubule order.

**Figure 2** Treatment of GFP-TUA expressing BY-2 cells with oryzalin. (a) Cortical microtubules before, during and after incubation with 20 µM oryzalin in a flow cell. Oryzalin was added at T-15 minutes and washed out again after 60 minutes by continuous washing with BY-2 culture medium. Scale bar is 10 µm. (b) Microtubule length density in oryzalin treatment over time (mean of 8 cells). The individual cells of the oryzalin treatments were aligned relative to the observation point at which no microtubules were visible after oryzalin addition, and to the point at which no microtubules were yet visible after oryzalin wash out. Both are referred to as $T_0$ in the text and figures. Imaging was continued during depolymerization and all images were checked for microtubules. At -16 ± 2 minutes (mean ± SE), 20 µM oryzalin was added and washed out again after 60 minutes. About 21 ± 6 minutes (mean ± SE) after oryzalin washout, the first cortical microtubules started to reappear. (c) Averaged angular distribution over time presented as the fraction of the total microtubule length at each measurement (mean of 8 cells). Just after addition of oryzalin at the start of recovery after wash out, diagonal microtubules are dominant. (d) Diagonal and transverse cortical microtubule ordering parameters, $D$ (green error
bars) and $T$ (red error bars), over time in oryzalin treatment experiments (means of 8 cells ± SE). Less than 2 minutes after oryzalin addition, $D$ (green circles) became dominant over $T$ (red squares) based on the intercept of the linear curve fittings of the individual data points (black lines). After oryzalin wash out, diagonal ordering became apparent and remained dominant for ~36 minutes, based on the intercept of the exponential curve fittings (black lines), followed by dominance of the transverse microtubule array.

**Figure 3** Free nucleations after cytokinesis and oryzalin wash out. (a and b) Polar histogram nucleation angles within the 30 minute period after the first cortical microtubules appear after cytokinesis in tobacco BY-2 cells (274 nucleations, 6 cells) (a) and oryzalin wash out (117 nucleations in 5 cells) (b). Angles in degrees, histogram scale is in number of observations.

**Figure 4** Oryzalin treatment in Arabidospsis root epidermal cells (a) Example of free nucleations after oryzalin wash out in Arabidopsis root epidermal cells. Dashes indicate microtubule minus-ends, arrowhead indicate microtubule plus-ends. Two out of four nucleations in this image sequence show a GCP2-3xGFP signal. Scale bar is 3 µm. (b) Bar graph of the fraction of nucleations where GCP2-3xGFP signal was detected or not. Results are shown for untreated cells Arabidopsis root cells (70 nucleations) and after oryzalin wash out (81 nucleations). (c) Cortical microtubules before, during and after incubation with 1 µM oryzalin in a flow cell. Oryzalin was added at -5 minutes and washed out again after 60 minutes by continuous washing. Scale bar is 5 µm. (d) Angular distribution over time presented as the fraction of the total microtubule length at each measurement. The orientational bias of the cortical microtubules after break down by orzyalin closely resemble the orientational bias before the oryzalin treatment.

**Figure 5** Results of the simulations. (a) Snapshots of representative microtubule configurations at 15 and 60 minutes after the start of the simulation both with (right) and without (left) persistent fragments. (b) The time evolution of the average diagonal $D$ (green error bars) and transverse $T$ (red error bars) cortical microtubule ordering parameters. The gray lines represent the average value and the error bars represent
one standard deviation. (c and d) Averaged angular distribution over time presented as the fraction of the total (optical) microtubule length from simulations without (c) and with (d) persistent microtubule fragments. (e) Diagonal and transverse cortical microtubule ordering parameters, $D$ (green error bars) and $T$ (red error bars), over time in the simulations. (f) The time evolution of the average (optical) density of microtubules for the simulations with and without persistent fragments, error bars show standard deviation. All simulation results are combined for 500 individual simulations.
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Figure 3

Orientation of microtubule initiation after cytokinesis

(a) Polar histogram nucleation angles within the 30 minute period after the first cortical microtubules appear after cytokinesis in tobacco BY-2 cells (274 nucleations, 6 cells) (a).

Orientation of microtubule initiation after oryzalin wash out

(b) Polar histogram nucleation angles within the 30 minute period after the first cortical microtubules appear after cytokinesis in tobacco BY-2 cells (274 nucleations, 6 cells) (a) and oryzalin wash out (117 nucleations in 5 cells) (b). Angles in degrees, histogram scale is in number of observations.
Figure 4 Oryzalin treatment in *Arabidopsis* root epidermal cells

(a) Example of free nucleation with microtubule ends in Arabidopsis root epidermal cells. Arrowheads indicate microtubule minus-ends, arrowhead indicates microtubule plus-ends. Two out of four nucleations in this image sequence show a GCP2-3xGFP signal. Scale bar is 3 μm. (b) Bar graph of the fraction of nucleations where GCP2-3xGFP signal was detected or not. Results are shown for untreated cells, Arabidopsis root cells (70 nucleations) and after oryzaolin wash out (81 nucleations). (c) Cortical microtubules before, during and after incubation with 1 μM oryzaolin in a flow cell. Oryzaolin was added at -5 minutes and washed out again after 60 minutes by continuous washing. Scale bar is 5 μm. (d) Angular distribution over time presented as the fraction of the total microtubule length at each measurement. The orientational bias of the cortical microtubules after breakdown by oryzalin closely resembles the orientational bias before the oryzalin treatment.
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