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Chapter 2

Actin-Microtubule Interaction in Plants

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Additional information is available at the end of the chapter

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Abstract

Interactions between actins and microtubules play an important role in many fundamental cellular processes in eukaryotes. Although several studies have shown actins and microtubules to be involved in specific cellular activities, little is known about how actins and microtubules contribute together to a given process. Preprophase band formation, which plays an essential role in plant division site determination, is a cellular process that lends itself to studies of actin-microtubule interactions and how they contribute to important cellular functions. Recently, we have analyzed microtubule-associated microfilaments during preprophase band formation in onion cotyledon epidermal cells using a combination of high-pressure freezing/freeze substitution and electron tomography. Quantitative analysis of our electron tomography data showed that relatively short single microfilaments form bridges between two adjacent microtubules in the process of narrowing of the preprophase microtubule band. Two types of microtubule-microfilament-microtubule connections are observed, and these microfilament-microtubule interactions suggest a direct role of F-actins in microtubule bundling. Based on these observations, we discuss how different actin-microtubule linkers might contribute to preprophase band narrowing and to other changes in microtubule organization in plant cells.

Keywords: actin-microtubule interaction, electron tomography, microtubule bundling, plant morphogenesis, preprophase band

1. