Facilitating cytokinesis by cross-linking antiparallel microtubules in the phragmoplast.

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Plant cytokinesis is brought about by the phragmoplast, which contains an antiparallel microtubule (MT) array. The MT-associated protein MAP65-3 acts as an MT-bundling factor that specifically cross-links antiparallel MTs near their plus ends. MAP65 family proteins contain an N-terminal dimerization domain and C-terminal MT interaction domain. Compared with other MAP65 isoforms, MAP65-3 contains an extended C terminus. A MT binding site was discovered in the region between amino acids 496 and 588 and found to be essential for the organization of phragmoplast MTs. The frequent cytokinetic failure caused by loss of MAP65-3 was not rescued by ectopic expression of MAP65-1 under the control of the MAP65-3 promoter, indicating nonoverlapping functions between the two isoforms. In the presence of MAP65-3, however, ectopic MAP65-1 appeared in the phragmoplast midline. We show that MAP65-1 could acquire the function of MAP65-3 when the C terminus of MAP65-3, which contains the MT binding site, was grafted to it. Our results also show that MAP65-1 and MAP65-3 may share redundant functions in MT stabilization. Such a stabilization effect was likely brought about by MT binding and bundling. We conclude that MAP65-3 contains a distinct C-terminal MT binding site with a specific role in cross-linking antiparallel MTs toward their plus ends in the phragmoplast.

INTRODUCTION

In plants, cytokinesis is executed by the phragmoplast, which contains a framework of two opposing sets of microtubules (MTs) with their plus ends facing each other at or near the cell division site (Staehelin and Hepler, 1996). Golgi-derived vesicles are transported by motors along MTs toward the plus end in order for the cell plate materials they carry to be deposited in the phragmoplast midzone (Jürgens, 2005). The phragmoplast MT array, which is derived from the spindle midzone MTs during late anaphase and telophase, exhibits high dynamicity (Zhang et al., 1990). Concomitant with cell plate formation, MT polymerization takes place at the phragmoplast periphery, while depolymerization happens in the central region where vesicle transport and fusion have already been completed. In other words, progressive MT turnover is accompanied by the expansion of the phragmoplast and cell plate toward the parental cell wall.

Phragmoplast MTs exhibit a high degree of dynamic instability that is required for the expansion of the array (Lloyd, 2011; Smertenko et al., 2011). The organization of the phragmoplast MT array is dependent on factors that regulate specific activities, such as MT nucleation, polymerization, and bundling. For example, mutations in genes encoding β-tubulin and augmin cause serious defects in MT nucleation and consequently result in collapse of the array in Arabidopsis thaliana (Pastuglia et al., 2006; Zeng et al., 2009; Ho et al., 2011b). Mutations in a gene encoding microtubule organization1/gemini pollen1 of the Xenopus laevis MT-associated protein215/defective in sister chromatid separation1 family cause fragmentation and branching of the phragmoplast MT array, suggesting that its role in promoting MT polymerization is critical for the dynamics of the array (Eleftheriou et al., 2005; Kawamura et al., 2006; Oh et al., 2010). The loss of the MT plus end tracking protein end binding1c also causes a similar phenotype upon challenge with low doses of the herbicide oryzalin (Liu et al., 2011). In addition, MT bundling activities conferred by the MT-associated proteins (MAPs) in the MAP65/anaphase spindle elongation1 (Ase1)/protein regulating cytokinesis1 (PRC1) family are also critical for the organization of the bipolar MT array (Liu et al., 2011). Besides the aforementioned proteins, the Kinesin-12 MT motors play critical roles in maintaining the MT plus ends in the phragmoplast midzone, likely by pushing newly polymerized MT segments apart (Lee et al., 2007).

It is believed that concerted roles of these regulatory proteins allow the phragmoplast MT array to sustain its bipolar configuration while expanding toward the cell periphery (Liu et al., 2011). However, it is intriguing how the two sets of antiparallel MTs are engaged dynamically through the rapid progression of cytokinesis. Electron microscopy tomography indicates that the majority of the antiparallel MTs do not interdigitate in the phragmoplast midzone (Austin et al., 2005). This study led to the hypothesis that MTs are anchored and stabilized by the
amorphous cell plate assembly matrix in the phragmoplast. However, this hypothesis contradicts observations that a number of proteins, including MAP65 members and Kinesin-12, conspicuously appear in the midzone in the phragmoplast (Lee and Liu, 2000; Smertenko et al., 2000, 2008; Pan et al., 2004). Among them, MAP65-3 plays a critical role in engaging the antiparallel MTs because mutations in the corresponding gene lead to a wider midzone and consequently result in frequent cytokinesis failures (Müller et al., 2004; Caillaud et al., 2008; Ho et al., 2011a).

Following the initial discovery of the −65-kD MAP65 protein in the tobacco (Nicotiana tabacum) (Chang-Jie and Sonobe, 1993), nine homologous proteins were predicted to be encoded by the Arabidopsis genome and shared 28 to 79% amino acid identity with the tobacco protein (Hussey et al., 2002). Proteins in this family form dimers that cross-link MTs (Smertenko et al., 2004; Li et al., 2007; Gaillard et al., 2008; Kapitein et al., 2008). Besides MAP65-3, other MAP65 isoforms, such as MAP65-1 and MAP65-5, also decorate the phragmoplast midzone as visualized by immunostaining using antibodies raised against antigens derived from the corresponding proteins (Smertenko et al., 2008). However, unlike MAP65-3, which is restricted in the midline where it is concentrated with MT plus ends, MAP65-1 shows a broader localization pattern along MTs in the phragmoplast midzone (Smertenko et al., 2008). On the other hand, a MAP65-4-green fluorescent protein (GFP) fusion associates with kinetochore fibers in mitotic spindles but not the phragmoplast midzone, despite the fact that the isoform is more closely related to MAP65-3 than to MAP65-1 or MAP65-5 (Hussey et al., 2002; Guo et al., 2009; Fache et al., 2010). Therefore, it remains unclear how the interaction between MAP65 isoforms and MTs of different arrays is established. Different patterns of MT association suggest Arabidopsis MAP65 isoforms may assume divergent functions. However, a recent study revealed possible functional redundancy among MAP65-1, MAP65-2, and MAP65-3 in Arabidopsis, and knocking out the genes encoding MAP65-1 or MAP65-2 did not seem to cause obvious cell division defects (Lucas et al., 2011; Sasabe et al., 2011).

