SPIRAL2 Determines Plant Microtubule Organization by Modulating Microtubule Severing

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Summary
One of the defining characteristics of plant growth and morphology is the pivotal role of cell expansion. While the mechanical properties of the cell wall determine both the extent and direction of cell expansion, the cortical microtubule array plays a critical role in cell wall organization and, consequently, determining directional (anisotropic) cell expansion [1–6]. The microtubule-severing enzyme katanin is essential for plants to form aligned microtubule arrays [7–10]; however, increasing severing activity alone is not sufficient to drive microtubule alignment [11]. Here, we demonstrate that katanin activity depends upon the behavior of the microtubule-associated protein (MAP) SPR2. Petiole cells in the cotyledon epidermis exhibit well-aligned microtubule arrays, whereas adjacent pavement cells exhibit unaligned arrays, even though SPR2 is found at similar levels in both cell types. In pavement cells, however, SPR2 accumulates at microtubule crossover sites, where it stabilizes these crossovers and prevents severing. In contrast, in the adjacent petiole cells, SPR2 is constantly moving along the microtubules, exposing crossover sites that become substrates for severing. Consequently, our study reveals a novel mechanism whereby microtubule organization is determined by dynamics and localization of a MAP that regulates where and when microtubule severing occurs.

Results and Discussion
SPR2 Promotes the Formation of Unaligned Microtubule Arrays by Inhibiting Katanin Activity
Cotyledon development results in the differentiation of several distinct cell types that make it an excellent system for studying microtubule (MT) organization (see Figure S1A available online). Pavement cells exhibit “net-like” arrays in which MTs exhibit no particular alignment, in contrast to adjacent petiole cells that exhibit anisotropic expansion and have clearly aligned arrays [10]. Several studies on Arabidopsis hypocotyls have demonstrated a role for katanin in releasing newly formed MTs from branch sites following their nucleation on an existing MT [12–15]. Such nucleation events are characterized by branched MTs. In the cotyledon, however, branched MTs indicative of nucleation are very rare, whereas new MTs frequently arise from the severing of existing MTs that occurs almost exclusively at sites where MTs cross one another. This provides a mechanism for removing unaligned MTs and drives MT alignment [10]. Consistent with this mechanism, petiole cells exhibit much higher rates of MT severing than pavement cells [10]. MT severing in plants is catalyzed by the enzyme katanin, which is composed of two subunits. In Arabidopsis, there is only a single gene for the p60 catalytic subunit (AtKatp60). Several AtKatp60 mutant alleles have been independently isolated, including fra2, lue1, erh3, and bott7–7 [7–9, 16]. Katanin mutants fail to form aligned MT arrays (Figure S1B) and exhibit cell expansion that is largely isotropic [7] (Figures S1B–S1D). A number of models have proposed to explain the basis of MT alignment [17–19], but to date, none explain the pivotal role of katanin in this process. Primordia formation in the shoot apical meristem has been shown to depend upon MT rearrangements that result from the perception of mechanical cues [20]. Katanin is essential for this process [21], confirming the idea that katanin is pivotal for most, if not all, MT rearrangements in plant cells. A recent report, based upon overexpression of both ROP6 small GTPase and its effector RIC1, suggests that activation of katanin by GTPase is a mechanism for generating local MT alignment in pavement cells [13]. The authors of that study, however, were unable to measure MT severing activity in pavement cells directly [13]. Furthermore, previous studies using inducible overexpression of katanin in pavement cells have demonstrated that increased MT severing activity resulted in shorter, more bundled MTs but no increase in MT alignment [11]. Consequently, katanin-dependent MT severing is necessary but not sufficient to drive MT alignment. In animal systems, katanin plays pivotal roles in processes such as neuronal growth and meiotic spindle organization [22–25]. In these systems, severing of MTs is frequently regulated by microtubule-associated proteins (MAPs) [22, 25]. To date, however, no such regulators of MT severing in plants have been reported. SPIRAL2 (SPR2) is a plant-specific MT binding protein that contains a series of HEAT repeats but no other homology to proteins of known function [26–28]. It has a complex distribution and localizes both at the growing ends of MTs and along their length (Figure S2A). It has also been reported that SPR2 is enriched at sites of MT crossover [28]. Due to the pivotal importance of MT crossovers as sites for MT severing, these data prompted us to examine more closely the effect of SPR2 on MT organization.

