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# Plant microtubule nucleating apparatus and its potential signaling pathway

Noriyoshi Yagi<sup>1</sup>, Satoshi Fujita<sup>2</sup> and Masayoshi Nakamura<sup>1,3</sup>

Plant cell cortical microtubules are located beneath the plasma membrane and direct the location of cellulose synthases during interphase, influencing cell morphology. Microtubule-associated proteins (MAPs) regulate these microtubules in response to growth and environmental stimuli. This review focuses on recent advances in understanding microtubule nucleation mechanisms in plants and the spatiotemporal regulation of cortical arrays via phytohormone signaling. Emphasis is placed on the conserved nature of the gamma-tubulin ring complex ( $\gamma$ TuRC) and plant-specific components. The discussion includes the role of the Augmin complex and the distinct function of the Msd1-Wdr8 complex in plants. We also explore the effects of hormone signaling, particularly brassinosteroids, on the microtubule regulatory apparatus. The interplay between hormone signaling pathways and microtubule dynamics, including phosphorylation events and post-translational modifications, is also addressed. Finally, the impact of environmental signals and the role of protein post-translational modifications in regulating microtubule organization are suggested for future research.

## Addresses

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## Introduction

Microtubules (MTs) are integral components of the eukaryotic cytoskeleton and carry out various functions, including organelle transport, chromosome segregation, and establishment of cell polarity, among other indispensable roles across numerous biological processes [1–3]. Within interphase plant cells, MTs are located beneath the plasma membrane, where they are involved in guiding cellulose synthase complexes at the cell surface [4,5]. This scaffolding is pivotal for shaping cell morphology during both developmental processes and physiological responses [6,7].

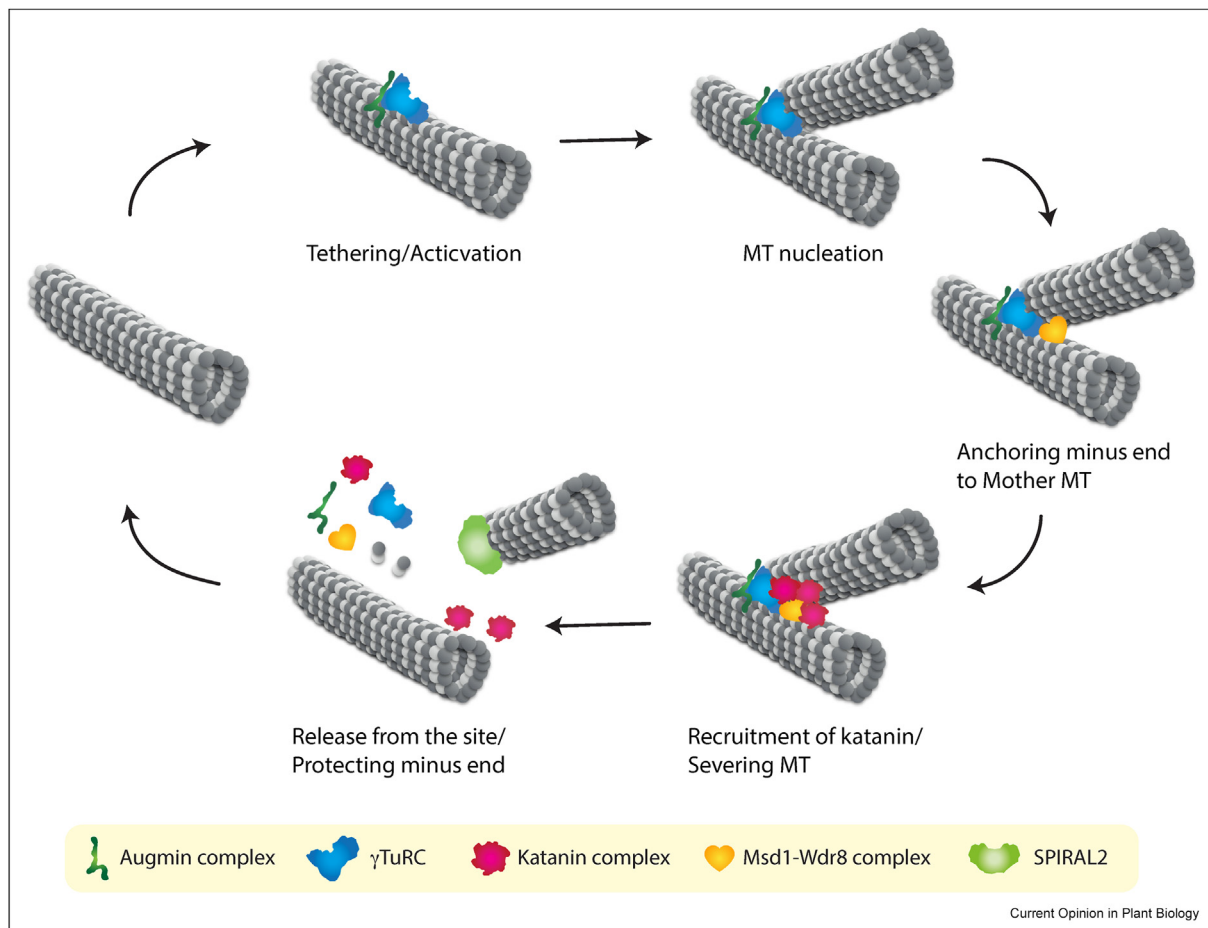
Pavement cells in the leaf epidermis are an example of the regulation of complex cell shapes by the cytoskeleton. In Arabidopsis and many other species, pavement cells exhibit a jigsaw puzzle shape, with lobes and necks that interlock with adjacent cells. As they mature into complex morphologies, MTs are primarily involved in the indentation, while another cytoskeleton, actin, is involved in the lobes [8].

The construction of the cortical MT pattern is organized by microtubule-associated proteins (MAPs), which bind MTs and govern MT nucleation, severing, and dynamics, without any other centralizing structure like a centrosome [9,10]. Despite the ongoing accumulation of knowledge regarding plant MAPs, key questions remain about the function and regulation of cortical MT array organization in plants. This review emphasizes recently elucidated mechanisms specific to plant MT nucleation and how cortical arrays are spatiotemporally regulated through plant hormone signaling. Additionally, we explore potential mechanisms through which signals regulate MTs and MAPs.

## Evolutionarily conserved MT nucleation apparatus and its plant-specific machinery

*De novo* formation of MTs prominently relies upon the gamma-tubulin ring complex ( $\gamma$ TuRC), a structural assembly comprising  $\gamma$ -tubulin and a cohort of gamma-tubulin ring protein (GRIP)-motif-containing proteins (GCPs) [11,12]. As a highly conserved member of the tubulin family,  $\gamma$ -tubulin is indispensable for MT nucleation. During interphase, plant cells lack obvious MT organizing centers (MTOCs), and MTs are

Figure 1



Major steps of cortical microtubule nucleation in plant cells.

In plant interphase cells, both the  $\gamma$ -TuRC (blue) and the augmin complex (green) tether onto the lattice of existing cortical microtubules, initiating a new microtubule branch. After nucleation, the Msd1–Wdr8 complex (yellow) is required to anchor branching microtubules and recruits the katanin complex (red) to release the daughter microtubule. SPIRAL2 plays a protective role at the minus end, which is exposed by severing.

nucleated from pre-existing cortical MTs through  $\gamma$ TuRC recruited to the existing lattice (Figure 1) [13]. Cryo-electron microscopy (cryo-EM) studies in combination with single-particle analysis have elucidated the molecular architecture of the native  $\gamma$ TuRCs, derived from both human and *Xenopus* egg cells [14–16]. The human  $\gamma$ TuRC intermediate, purified from insect cells, has a distinctive six-spoke arrangement of one copy of  $\gamma$ -tubulin small complex unit, a GCP4–GCP5 heterodimer, and a GCP4–GCP6 heterodimer. Notably, the N-terminal segment of GCP6 reinforces the stability of the six-spoke intermediate through the recruitment of Mozart (MZT) 1 to form a luminal bridge with GCP3 [17]. Recently, investigations have demonstrated that  $\gamma$ TuRCs in yeast and animal cells undergo a conformational transition to a closed ring structure, which is the active form capable of nucleating exclusively 13-protofilament MTs [18–21].

Considering the conservation of fundamental  $\gamma$ TuRC proteins in *Arabidopsis* [13,22], the configuration of the *Arabidopsis*  $\gamma$ TuRC may resemble those observed in their human and *Xenopus* counterparts. However, human cells also contain novel factors that influence the geometry and assembly of the  $\gamma$ TuRC, such as actin and RuvB-like protein 1 [17,23], so it is imperative to identify the inherent elements and to determine their structures in plants. Genetic loss of the GCP4 subunit results in a lethal phenotype in *Arabidopsis* as well as in mice, suggesting the pivotal role of  $\gamma$ TuRC in MT initiation across diverse organisms [24,25]. However, a recent investigation has shown that mutants deficient in *Arabidopsis* GCP6 maintain viability despite displaying aberrations in spindle organization, indicating that MT nucleation can occur independently of the canonical  $\gamma$ TuRC but in a  $\gamma$ -tubulin-dependent manner [26].

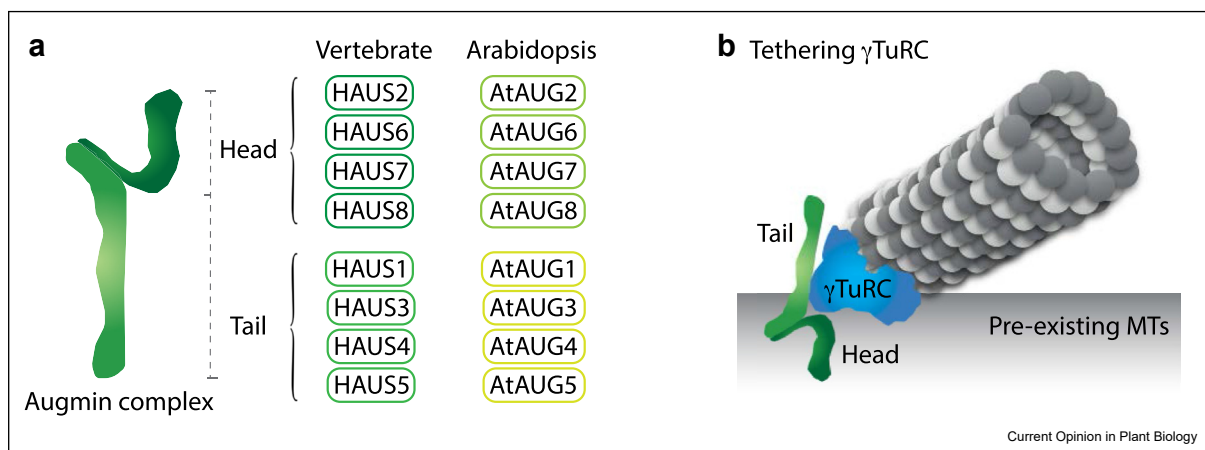
The spatiotemporal dynamics of the  $\gamma$ TuRC, and in particular its recruitment sites, exert a critical influence on the pattern of MT arrays [27,28]. GCPs were also recruited to the plasma membrane independently of MTs, but they were rarely activated and lacked essential cofactors such as GIP1s [13,22]. While several factors have been implicated in the recruitment of  $\gamma$ TuRC at MT nucleation sites, including GCP-WD/NEDD1 and the Augmin complex, consisting of 8 HAUS proteins [29,30], the regulatory mechanism orchestrating their modulation of  $\gamma$ TuRC remains to be elucidated. In human cells, the Augmin complex enhances spindle formation and functionality by recruiting  $\gamma$ TuRC onto pre-existing MTs through its interaction with NEDD1 [31,32]. Recent structural analyses of the vertebrate Augmin octamer complex have unveiled a composite MT-binding site characterized by a positively charged disordered region in HAUS8/Hice1 and two calponin homology domains in HAUS6/FAM29A and HAUS7 exhibiting globular structure in the head region, thereby providing a structural basis for a role of the Augmin complex in nucleation of MT branches (Figure 2) [33–35]. The Arabidopsis Augmin complex comprises the AtAUG1-AtAUG8 proteins, counter parts of the HAUS subunits. Among these subunits, AtAUG8 and AtAUG3 are able to bind to MTs [36,37]. AtAUG6, which is the counterpart of HAUS6, did not show cortical MT localization when solely over-expressed in tobacco epidermal cells [36]. Further investigation is warranted to explore the structures and potential roles of the AUG proteins in cortical array formation.

Recently, the plant-specific protein MACERATOR 4 (MACET4)/CORTICAL MICROTUBULE

DISORDERING 4 (CORD4) was shown to interact with AtAUG7 *in vitro* and to tether AtAUG7 to the phragmoplast during anaphase [38], suggesting that plants have evolved a unique Augmin complex recruitment system that directs proper MT structure formation through branching MT nucleation. However, it is noteworthy that the localization of  $\gamma$ -tubulin in the phragmoplast was not significantly altered between wild-type and the *cord4 cord5* mutants [39]. The functional relevance of the  $\gamma$ -tubulin and the Augmin complexes in the phragmoplast requires further investigation. The phenotype of *cord4 cord5* phragmoplasts resembles that of plants that have lost an MT severing protein, indicating that CORD4 functions in the proper recruitment of katanin to the phragmoplasts as well as tethering of AtAUG7.

The MT nucleation apparatus characteristic of plants begins with the initiation of nascent MT from  $\gamma$ TuRC at the MT lattice and is completed when the MT is severed from the nucleation site by the katanin complex [7,13,40]. The precise localization of this distinctive MT severing event is determined by a complex of two proteins, mitotic spindle disanchored 1 (Msd1) and a WD40 repeat-containing protein (Wdr8). The Msd1-Wdr8 complex was initially identified as the complex that anchors  $\gamma$ TuRC to the MTOC during mitosis in fungi and vertebrates [41–43], but this complex also controls the recruitment of katanin to the MT nucleation site in plants [44]. It is noteworthy that the Msd1-Wdr8 complex does not participate in the recruitment process to the MT crossover, another cleavage target site for katanin in plant cells [44]. In interphase cells of Arabidopsis, which lack the MTOC, the Msd1-Wdr8 complex plays a pivotal role in tethering the minus end

Figure 2



Augmin complex in microtubule branching nucleation.

(a). Molecular components of the augmin complex as described by Travis *et al.*, 2023, along with a list of vertebrate and Arabidopsis components in the augmin complex. Complex purification from plant cells show all components of AtAUGs are conserved [36]. (b). A diagram depicting augmin and  $\gamma$ TuRC binding to pre-existing microtubules to initiate daughter microtubules.

of the newly generated daughter MT at the precise site of MT nucleation. This stabilized branched structure provides a recruitment locus for katanin, facilitating subsequent cleavage and release of the daughter MT. A comprehensive understanding of the plant-specific function of the evolutionarily conserved Msd1-Wdr8 complex, such as the recruitment of katanin, will provide valuable insights into the distinct mechanisms underlying MT nucleation in plants.

In fission yeast, it is postulated that the recruitment of the Msd1-Wdr8 complex to MTOCs is accomplished through the interaction with the kinesin Pkl1 [43]. In yeast and vertebrates, MTs are anchored to MTOCs such as spindle pole bodies and centrosomes, whereas in plants, MTs need to be anchored to pre-existing MTs rather than MTOCs. There is likely a plant-specific molecular mechanism, although at this point, the mechanism through which the plant Msd1-Wdr8 complex is recruited to the MT nucleation site remains to be elucidated. The minus end of the MT, released by the katanin activity, is protected from rapid depolymerization through the intervention of the plant MAP SPIRAL2 (SPR2) [45,46]. The N-terminus of SPR2 resembles the tumor overexpressed gene (TOG) domain, which is known for its MT-binding affinity, while the C-terminus is structurally homologous to the C-terminus of the katanin p80, as elucidated by structural analysis [47–49]. It is not yet known how these domains contribute to the localization of SPR2 at the MT minus end. Considering the similarities in structure, elucidating the interaction dynamics between SPR2 and katanin is poised to be fascinating. In addition, the processes responsible for the rapid depolymerization of the minus end in the absence of SPR2 and the intricate construction of the minus end in plants still need to be investigated.

### Hormone signaling to MT regulatory apparatus

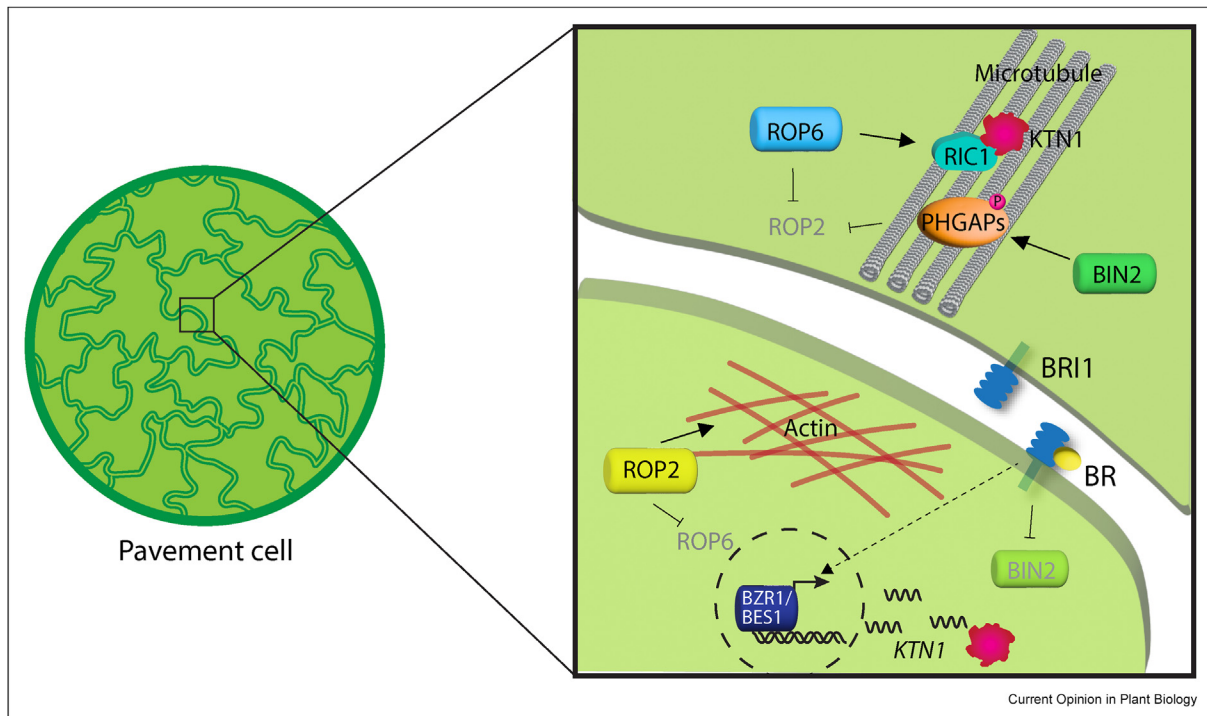
To understand how MT orientation and reorganization are managed, the spatiotemporal mechanisms governing the apparatus responsible for constructing cortical array patterns must be defined, including both nucleating and severing complexes [50]. However, the mechanisms underlying the location, frequency, and angle of MT nucleation as well as the frequency of MT severing are major enigmas yet to be understood. MT severing is executed by katanin, a heterohexamer containing a p60 catalytic subunit (KTN1) and a p80 regulatory subunit, with the spatial determinants of severing controlled by the p80 subunit [51,52]. Notably, recent findings highlight the regulatory impact of brassinosteroid (BR) signaling on the expression levels of the p80 subunit, influencing reproductive organ morphology. BRASSINAZOLE RESISTANT1 (BZR1) and BRI EMS SUPPRESSOR1 (BES1), both central transcription factors in BR signaling, directly bind to the p80 promoter region.

This coordinated interaction sustains the intracellular abundance of the MT severing machinery, contributing to the establishment of the orientation of the cortical MT arrays [53].

The BZR1 and BES1 transcription factors are integral to the BR–BR INSENSITIVE1 (BRI1)–BR INSENSITIVE2 (BIN2) signaling pathway and are subject to regulation through the phosphorylation signaling cascade [54,55]. The BR-BRI1-BIN2 pathway directs characteristic MT organization in leaf epidermal pavement cells by modulating signaling of the small GTPase Rho-of-plants (ROP) in Arabidopsis. Recent reports demonstrated that two ROP GTPase-activating proteins (ROPGAP) with Pleckstrin Homology (PH) domain, PHGAP1/2, locally downregulate ROP2, altering pavement cell shapes, and that their activities were regulated by BR signaling [56,57]. In the absence of BRs, the kinase BIN2 phosphorylates and activates PHGAPs, stabilizing PHGAP localization at indented regions of the cell in a cortical MT-dependent manner. Consequently, the PHGAPs inactivate ROP2, and the ROP6–RIC1–KTN1 module activates around the indentation regions, leading to well-ordered MT alignment [56–58]. Conversely, when BR signaling is activated, BIN2 becomes inactive, releasing ROP2 from PHGAP inhibition [57]. As mentioned above, BRs also positively regulate the katanin p80 subunit expression. Hence, it is reasonable to assume that BRs promote the jigsaw puzzle shape of pavement cells through controlling the abundance of the katanin complex along with regulating ROP signaling (Figure 3) [53,59]. Although their significance is still unclear, some reports pointed out that BRs or their signaling components induce post-translational modification on tubulins or MT nucleating complex components. For example, *in vitro* experiments demonstrated that the BIN2 kinase phosphorylated both  $\alpha$ -tubulin 6 and  $\beta$ -tubulin 4 [59]. A recent proximal labeling analysis using the turboID technique and *in vitro* experiments revealed that the  $\gamma$ -tubulin complex component GCP-WD/NEDD1 is phosphorylated through the BIN2 signaling network [60]. Beyond the regulation of MAPs, BIN2 negatively regulates cellulose synthesis, which may also influence the MT network [61]. These facts could open a new avenue connecting phytohormone signaling and MT regulation by post-translational modifications.