Because the loss of MAP65-3 alone causes severe defects in cytokinesis, we were prompted to further analyze the structural feature that enables the protein to play its critical role in the phragmoplast. Sequence alignment of Arabidopsis MAP65 family proteins shows relatively conserved N-terminal halves and variable C-terminal halves (Smertenko et al., 2008). The N-terminal part is required for protein dimerization (Smertenko et al., 2004; Kapitein et al., 2008). When the C-terminal halves of MAP65 proteins were further divided into the MTB1 (for MT binding domain 1) and MTB2 domains, the C-terminal-most MTB2 domains exhibited the most divergence (Smertenko et al., 2008). For simplicity, MTB1 and MTB2 domains of the C terminus are referred in this article as C1 and C2, respectively. Biochemical studies showed that the C1 domains of MAP65-1, -4, and -5 possess MT binding activity (Smertenko et al., 2004; Gaillard et al., 2008; Fache et al., 2010). A recent structural analysis of the human counterpart of MAP65, PRC1, also showed that its most C-terminal region facilitated MT binding (Subramanian et al., 2010). Compared with other isoforms, MAP65-3 contains an extended C2 domain and is responsible for the slow turnover rate (half-time = 143 s) of the protein in the phragmoplast (Smertenko et al., 2008). However, it is unclear whether this domain is responsible for the distinct localization pattern of the protein and critical for its role in cytokinesis.

To examine whether the divergent C2 domain of MAP65-3 might contribute to its unique role, we expressed full-length and several truncated MAP65-3 proteins in the dyc283 map65-3 null mutant background. When compared with a functional full-length MAP65-3 fusion protein, the deletion of the C2 domain abolished the localization and function of MAP65-3. MAP65-1 was also tested as a substitute for the role of MAP65-3 in cytokinesis. Only when the C2 domain of MAP65-3 was fused to MAP65-1 did the fusion protein decorate the phragmoplast midline and largely restore successful cytokinesis. We report here that the C2 domain of MAP65-3 contains a MT binding site that enables specific activity in the phragmoplast midzone.

RESULTS

The Function of MAP65-3 Is Dependent on Its C-Terminal Domain

Among members of the MAP65 family, the C-terminal halves are highly variable compared with the more or less conserved N-terminal halves (Smertenko et al., 2008). As reported by others (Müller et al., 2004; Caillaud et al., 2008), the loss of MAP65-3 caused obvious defects in cell division and seedling growth that can be reflected by dwarf shoots and shortened roots. The defects were completely suppressed by the expression of full-length MAP65-3 fused with the FLAG epitope and expressed under the control of its native promoter (Figures 1A to 1D). Because the C2 domain in the C-terminal half has a 119-amino acid region that is not present in MAP65-1, we asked whether the extended sequence was responsible for the functional difference between MAP65-3 and MAP65-1. MAP65-1−588, resulting from the deletion of this 119-amino acid region, was expressed in fusion with FLAG under the control of the MAP65-3 promoter. The fusion protein almost completely rescued the defects in root growth at the seedling stage and shoot growth at the mature stage caused by the dyc283 mutation (Figures 1A to 1D). The deletion of the entire C2 domain, however, failed to rescue the dyc283 mutation, indicating that this MAP65-3−4495-FLAG fusion protein did not retain the function of MAP65-3 (Figures 1A to 1D).

A published study showed that MAP65-1 appeared in the phragmoplast midzone in cultured Arabidopsis cells (Smertenko et al., 2008). A more recent genetic study suggested that MAP65-1 and MAP65-3 exhibited functional redundancy (Sasabe et al., 2011). Therefore, we tested whether expression of MAP65-1 could substitute MAP65-3 in performing its antiparallel MT binding function. Overall growth defects caused by the dyc283 mutation were partially suppressed, albeit not to a high degree, when MAP65-1 was expressed under the control of the MAP65-3 promoter (Figures 1A to 1D). Because the C2 domain is required for the function of MAP65-3, we swapped the C-terminal fragment of MAP65-1 for the C2 domain of MAP65-3 and expressed a FLAG-fusion of this chimeric protein under the control of the MAP65-3 promoter. This MAP65-1−20850 fusion protein was able to significantly rescue, although incompletely, the growth defects of the dyc283 mutant (Figures 1A to 1D). Collectively,
the dyc283 seedlings formed stunted roots. While MAP65-31-495 could not enhance root growth, MAP65-1 slightly restored root length to 25.0% of the wild-type control from 14.8% in dyc283 mutant (Figures 1B and 1C). MAP65-1SWAP restored the root growth to 44.8% and MAP65-31-588 restored it to 66.6% (Figures 1B and 1C). Consistent with the root growth phenotypes, mature plants also showed corresponding suppression effects by expressing these versions of proteins. While MAP65-31-495 and MAP65-1 hardly made any difference when expressed in the dyc283 mutant, MAP65-1SWAP and MAP65-31-588 enabled the production of mature plants that were significantly healthier than dyc283. These results suggest that the C2 domain is critical for the function of MAP65-3. This domain could enable MAP65-1 to acquire the function of MAP65-3 in the phragmoplast.

Remediation of Defects in Phragmoplast MT Organization and Cytokinesis by MAP65 Proteins and Derivatives

Published studies showed that the loss of MAP65-3 led to a disorganized MT array in the phragmoplast that showed a wider gap in the phragmoplast midzone when compared with wild-type cells (Müller et al., 2004; Ho et al., 2011a). When phragmoplast MTs were revealed by immunofluorescence, clear differences were detected in the dyc283 mutant cells compared with the wild-type control (Figure 2A, panels a and b). While MT bundles are tightly packed and organized in the wild-type phragmoplasts, the mutant phragmoplasts possessed bundles that were loosely packaged and disorganized. Besides the wide gap in the phragmoplast midzone, the overall length of the phragmoplast MT array increased to 4.66 µm in dyc283 cells (n = 26) compared with 2.52 µm in wild-type cells (n = 23) (Figure 2B). The width of the antitubulin dark gap (right bracket, Figure 2A, panel b) increased to 1.15 µm from 0.42 µm (Figure 2C). In addition, eminent MTs emanated from the surface of the reforming daughter nuclei (arrow, Figure 2A, panel b). Both the full-length MAP65-3 and truncated MAP65-31-588 fusions restored phragmoplast MT arrays to the normal organization patterns seen in the control cells (Figure 2A), and their phragmoplast length and gap were comparable to those of the wild-type control cells (Figures 2B and 2C). However, the expression of MAP65-31-495 or MAP65-1 did not significantly alter the loose and disorganized phragmoplast MTs when compared with the dyc283 root cells (Figure 2A). The widths of the dark gaps were similar to those in dyc283 cells (Figure 2C). However, the length of the phragmoplast MT array was shorter in cells expressing MAP65-31-495 than in dyc283 cells (Figure 2B). The expression of either MAP65-31-495 or MAP65-1 also did not alter the appearance of abundant MTs

Figure 1. Suppression of dyc283 by MAP65 Variants.

(A) Bar graphs represent MAP65-3, MAP65-1, and their derivatives. Under the control of the endogenous MAP65-3 promoter, they were expressed in fusions with the FLAG tag at the C terminus in the dyc283 mutant background. The numbers shown are the amino acid positions in the polypeptides. The C-terminal half is divided into the C1 and C2 domains.

(B) Growth phenotypes of 5-d-old seedlings of the control wild-type (WT), dyc283, and transformants expressing the designated FLAG fusion proteins. Note the difference in root lengths. Bar = 1 cm.

(C) Quantitative analysis of root lengths of 5-d-old seedlings of the control and transformants (n = 57, 50, 72, 66, 77, and 51 for wild-type, dyc283, and mutant plants expressing MAP65-3, MAP65-31-588, MAP65-31-495, MAP65-1, and MAP65-3swap, respectively). Error bars represent SD.

(D) Growth phenotypes of mature plants of wild-type, dyc283, and mutant plants expressing MAP65-3, MAP65-31-588, MAP65-31-495, MAP65-1, and MAP65-3swap. Note that MAP65-1SWAP suppresses the dyc283 mutation.
Figure 2. Assays of Phragmoplast MT Organization and Cytokinesis in Root Cells in the Wild Type, dyc283, and Various MAP65 Transformants.

(A) The C2 domain of MAP65-3 is important for the integrity of the phragmoplast MT array. At similar stages of cytokinesis, cells of the wild type (WT) (a), dyc283 (b), and dyc283 plants expressing MAP65-3 (c), MAP65-31-588 (d), MAP65-31-495 (e), MAP65-1 (f), and MAP65-1swap (g) exhibit different patterns of the phragmoplast array. In (b), the bracket on the left represents the phragmoplast length for the measurement presented in (B) and that on the right for the gap width for the measurement presented in (C). Eminent MTs radiating from the two reforming daughter nuclei (arrows in [b], [e], and [f]) are observed in cells of dyc283 plants and dyc283 plants expressing MAP65-31-495 and MAP65-1 proteins.

(B) Quantitative assessment of the phragmoplast length in the cells of the aforementioned lines. The phragmoplasts in MAP65-31-495 (n = 29) and MAP65-1swap (n = 30) are significantly reduced compared with that in the dyc283 mutant. Error bars represent s.d.

(C) Comparison of the width of the phragmoplast midline in wild-type (n = 23), dyc283 (n = 26), and dyc283 plants expressing MAP65-3 (n = 34), MAP65-31-588 (n = 29), MAP65-31-495 (n = 29), MAP65-1 (n = 22), and MAP65-1swap (n = 30). The midline is much narrower in cells expressing MAP65-1swap than in those of the dyc283 mutant expressing MAP65-1. Error bars represent s.d.

(D) The root cell profiles of 5-d-old seedlings of wild-type (n = 34), dyc283 (n = 20), and dyc283 plants expressing MAP65-3 (n = 36), MAP65-31-588 (n = 35), MAP65-31-495 (n = 21), MAP65-1 (n = 28), and MAP65-1swap (n = 25). Cell wall stubs can be observed in dyc283 and dyc283 cells expressing MAP65-31-495, MAP65-1, and MAP65-1swap (asterisks) but not those expressing MAP65-3 or MAP65-31-588.
radiating from the surface of the nuclear envelope (arrows, Figure 2A, panels e and f). The expression of MAP65-1SWAP-FLAG further reduced the phragmoplast length and caused the dark gap in the phragmoplast midzone to be significantly reduced but not to the level seen in wild-type cells (Figures 2B and 2C). These data suggest that redirection of MAP65-1 by the addition of the C2 domain of MAP65-3 significantly restored MT organization in the phragmoplast.

Furthermore, we investigated how the aforementioned changes in phragmoplast MT organization might correlate to how well cytokinesis was brought about. The wild-type roots contained organized cell profiles, as indicated by intact cell walls separating individual cells (Figure 2D). In the roots of the dyc283 mutant, however, cell wall stubs were frequently found, indicating failures in cytokinesis (asterisk, Figure 2D). In fact, 38.1% of the dyc283 root cells contained cell wall stubs (Figure 2E). We found that the seed germination rate dropped to 66.8% (n = 244) in the dyc283 mutant compared with 99.1% (n = 222) in the wild-type control. Frequent cytokinetic failures might have caused such defects. The expression of either MAP65-3 or MAP651-588 resulted in root cell profiles similar to those in wild-type roots and hardly had any cells show cytokinesis defects (Figures 2D and 2E). Surprisingly, although MAP65-31-495 and MAP65-1 did not appear at the phragmoplast midzone, expression of both of these proteins significantly suppressed the cytokinetic defect caused by the dyc283 mutation (Figures 2D and 2E). MAP65-1 seemed to exert a greater rescuing effect than MAP65-31-495 (Figure 2E). The expression of MAP65-1SWAP even restored cytokinesis to a greater extent, with <5% of root cells showing cell wall stubs (Figures 2D and 2E). These results suggest that the function of MAP65-3 at the phragmoplast midzone is essential for MT arrangement but that of MAP65-1 elsewhere also contributes to MT organization in the phragmoplast, although to a lesser extent.

Localization of MAP65-3-FLAG and Its Derivatives in the Phragmoplast

We first examined the localization of the MAP65-3-FLAG fusion protein when expressed in dyc283 mutant cells. Consistent with the complete rescue of the mutant, the localization of full-length MAP65-3 fusion protein recapitulated that of the native protein (Ho et al., 2011a). In cells bearing an early phragmoplast, the fusion protein appeared intensively in the phragmoplast midline, where the antitubulin signal was seen weakly as a dark line in fluorescent images (Figure 3A, panels a to c). This localization pattern persisted throughout cytokinesis (Figure 3A, panels d to f). We then examined the localization of the MAP65-31-588-FLAG fusion in cells undergoing cytokinesis. In a cell at the early stage of cytokinesis, the fusion protein was detected in the midline of the phragmoplast, highlighting the dark line of the antitubulin immunofluorescence (Figure 3B, a to c) and similar to what was observed for the full-length fusion. Compared with the full-length protein, the truncated protein showed an extended localization pattern, decorating MTs beyond the phragmoplast midline (arrows, Figure 3B, panel d), suggesting that it was probably associated with parallel MTs as well. Unlike the full-length and MAP651-588 fusions, the complete removal of the C2 domain resulted in the MAP65-31-495-FLAG fusion protein no longer being concentrated at the phragmoplast midline (Figure 3C, panels a to f). Instead, the anti-FLAG signal became dispersed across the cytoplasm at both the early (Figure 3C, panels a to c) and late (Figure 3C, panels d to f) stages of cytokinesis. The phragmoplast MT array consisted of two fan-shaped halves (Figure 3C, panel b). The abnormal MT organization pattern in mutant plants expressing MAP65-31-495-FLAG (Figure 1B) was consistent with the result that expression of this truncated protein did not rescue the dyc283 mutant. Thus, the results suggest that the localization of MAP65-3 at the phragmoplast midline is essential for its function. Upon the loss of its localization by the deletion of the C2 domain, its function in the phragmoplast is abolished.

Because MAP65-3 is required for the localization of Kinesin-12 (Ho et al., 2011a), we examined whether the new localization pattern of MAP651-588 would change the localization of the motor. We also wondered whether the extending MAP651-588 localization zone represented a broadened overlapping region of antiparallel MTs in the phragmoplast. Unlike MAP65-31-495-FLAG, which showed a wide localization pattern, especially toward the phragmoplast periphery (arrows, Figures 4A and 4D), Kinesin-12 remained at the phragmoplast midline (Figures 4B and 4E). Moreover, MAP651-588, but not Kinesin-12, appeared conspicuously at the expanding front of the phragmoplast array. This result suggests that MAP65-31-495-FLAG retained the native protein's function in allowing proteins like Kinesin-12 to specifically interact with MT plus ends in the phragmoplast midline, albeit losing its highly restricted localization in the phragmoplast midline. Again, this is consistent with the complementation of the dyc283 mutation by expressing this truncated version of MAP65-3. The result also indicated that the region of amino acids 589 to 707 is required for MAP65-3 to define its localization specifically at or near MT plus ends.

Figure 2. (continued).

(E) Quantitative analysis of cells with cell wall stubs caused by defective cytokinesis. Compared with the dyc283 mutant, which has the most severe cytokinesis defects, the transformant expressing MAP65-31-495 showed significant improvement. Greater improvement can be seen with MAP65-1 expression. The expression of MAP65-1SWAP reduces cytokinesis defects to an even greater extent. Less than 1% of wild-type, MAP65-3, and MAP65-31-588 cells exhibited cell wall stubs caused by incomplete cytokinesis. Error bars represent So. Bars = 5 µm in (A) and 50 µm in (D).
whether these differences were related to different localization patterns of the two proteins. Immunostaining of the MAP65-1 fusion protein by the anti-FLAG antibody showed punctate signals along MTs but flanking the phragmoplast midline (Figure 5A, panel a). While the conspicuous MAP65-1-FLAG signal was detected in the phragmoplast, these dyc283-derived cells did not possess obvious MAP65-3 signals when probed with anti-MAP65-3 specific antibodies (Figure 5B, panel b). This result was surprising because previously published data suggested MAP65-1 locates in the spindle and phragmoplast midzones (Smertenko et al., 2004, 2008; Mao et al., 2005a). We then tested whether the localization of MAP65-1 to the phragmoplast midzone was dependent on MAP65-3. When the MAP65-1-FLAG fusion was expressed in the wild-type background, in other words in the presence of MAP65-3, MAP65-1 was detected in the phragmoplast midline at all stages of cytokinesis (Figure 5C). These results suggested MAP65-1 localization to the phragmoplast midline requires MAP65-3 under this artificial condition.

A likely scenario was that the ectopically expressed MAP65-1 protein directly interacted with the endogenous MAP65-3. To test this possibility, we employed the bimolecular fluorescence complementation (BiFC) technique using vectors that express target proteins at low levels in *Nicotiana benthamiana* leaf cells. Because the dimerization domain is located in the N-terminal part of MAP65 proteins (Smertenko et al., 2004), we expressed the nCitrine fusion proteins with truncated MAP65-11-339 and MAP65-31-340. BiFC signal highlighting parallel MTs was observed due to the self-interactions between MAP65-11-339-nCitrine and MAP65-1-cCitrine and between MAP65-31-340-nCitrine and MAP65-3-cCitrine (Figures 6A and 6D). Fluorescence was also established along cortical MTs when MAP65-31-340-nCitrine and MAP65-1-cCitrine were expressed (Figure 6B). Reciprocally, expression of MAP65-11-339-nCitrine and MAP65-3-cCitrine also rendered a positive BiFC (Figure 6C). To confirm the specificity of the interactions and ascertain that the observed fluorescence was not due to accidental association between nCitrine and cCitrine, the motor domain of Kinesin-12A that harbors a MT binding site was used in place of full-length MAP65-1 or MAP65-3. No fluorescence was observed when Kinesin-12A61-425-cCitrine was coexpressed together with either MAP65-11-339-nCitrine or MAP65-31-340-nCitrine (Figures 6E and 6F). These results further suggest that MAP65-1 and MAP65-3 would likely interact with each other when their expressions were temporally coordinated. Because of such an interaction, MAP65-3 could bring MAP65-1 to the phragmoplast midline when both were present during cytokinesis.

![Figure 3. The C2 Domain Is Required for MAP65-3 Localization to the Phragmoplast Midline.](image)

The merged images have fusion proteins of MAP65-3 and its derivatives in green, MTs in red, and DNA in blue. Bar = 5 µm. (A) Localization of MAP65-3-FLAG. During early (a) to (c) and late (d) to (f) stages of cytokinesis. MAP65-3 consistently associates with the phragmoplast midline. (B) Localization of MAP65-31-588-FLAG. (a) to (c) The fusion protein localizes to the phragmoplast midline during the early stage of cytokinesis. (d) to (f) In a mature phragmoplast, MAP65-31-588-FLAG exhibits a broader localization pattern (arrows in [d]) along phragmoplast MTs and across the midline. (C) Localization of MAP65-31-495-FLAG. The fusion protein is detected as diffuse signals across the cytoplasm in both the early (a) to (c) and late (d) to (f) stages of cytokinesis.
The C2 Domain of MAP65-3 Can Redirect MAP65-1 to Bundled Antiparallel MTs in the Phragmoplast Midzone

Because MAP65-1SWAP but not MAP65-1 was able to suppress the dyc283 mutation, we wondered whether MAP65-1SWAP had a different localization pattern than MAP65-1. Indeed, the MAP65-1SWAP-FLAG fusion protein conspicuously appeared at the midzone of the early phragmoplast (Figure 7A, panels a to c). At later stages of cytokinesis, the protein continued to decorate the areas that bridged MT bundles from the opposite sides of the phragmoplast (arrowheads, Figure 7A, panels d to f). This result suggested that the fusion protein most likely bundled antiparallel MTs in the phragmoplast midzone and suppressed the defects in cytokinesis caused by the dyc283 mutation. Because these cells lacked MAP65-3, no obvious signals were detected by the anti-MAP65-3 immunofluorescence (Figure 7B, panels a to c). These results suggested that, in the absence of MAP65-3, the addition of the MAP65-3 C2 domain was sufficient to redirect MAP65-1 to engage antiparallel MTs in the phragmoplast midzone.