Examination of cortical MTs in pavement cells of spr2-1 clearly revealed well-aligned arrays that resembled the organization present in petiole cells, in contrast to the net-like organization in wild-type pavement cells (Figure 1). Furthermore, the petiole cells of spr2-1 appeared to exhibit “hyperaligned” MTs, even compared to the already well-aligned array in wild-type petiole cells (Figure 1). To determine whether katanin and SPR2 regulate MT organization in the same pathway, we quantified rates of MT severing. Consistent with previous
reports [10], wild-type (WT) pavement cells exhibited low severing rates; however, severing frequency in spr2-1 pavement cells was greatly increased and was comparable to that of petiole cells (Figure 2A; Table S1). Furthermore, the hyperaligned MT organization seen in spr2-1 is dependent upon a functional katanin, since double mutants exhibited solely unaligned arrays in all cell types (Figure 1), similar to those found in katanin single mutants (Figure S1B). We use the bot1-7 alleles to be consistent with previous studies; however, bot1-7 is in the Ws background, whereas spr2-1 is in the Landsberg background. Consequently, we also constructed a double mutant using the lue1 allele of katanin p60 and the spr2-2 allele, which are both from the Columbia background. We found that these plants exhibited patterns of MT organization very similar to bot1-7, spr2-1 (Figures 1 and S1E, S1F).

In this study and in a previous report, we found that nearly all severing events occurred at MT crossovers, and as such, the number of MT crossovers is a reflection of the number of potential substrates for MT severing. Within a single petiole cell, we were able to identify regions of differing MT alignment with consequent variation in crossover density (Figure 2B). When we compared the crossover density with the severing frequency, we found a clear linear relationship (Figure 2C), consistent with MT crossovers being the substrate sites for MT severing. Furthermore, when we compared severing frequency in different cell types and different genotypes, it showed that severing was low in WT pavement cells, even though the number of potential substrates was very high (Figure 2D), and that high rates of severing occurred in spr2 pavement cells, even though the number of crossovers was low in these cells (Figures 2A and 2D).

Together, these data strongly suggest that the increased alignment of the MT array observed in spr2-1 is caused by a specific increase in severing at MT crossovers and reveal that both cell types in wild-type cotyledons possess MT-severing activity, but that in pavement cells, severing is inhibited by the presence of SPR2. The data also provide additional support for a mechanism by which increased katanin-dependent severing at MT crossover sites drives MT alignment [10], whereas in pavement cells, the presence of SPR2 is able to inhibit this katanin-based severing and explains why overexpression of katanin alone does not drive MT alignment in pavement cells [11].

Regulation of Microtubule Severing by Katanin Is Dependent upon SPR2 Dynamics and Localization

To address why severing occurs at much higher rates in wild-type petiole cells as compared to pavement cells, we performed live-cell imaging using a previously described SPR2:GFP fusion [28]. Average intensity of time-lapse series showed that SPR2 accumulated to similar levels in both pavement and petiole cells, but in pavement cells, SPR2 accumulated at sites where MTs cross one another, whereas in petiole cells, the distribution was more even along the MT (Figure S2B). SPR2 has been reported to preferentially bind MT plus ends [28]. We further observed in both pavement and petiole cells that, shortly after the growing MT plus end crossed another MT, it rapidly deposited SPR2 to the newly formed MT crossovers (Figure 3A; Movie S1) and so contributes to a mechanism for distributing SPR2 around the MT array.

It has previously been shown that SPR2 particles move along the MT lattice [29]. In order to understand how the difference in SPR2 distribution between pavement and petiole is
generated, we analyzed the dynamics of SPR2-GFP particles in pavement and petiole cells. To investigate whether there were differences in SPR2 dynamics, we monitored the behavior of individual particles along single MTs in a time-lapse image series using kymographs. A representative kymograph from pavement cells shows a series of straight lines indicating that the SPR2-GFP particles remained stationary during the course of the time-lapse series (100 s; Figure 3B). Conversely, diagonal lines in kymographs from petiole cells indicate that SPR2 particles were constantly moving. SPR2 particles move by alternating pauses and dynamic moments [28] (Figure S2D; Movie S2). To quantify these differences, we measured the movement of SPR2 particles. The mean velocity of SPR2 particles was 10-fold higher in petiole cells (0.24 ± 0.02 versus 2.4 ± 0.06 µm/min). In pavement cells, 64% of the total particles had a velocity lower than 0.25 µm/min, whereas in petiole cells, 61% of the particles had a velocity higher than 2 µm/min (Figure 3C). The distribution of SPR2-GFP particle velocity in petiole cells was significantly higher than in pavement cells (Kolmogorov-Smirnov test; p < 0.001). Altogether, the data suggest that the differences in the ability of SPR2 to prevent severing in different cell types are correlated with large differences in SPR2 dynamics in the two cell types. Yao et al. [28] previously suggested a possible mechanism of SPR2 mobility including binding to growing or shrinking ends of MTs. Our previous studies, however, have shown broadly similar rates of MT growth and shrinkage in petiole and pavement cells [10]. Consequently, the difference in SPR2 mobility is unlikely to be explained by differences in MT dynamics, and given the complex nature of SPR2 movement, it seems likely that a MT-based motor is involved.

To further address the relationship between SPR2 and severing in petiole cells, we used kymograph analysis to monitor the level of SPR2-GFP at MT crossovers in petiole cells, it appeared to be much rarer in pavement cells. To quantify these differences, we measured the movement of SPR2 particles. The mean velocity of SPR2 particles was 10-fold higher in petiole cells (0.24 ± 0.02 versus 2.4 ± 0.06 µm/min). In pavement cells, 64% of the total particles had a velocity lower than 0.25 µm/min, whereas in petiole cells, 61% of the particles had a velocity higher than 2 µm/min (Figure 3C). The distribution of SPR2-GFP particle velocity in petiole cells was significantly higher than in pavement cells (Kolmogorov-Smirnov test; p < 0.001). Altogether, the data suggest that the differences in the ability of SPR2 to prevent severing in different cell types are correlated with large differences in SPR2 dynamics in the two cell types. Yao et al. [28] previously suggested a possible mechanism of SPR2 mobility including binding to growing or shrinking ends of MTs. Our previous studies, however, have shown broadly similar rates of MT growth and shrinkage in petiole and pavement cells [10]. Consequently, the difference in SPR2 mobility is unlikely to be explained by differences in MT dynamics, and given the complex nature of SPR2 movement, it seems likely that a MT-based motor is involved.

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Regulation of Microtubule Severing by SPIRAL2

Figure 3. Variation in SPR2 Dynamics in Different Cell Types
(A) SPR2 is deposited at newly formed MT crossovers. The sequence shown was taken in WT Ler pavement cells. SPR2 is localized at the MT plus end and moves to the crossover as the plus end crosses another MT. Arrowheads point to the MT plus end and the SPR2 particles it deposits at crossovers. Arrow points at a complex MT crossover, which the growing MT will join following a 32.3° reorientation from the initial trajectory (dotted line). Scale bar represents 2 μm.
(B) Kymograph analysis of SPR2 behavior in SPR2-GFP pavement and petiole cells. The top panels show a projection of a 100 s movie sequence. The white line indicates the MT that was selected for the corresponding kymograph analysis shown at the bottom (9.4 μm for petiole cells; 14.4 μm for pavement cells). Arrows indicate particles that are stationary during the course of the movie in pavement cells and mobile particles in petiole cells.
(C) Quantitative analysis of SPR2-GFP particle movement in pavement cells (n = 253 particles; n = 18 cells) and petiole cells (n = 258 particles; n = 16 cells). The average particle velocity is 0.24 μm/min in pavement cells versus 2.4 μm/min in petiole cells.
See also Figure S2 and Movies S1 and S2.

SPR2 Promotes Unaligned Arrays in Pavement Cells by Promoting Formation of Complex Microtubule Crossovers

The relationship between SPR2 and MT crossover points was further exemplified when we examined what we describe as complex crossover sites, where more than two MTs appeared to cross at the same point. In WT pavement cells, there is a linear relationship between the intensity of SPR2-GFP at crossover sites and the number of MTs per crossover (Figure 4A). While monitoring SPR2 dynamics, we frequently observed events in which complex crossovers involving three or more MTs were favored over simple crossovers that only involved two MTs. Examples of this behavior are shown in Figure 4A and Movies S3 and S4, where a growing MT changes trajectory following a simple crossover formation to establish a complex MT crossover. To examine whether SPR2 itself might contribute to the formation and/or stability of complex MT crossovers, we analyzed the number of crossovers in spr2-1 mutants and found significantly fewer MTs per crossover in the mutant as compared to the wild-type (Figure 4B). The opposite is true in katanin mutants, where there is a large shift toward complex MT crossovers (Figure 4B). To ensure that decreases in formation of complex crossovers seen in spr2-1 were not a result of alteration in MT organization, we compared MTs in katanin (bot1-7) mutants with those of bot1-7, spr2-1 double mutants, since both lines exhibit the same distribution of MT orientation (Figures 1 and S1B). Consistent with the idea that mutation in spr2-1 results in fewer complex MT crossovers, the proportion of simple crossovers involving two MTs in bot1-7, spr2-1 double mutants (0.48) was approximately twice that found in bot1-7 single mutants (0.26; Figure 4B), although these differences were not statistically significant. These data support an additional role for SPR2 in promoting the formation of complex MT crossovers in a manner that is independent of its role in MT alignment and support a role of SPR2 in the formation of a robust crosslinked network of MTs in pavement cells.

Conclusion
In neurons, the MT-associated protein Tau protects axon MTs from katanin-mediated severing [22], indicating that a convergent mechanism for regulating katanin has likely evolved in...
both plants and animals. However, the mechanism defined in this study, in which binding and dynamics of a MAP regulate katanin activity, is unique. SPR2 is clearly a multifunctional MAP. In cells that exhibit unaligned arrays, such as pavement cells, it binds to and promotes MT crossover formation and remains relatively stationary at these sites, preventing katanin-based MT severing. Binding of SPR2 to growing ends may also be a means of distributing SPR2 to sites of MT crossover in these cells. In contrast, in cells with well-aligned MT arrays, SPR2 is highly mobile along the MT and consequently exposes MT crossover sites to MT severing. Such a mechanism allows tissue-specific katanin regulation while maintaining the function of SPR2 in promoting uninterrupted MT growth \[28\]. Hence, SPR2 dynamics provides an efficient means of cytoskeletal regulation by directly modulating the accessibility of MT crossovers to katanin.

During plant development, the cytoskeleton is rearranged in response to a number of external cues, including mechanical forces that have recently been shown to be essential signals in the shoot apical meristem \[20\]. How these mechanical forces are perceived and transduced into cytoskeletal rearrangements is unclear, but in common with most, if not all, cytoskeletal rearrangements, it is dependent upon katanin \[21\]. Given that we have demonstrated the pivotal role of SPR2 in regulating katanin-based MT severing, it is likely that interactions between katanin and SPR2 provide the basis for a rapid and flexible system for transducing the signals that drive cytoskeletal rearrangements.

**Supplemental Information**

Supplemental Information includes three figures, one table, Supplemental Experimental Procedures, and four movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.07.061.

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