Other plant hormones seem to also have specific roles in regulating MT formation. In the context of stomatal closure, ABA signaling induces the phosphorylation of Ser6 in the MAP SPIRAL 1 (SPR1) by OPEN STOMATA 1/SNF1-related protein kinase 2.6 (OST1/SnRK2.6). This phosphorylation enhances the affinity of SPR1 to tubulin heterodimers, thereby inhibiting MT polymerization [62]. Similarly, in root halotropism,

Figure 3



#### Brassinosteroid signaling influences the arrangement of the cytoskeletal network in pavement cells.

Microtubule-actin network organization is largely dependent on ROP signaling modules in pavement cells. Pavement cells have an irregular shape, with neighbor cells sharing a lobe and indentation across the cell walls. PHGAPs downregulate ROP2 activity and are primarily located on cortical microtubules, specifically in indentation regions (upper right cell) along the cell periphery. Phosphorylation of PHGAPs by BIN2 leads to ROP2 downregulation around indentations, activating the ROP6-RIC1-KTN1 module. In contrast, around lobe areas (lower left cell), where BR signaling is activated, BIN2 is inactive, which releases ROP2 from inhibition by PHGAPs, promoting actin network formation. BR signaling is also critical to sustain BES1/BZR1-dependent katanin expression, impacting microtubule alignment at indentation sites.

phosphorylation of Ser406 in SP2L by the ABA-SnRK2s signal cascade is essential for evading detrimental salinity conditions [63].

#### Outlooks

The investigation of the  $\gamma$ -TuRC in animal and yeast cells is more advanced than in plants. Centrosomin motif 1 (CM1), found in CDK5RAP2 and SPC110, is crucial for the structural rearrangement and recruitment of the  $\gamma$ -TuRC [64]. However, in plants, CM1 is absent at the primary amino acid sequence level. Thus, the isolation of novel related factors through complex purification by mass spectrometry becomes increasingly valuable, as functional similarities, such as GCP8/MZT2 [65], may be identified. Despite the relatively low sequence identity and similarity of AUGMIN components in some HAUS subunits across species, AlphaFold2 predicts similar structures for the H1/4 and H3/5 dimers and for the head and tail domains [33]. Comprehensive structural analyses are required. Further elucidation of plant-specific molecular mechanisms,

such as katanin recruitment and the anchoring mechanism of the Msd1-Wdr8 complex to MTs, is necessary.

As discussed above, plant hormones alter MT formation. While the signaling mechanisms mediated by BRs are relatively well-understood, other signaling pathways, such as ABA signaling and response to salt stress, also clearly alter MTs [62,63,66]. These instances exemplify how kinase-mediated phosphorylation of MAPs functions as a sensor of environmental changes that is perceived by the MT machinery. Recent proteomic analyses have revealed that many plant MAPs are O-GlcNAcylated [67,68]. Although the physiological significance of O-GlcNAcylation in plants remains to be fully elucidated, glycosylation of proteins involved in MT nucleation and severing presents a promising avenue for future research. Additionally, studies into the regulation of MT dynamics through post-translational modification of tubulins, known as the tubulin code, are expected to yield critical insight [69]. Investigating how these post-translational modifications are perceived in the environment and

how they influence the organization of cortical MT patterns will be of significant interest in future studies. Exploring the complex spatiotemporal interactions between MAPs and signaling pathways, such as hormones, has great potential for advancing our understanding of cell morphology and growth regulation, ultimately leading to enhanced plant resilience.

### Author contributions

All authors participated in writing the text and preparing the figures.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Data availability

No data was used for the research described in the article.

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- \*\* of outstanding interest

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