A MT Binding Domain Resides in the C2 Domain of MAP65-3

To further understand how MAP65-3 interacts with MTs, GFP fusions of MAP65-3 and its derivatives (Figure 8A) were expressed in tobacco leaf cells under the control of a 35S promoter. The ectopically expressed MAP65-3-GFP fusion appeared in linear thick bundles, resembling MT bundles (Figure 8B, panel a). Similar results were obtained when the truncated MAP65-31-588-GFP was expressed (Figure 8B, panel b). MAP65-31-496-GFP, however, exhibited a diffuse localization pattern in the cytoplasm (Figure 8B, panel c). Because a published study showed that the MT binding site in MAP65 family proteins is located at the C-terminal half of the protein (Smertenko et al., 2004), we first tested the entire C-terminal half consisting of both C1 and C2 domains. The resulting MAP65-3C-GFP fusion decorated a filamentous meshwork at the cell cortex (Figure 8B, panel d). Such a distribution pattern was also detected by the MAP65-3C2-GFP containing the C2 domain alone, but not MAP65-3C1-GFP with the C1 domain (Figure 8B, panels e and f). This result further suggested that the MT binding site resides in the C2 domain. Because of the difference in suppression of the dyc283 mutation by MAP65-31-588 and MAP65-31-496, we asked whether the region of amino acids 496 to 588 in MAP65-3 constituted a MT-interacting site. Using this region, the MAP65-3C2N-GFP fusion also decorated a MT-like meshwork at the cell cortex (Figure 8B, panel g). These results support the notion that the fusion proteins can directly bind to cortical MTs. The peptides of MAP65-3C1, MAP65-3C2, and MAP65-3C2N have calculated isoelectric points of 5.15, 10.24, and 11.38, respectively. This result indicates that the interaction between MAP65-3C2N and MTs is likely charge dependent and the acidic nature of the MAP65-3C1 domain prevents it from decorating MTs. However, such a mechanism would not cause MAP65-3 to selectively bundle antiparallel MTs only near their plus ends in the phragmoplast midzone.

To test whether the filamentous networks decorated by the MAP65-3-GFP fusions truly represented cortical MTs, they were coexpressed with the MT binding domain derived from CKL6 (for casein kinase 1-like 6) in an mCherry fusion (Ben-Nissan et al., 2008). The results indicate that all filamentous networks highlighted by the GFP fusions colocalize perfectly with mCherry-marked cortical MTs (see Supplemental Figure 1 online). In cells showing diffuse signals of GFP alone, MAP65-31-496-GFP, and MAP65-3C1-GFP, cortical MTs were still detected by the CKL6-mCherry fusion (see Supplemental Figure 1 online).

Published work shows that ectopic expression of MAP65-1 in interphase cells increases the resistance of cortical MTs to challenges of MT-depolymerizing agents (Van Damme et al., 2004b). We asked whether a similar effect was conferred by MAP65-3 by examining the turnover of the MAP65-3-GFP fusion proteins. Fluorescence recovery after photobleaching (FRAP) analysis was conducted on the GFP signals decorating cortical MTs. Both MAP65-3-GFP and MAP65-31-588-GFP had a stable appearance with low turnover rates of half-time values >90 s (Figures 8A and 8C). However, the complex network of fine cortical MTs decorated by MAP65-3C-GFP, MAP65-3C2, or MAP65-3C2N all had greater turnover rates with half-time values <2 s (Figures 8A and 8C). To further test whether MTs became stabilized upon MAP65-3 expression, we challenged the tobacco leaf cells with oryzalin for 30 min before they were observed by confocal microscopy. Compared with cells expressing full-length MAP65-3 that showed bundled MTs decorated by MAP65-3-GFP (see Supplemental Figure 2A online), cells expressing MAP65-3C that lacked the N-terminal dimerization domain had cortical MTs organized into a fine and complex network decorated by MAP65-3C-GFP (see Supplemental Figure 2B online). Upon oryzalin treatment, cortical MTs remained highly bundled in cells expressing MAP65-3-GFP (see Supplemental Figure 2C online). However, the fine and dense cortical MT network was replaced with scarce and wavy filaments in oryzalin-treated cells expressing MAP65-3C-GFP.
Supplemental Figure 2D online). Based on the results summarized above, we conclude that the dimerization effect conferred by the N-terminal half of MAP65-3 likely contributed to bundling and stabilization of cortical MTs when it was expressed in interphase cells.

A remaining question is whether the truncated MAP65-3 that lacked the dimerization domain exhibited lower affinity to MTs. To answer this question, 6XHis-tagged fusion proteins of MAP65-1-588 and MAP65-3-341-588 were expressed in bacteria and purified for in vitro MT binding assays. Both fusion proteins cosedimented with taxol-stabilized MTs after centrifugation, while the negative control of BSA remained in the supernatant (Figure 8D). To test whether the two fusion proteins exhibited different affinities to MTs, we examined the binding of fixed amounts of the two fusion proteins at various concentrations of MTs (see Supplemental Figure 3 online). The percentage of proteins bound to MTs was plotted against MT concentration (Figure 8E). The MT binding affinities of the two fusion proteins were similar if not identical (Figure 8E). This result is consistent with the fact that the two fusion proteins contain an identical MT binding domain.

**DISCUSSION**

Our results show that *Arabidopsis* MAP65-3 possesses a single MT binding site in the C2 domain, and this site is critical for the interdigitation/cross-linking of antiparallel MTs toward their plus ends in the phragmoplast. MAP65-3 function is critical for the organization of the phragmoplast MT array, which directly influences the outcome of cytokinesis. In comparison, the related MAP65-1 protein in the same family cannot substitute the role of MAP65-3 in bridging the antiparallel MTs in the phragmoplast. However, grafting of the C2 domain allowed MAP65-1 to acquire MAP65-3’s role in phragmoplast MT organization. We

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**Figure 5.** The Midline Localization of MAP65-1 Depends on MAP65-3.

(A) Expressed in a dyc283 mutant cell, the MAP65-1-FLAG fusion protein appears in a punctate localization pattern (a) along phragmoplast MTs (b). The merged image (c) has MAP65-1-FLAG in green, MTs in red, and DNA in blue. Bar = 5 µm.

(B) MAP65-3 (b) is absent in the dyc283 cell expressing MAP65-1-FLAG (a).

(C) In the wild-type background, MAP65-1-FLAG ([a] and [d]) colocalized with MAP65-3 ([b] and [e]) in early ([a] to [c]) and late ([d] to [f]) phragmoplasts.

**Figure 6.** BiFC Assay of the Interaction between MAP65-1 and MAP65-3.

Positive BiFC demonstrated by the dimerizations of MAP65-1-cCitrine and MAP65-1-1-339-nCitrine (A), MAP65-1-cCitrine and MAP65-31-340-nCitrine (B), MAP65-3-cCitrine and MAP65-1-1-339-nCitrine (C), and MAP65-3-cCitrine and MAP65-31-340-nCitrine (D). No fluorescence was observed when MAP65-1-1-339-nCitrine and Kinesin-12A61-425-cCitrine (E) or MAP65-31-340-nCitrine and Kinesin-12A61-425-cCitrine (F) were expressed together. Bar = 20 µm.
conclude that the C2 domain specifies the function of MAP65-3 in maintaining the integrity of the phragmoplast MT array during cytokinesis.

The C2 Domain Distinguishes MAP65-3 from Other MAP65 Family Proteins

The MAP65/Ase1/PRC1 family of proteins act in dimers to cross-link and bundle MTs (Walczak and Shaw, 2010). They possess a dimerization domain toward the N terminus and MT binding domain(s) toward the C terminus (Smertenko et al., 2004). Both monocot and dicot plants produce a large number of proteins in this family (Smertenko et al., 2008; Guo et al., 2009). These MAP65 isoforms can be nicely aligned according to their sequence homology except for the very C-terminal regions (Smertenko et al., 2008). The divergence of their C termini may be responsible for the diversity of their intracellular localization patterns (Van Damme et al., 2004b; Mao et al., 2005b) and consequently for their varied functions. A recent structural analysis presented a model showing that the human MAP65 homolog PRC1 uses its spectrin-featured domain to bind to MTs and decode their polarity so that it can specifically mark the overlapping region of the anaphase spindle (Subramanian et al., 2010). Earlier studies delineated two MT binding sites, namely, MTB1 and MTB2, in MAP65-1 (Smertenko et al., 2004). However, our results showed that only the MTB2/C2 region of MAP65-3 and not the MTB1 region binds directly to MTs. Thus, MAP65-3 may have adopted a MT binding mechanism different from that of MAP65-1 or other isoforms.

We demonstrated that the presence of the C2 domain was most critical for the interaction of MAP65-3 with antiparallel MTs in the phragmoplast. Compared with MAP65-3, MAP65-1 does not contain a similar homologous region, but mixed results have been published on MAP65-1 localization in the phragmoplast. Antibodies raised against a MAP65-1 region decorate the phragmoplast midline in dividing suspension cultured cells (Smertenko et al., 2008). When expressed in GFP fusions under the control of the 35S promoter in tobacco cells, MAP65-1 does not appear in the midline of mature phragmoplasts, except for in the spindle midzone at anaphase (Van Damme et al., 2004a; Mao et al., 2005a; Gaillard et al., 2008). This discrepancy could have arisen from the differences in cell types and/or expression levels of the protein under different conditions. A recent study had MAP65-1-GFP expressed under its native promoter (Lucas et al., 2011). In root meristematic cells, this fusion protein uniformly decorates the entire phragmoplast MT array and does not appear predominantly in the phragmoplast midzone (Lucas and Shaw, 2012). In this study, we showed that MAP65-1 alone was insufficient to cross-bridge antiparallel MTs in the phragmoplast. It would appear at the phragmoplast midline when expressed under the control of the MAP65-3 promoter and in the presence of MAP65-3. This result may arise from two scenarios. First, the expressed MAP65-1 fusion protein might have interacted with the endogenous MAP65-3 protein to be localized at the midline. This notion is supported by our BiFC data that showed that the two proteins interact directly when their expression is temporally coordinated. The other possibility is that the elevated expression level of MAP65-1 at the mitotic stages would allow it to appear in the phragmoplast midzone in the presence of interdigitated antiparallel MTs cross-linked by MAP65-3.

Compared with MAP65-1 and MAP65-3, other MAP65 isoforms also exhibit distinct localization patterns. For example, MAP65-6 decorates mitochondria (Mao et al., 2005b). At prophase, MAP65-5 decorates both the prophase spindle and the preprophase band, while MAP65-1 only appears at the preprophase band (Gaillard et al., 2008). Unlike MAP65-1, which appears at the spindle midzone, MAP65-4 is specifically associated with the kinetochore fibers but not the preprophase band (Fache et al., 2010). It is intriguing how these related proteins achieve such dynamic localization patterns at particular stages of cell division. Detailed structural and functional dissections are

Figure 7. The MAP65-3 C2 Domain Redirects MAP65-1 to the Phragmoplast Midline.

(A) The localization of MAP65-1SWAP-FLAG (green) at different stages of cytokinesis. The merged image has MAP65-1SWAP-FLAG in green, MTs in red, and DNA in blue. The MAP65-1SWAP-FLAG signal is concentrated between two sets of antiparallel MTs during late anaphase/telophase (a to c). The signal is pronounced in the midline (arrowheads in [a], [d], and [g]) of cells bearing mature phragmoplasts. Bar = 5 µm.

(B) In the dyc283 cell expressing MAP65-1SWAP-FLAG, which was detected at the phragmoplast midline (arrowhead in [a]), MAP65-3 is absent (b). The merged image has MAP65-1SWAP-FLAG in green, MAP65-3 in red, and DNA in blue (c).
Figure 8. A MT Binding Site in the C2 Domain of MAP65-3.

(A) Diagrams representing truncations used in the MT binding assays shown in (B) and (C). Full-length and truncated MAP65-3 fragments are expressed in fusions with GFP at their C terminus under the control of the 35S promoter. $t_{1/2}$, time in seconds (s) for the recovery of the GFP signal intensity to 50% of the maximum fluorescence; n, number of cells examined by FRAP.
required to further characterize their activities in rapidly growing plant cells.

Functional Redundancy among the MAP65 Isoforms

The sequence similarities among the MAP65 family proteins would imply functional redundancies. Indeed, a recent work showed that MAP65-1, MAP65-2, and MAP65-3 were substrates of an important MAP kinase in cytokinesis (Sasabe et al., 2011). It has been shown that mutations in genes encoding either MAP65-1 or MAP65-2 would enhance the phenotype by adding a mutation in the MAP65-3-encoding gene in Arabidopsis (Sasabe et al., 2011). Our results support such a genetic interaction. The expression of MAP65-1 significantly suppressed the cytokinetic defects and root growth problems caused by the dyc283 mutation. This suppression phenomenon may be beyond the localization in the phragmoplast midline because the ectopically expressed MAP65-1 did not appear at the site normally occupied by MAP65-3. It implied that MAP65-3 might play a role in aspects other than cross-linking antiparallel MTs, and such a role could be shared with MAP65-1. If this were true, the suppression would be brought about by the presence of an increased amount of MAP65-1.

Due to the high sequence similarity between MAP65-3 and MAP65-4, one would expect that they may share similar functions. However, the ectopically expressed MAP65-4 in tobacco Bright Yellow-2 cells that contain a putative ortholog(s) of MAP65-3 does not appear in the phragmoplast (Fache et al., 2010). In other words, it might not be able to interact with MAP65-3. Some intrinsic features embedded in the sequence differences between MAP65-3 and MAP65-4 must demarc the drastically different localization patterns of these two highly similar proteins.

A Generalized Role in MT Stabilization by MAP65 Family Proteins

Published data show that non-MAP65-3 isoforms of MAP65 stabilize cortical MTs when they are ectopically expressed (Van Damme et al., 2004b; Li et al., 2009). Here, we showed that ectopically expressed MAP65-3 cause hyperbundling of cortical MTs with low turnover rates. The result suggests that the underlying cortical MTs became stabilized after being cross-linked together into bundles. There is no evidence showing that the endogenous MAP65-3 protein interacts with cortical MTs. This may be a result of its cell cycle-dependent expression pattern. The ectopic expression of all tested MAP65 family proteins in interphase cells all leads to hyperbundling of cortical MTs that are aligned largely in parallel to each other. Such an effect was not observed when MAP65-1 and MAP65-2-GFP fusions were expressed under the control of their endogenous promoter (Lucas et al., 2011). In vitro biochemical studies showed that two MAP65 isoforms and the human PRC1 bind to individual MTs in monomeric forms followed by a zipper effect to allow antiparallel MTs to be bundled together (Gaillard et al., 2008; Lloyd, 2011). Taking the FRAP data into account, we believe that the stabilization of cortical MTs was likely brought about by the bundling effect of overexpressed MAP65-3 in interphase cells. In the phragmoplast, ectopically overexpressed MAP65-1 decorates MTs flanking the midline (Gaillard et al., 2008). In a halved phragmoplast, MTs are arranged in a parallel fashion (Euteneuer and McIntosh, 1980). Such a pattern is essential for unidirectional transport of Golgi-derived vesicles toward MT plus ends to form the cell plate. If MAP65-1 only bundled antiparallel MTs, the phragmoplast of cells overproducing MAP65-1 would have antiparallel MTs present in both phragmoplast halves. Therefore, it would be interesting to examine MT polarity in the phragmoplasts of cells containing high concentrations of MAP65-1.

Unlike MAP65-1, ectopically overexpressed MAP65-3 only appears in the phragmoplast midline (Van Damme et al., 2004a). However, it decorated cortical MTs evenly and without any preference in interphase cells in our study. MAP65-3, when expressed under the control of its native promoter, transiently interacted with cortical MTs when cells were ready to enter the M phase (Caillaud et al., 2008). Collectively, these results suggest that the interaction of MAP65-3 with phragmoplast MTs is different from that with cortical MTs in interphase cells. Its activity in bundling antiparallel MTs near their plus ends could be cell cycle dependent. Such an activity could be activated upon a posttranslational modification of the protein.

Regulation of MAP65 Functions in the Phragmoplast

A number of protein kinases appear in the phragmoplast and play critical roles in regulating the progression of cytokinesis (Oh et al., 2005; Sasabe and Machida, 2006; Krupnova et al., 2009). Among them, the TWO-IN-ONE kinase and kinases of the NACK-PQR pathway specifically appear at the phragmoplast midline (Nishihama et al., 2002; Kosetsu et al., 2010). Currently, the substrate of TWO-IN-ONE during cytokinesis is unknown. However, the MAP kinase of the NACK-PQR pathway NPK1 phosphorylates MAP65-1 and...

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Figure 8. (continued).

(B) A MT binding site resides in the region of amino acids 496 to 588 of MAP65-3. MAP65-3- and MAP65-3-1-588-GFP fusions decorate thick and parallel MT bundles. GFP fusions of MAP65-3-ć, MAP65-3-ćć, and MAP65-3ć-ćN highlight a fine filamentous network of cortical MTs. However, the GFP fusions of MAP65-3ć-ć-465 and MAP65-3ć-ć- give diffuse signals in the cytoplasm. Cells expressing GFP alone are used as the control. Bar = 20 μm.

(C) The N-terminal region of MAP65-3 contributes to the stable association with bundled cortical MTs as revealed by the FRAP experiment. GFP fusions of MAP65-3 and MAP65-3ć-ć-468 have half-life times >90 s. In comparison, MAP65-3ć, MAP65-3ćć, and MAP65-3ć-ćN GFP fusions have half-life times of <2 s.

(D) MT cosedimentation of MAP65-3 derivatives. After incubation of MAP65-3ć-ć-468 (1), MAP65-3ćć-ć-ćN (2), and BSA (3) with taxol-stabilized MTs, supernatant (s) and pellet (p) fractions were analyzed by SDS-PAGE. Positions of MAP65-3ć-ć-468, MAP65-3ćć-ć-ćN, and BSA are indicated by arrowheads and that of tubulins by an asterisk on the right. Molecular mass markers in kilodaltons are shown on the left.

(E) Equilibrium binding assays of MAP65-3ć-ć-468 (circles, solid line) and MAP65-3ćć-ć-ćN (triangles, broken line) with MTs. BSA (diamonds, dashed line) was used as a negative control. Error bars represent so of three replicates.
negatively regulates its MT-bundling activity in the phragmoplast in tobacco cells (Sasabe et al., 2006; Komis et al., 2011). Recently, it was found that in Arabidopsis not only MAP65-1 but also MAP65-2 and MAP65-3 are substrates of the MAP kinase MPK4 (Kosetsu et al., 2010; Sasabe et al., 2011). Redundant roles were also suggested for these three MAP65 isoforms. Thus, the MT-stabilizing function of these MAPs has to be downregulated upon the completion of cell plate assembly so that phragmoplast MTs would be ready to undergo depolymerization. It was shown that Ser/Thr-to-Ala mutations in the NRK1 phosphorylation sites led to the formation of multinucleate cells caused by failures of cytokinesis (Sasabe et al., 2006).

Conversely, in human cells, the MAP65-3 counterpart, PRC1, was found to be phosphorylated at prophase to metaphase and dephosphorylated at anaphase (Zhu et al., 2006). The dephosphorylation event is critical for PRC1 to be localized to the spindle midzone and to oligomerize there. Thus, different mechanisms may regulate MAP65/PRC1 function in different organisms.

The Requirement of MAP65-3 for Other Proteins to Interact with MT Plus Ends in the Phragmoplast

It is intriguing that the MT motors Kinesin-12 and PAKRP2 require MAP65-3 to interact with MT plus ends in the phragmoplast (Ho et al., 2011a). A similar function can be found for its human counterpart, PRC1, for the localization of the kinesin family protein KIF14 in the spindle midzone (Gruneberg et al., 2006). In both fission yeast and animal cells, Ase1p and PRC1 interact with a number of kinesin motors (Kurasawa et al., 2004; Zhu and Jiang, 2005; Gruneberg et al., 2006). In human cells, the kinesin Kif4 plays a role in translocating PRC1 to the spindle midzone (Zhu and Jiang, 2005). In our experiment, the loss of two Kinesin-12 motors that led to phragmoplast MT disorganization and cytokinesis failures abolished the midzone formation and MAP65-3 distribution along the phragmoplast MTs, suggesting that MAP65-3 and Kinesin-12 are interdependent.

The localized activity of MAP65-3 in the phragmoplast may also have a regulatory role in the MT bundling function in the phragmoplast proper. During cytokinesis, newly polymerized MTs would be brought together by the fortified phragmoplast MT array. In the absence of MAP65-3, however, MTs nucleated elsewhere, such as on the envelope of the reforming daughter nuclei, would remain discrete. This is probably why MTs polymerized in places other than the phragmoplast proper in cells lacking a functional MAP65-3.

METHODS

Plant Materials, Growth conditions, and Transformation

The Arabidopsis thaliana materials used in this study include the control Wassilewskija plants and the dcy283 mutant (Caillaud et al., 2008). Plant growth conditions and Agrobacterium tumefaciens–mediated transformation were performed as described previously (Kong et al., 2010; Ho et al., 2011a).

For observation of various GFP fusions, Agrobacterium with the p19 and testing plasmids (see below) were grown overnight in lysogeny broth medium at 30°C. The culture was harvested by centrifugation and resuspended in the infiltration medium (10 mM MgCl₂, 10 mM MES, pH 5.6, and 200 μM acetosyringone) to a final OD₆₀₀ of 0.6 to ~1.0. The agrobacterial suspension was incubated at 22°C for at least 3 h and then used for filtration of Nicotiana benthamiana leaves according to a published protocol (Popescu et al., 2007).

Construction of Expression Vectors

A previously constructed plasmid containing full-length MAP65-3 (i.e., ProMAP65-3:MAP65-3–FLAG) (Ho et al., 2011a) was used as template for the following constructs. To make the ProMAP65-3:MAP65-3–1588–FLAG and ProMAP65-3:MAP65-3–1588–FLAG constructs, promoter and coding sequences were amplified by PCR using the forward primer AtMAP65 1 (5’-CACCCACACTTCTCCTACACAAAAACCGC-3’) and the reverse primer AtMAP65 588r (5’-GGGGAATACCGTTGCACTAAATG-3’), and AtMAP65 1 and AtMAP65 495r (5’-TGTTCGTCGCTATGAGCTGTCGTTTGAACG-3’). To make the ProMAP65-3:MAP65-1–SWAP and ProMAP65-3:MAP65-1–SWAP constructs, the MAP65-3 promoter was first amplified by PCR using the AtMAP65 1 and AtMAP65 22 (5’-TCTGAAATGCT-TAAGCGCTGACACCCC-3’) primers. The full-length MAP65-1 DNA sequence was amplified by PCR from the cDNA clone M78N02STM using the forward primer AtMAP-1a (5’-CCCTTGTACCGGTTAAGGTTTCAGGCGCAAA-3’) and the reverse primer AtMAP-1b (5’-TGCGTAGCGTGAAGCTGATACGCTGAC-3’). The MAP65-3 promoter and MAP65-1 fragments were fused together by PCR using the primers AtMAP65 1 and AtMAP65 1b. To make the ProMAP65-3:MAP65-1–SWAP construct, two fragments in addition to the MAP65-3 promoter were amplified individually. The MAP65-1 truncated fragment was amplified using the primers AtMAP-1a and AtMAP-1c (5’-GGGTTGACCGCTTAAGTGGCCTTCGCTGCTATGAGCTGTCGTTGAC-3’). The fragment encoding the MAP65-3 C2 domain was amplified with the primers AtMAP65 21 (5’-GGAGGACTTACGCTCAGCAACCC-3’) and AtMAP65 8 (5’-AACCCAGACACCTTACATGATCCG-3’). The entire fused ProMAP65-3:MAP65-1–SWAP fragment was amplified by an additional PCR reaction using the primers AtMAP65 1 and AtMAP65 8 and the three fragments as templates. The aforementioned PCR products were cloned into the Gateway PENTR/TOPO vector (Invitrogen), followed by LR recombination reactions with the destination vector pGW10 (Nakagawa et al., 2007) to render the final vectors that would allow the expression of C-terminal FLAG fusion proteins. These plasmids were transformed into the dcy283 mutant.

Constructs for tobacco (Nicotiana tabacum) leaf filtration experiments were made as following. The cDNA fragments encoding full-length MAP65-3 and truncated MAP65-3–1588 and MAP65-3–1588 proteins were amplified by the forward primer AtMAP65-3 33 (5’-CACCAATGCATGTGGTCAAAAAATCCCGATTCTTC-3’) and reverse primers AtMAP 8, AtMAP 588r, and AtMAP 495r. The entire fused ProMAP65-3:MAP65-1–SWAP fragment was amplified by an additional PCR reaction using the primers AtMAP65 1 and AtMAP65 8 and the three fragments as templates. The aforementioned PCR products were cloned into the Gateway PENTR/TOPO vector (Invitrogen), followed by LR recombination reactions with the destination vector pGW10 (Nakagawa et al., 2007) to render the final vectors that would allow the expression of C-terminal FLAG fusion proteins. These plasmids were transformed into the dcy283 mutant.

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Fluorescence and Confocal Microscopy

Root tip cells were prepared for indirect immunofluorescence staining as described previously (Lee and Liu, 2000). Primary antibodies used in this...
study included anti-FLAG C2 (Shanghai Genomics), polyclonal anti-tubulin (Cytoskeleton), anti-MAP65-3 (Ho et al., 2011a), and anti-PAKRP1-C for Kinesin-12 (Lee and Liu, 2000). Secondary antibodies were fluorescein isothiocyanate–conjugated donkey anti-mouse IgG, fluorescein isothiocyanate–conjugated donkey anti-rabbit IgG, and Texas Red–conjugated donkey anti-goat IgG (Rockland Immunocochemicals).

Slides were observed under an Eclipse E600 microscope equipped with epifluorescence optics (Nikon) and ET filter sets (Chroma Technology). Images were captured by an Orca charge-coupled device camera (Hamamatsu Photonics) driven by the MetaMorph software package (Molecular Devices) before being assembled by Photoshop software (Adobe).

The signals of GFP fusions with the full-length MAP65-3 protein and various truncations were visualized in N. benthamiana leaves 3 to 4 d after agro bacterial infiltration using an FV1000 laser scanning confocal microscope with a PLAPON ×60 oil objective lens (Olympus USA). The GFP signal was collected at 500 to 550 nm. The FRAP experiments were performed using the built-in function in FV1000. The GFP and mCherry signals were excited by the 488- and 543-nm lasers and collected at the 500- to 550-nm and 550- to 600-nm ranges, respectively. Acquired images were processed by MetaMorph and Photoshop (Adobe).

The cell profiles in the root tip regions were observed according to the fluorescent signal of propidium iodide infiltrated into the cell wall. Briefly, 5-d-old seedlings were incubated in 20 µg/mL propidium iodide solution and observed under a TCS SP2 (Leica) confocal microscope with an HCX PL APO ×63 water or an HC PL APO ×20 objective lens. Two cell files in regions of 80 to 240 µm from the root tip were used to calculate the degree of cytokinetic defects.

BiFC Experiments

The BiFC experiments were performed using the yellow fluorescent protein variant Citrine as reported (Caplan et al., 2008). Instead of the cauliflower mosaic virus 35S promoter, the much weaker NOS promoter was used for all constructs used in BiFC. Briefly, the full-length MAP65-1–coding sequence was cloned into SPDK919 (pNOS-MCS-Citrine) at the MluI and XbaI sites. The fusion proteins were expressed and purified proteins at the concentration of 2 µM were subjected to a MT cosedimentation assay as described previously (Lee et al., 2001). Proteins were analyzed by 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue G250 prior to being imaged for density analysis.

Accession Numbers

The Arabidopsis Information Resource locus identifier for the genes mentioned in this article are At5g51600 for MAP65-3 and At5g55230 for MAP65-1. The cDNA clone M78N02STM (GenBank accession number BE530728) contains the full-length coding sequence of MAP65-1.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Colocalization of MAP65-3 and Its Derivatives with Cortical MTs.

Supplemental Figure 2. Cortical MTs upon Expression of MAP65-3 or MAP65-3Δ351–702 with and without Oryzalin Treatment.

Supplemental Figure 3. MT Binding Assays of MAP65-31–588 and MAP65-3Δ341–588.

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AUTHOR CONTRIBUTIONS


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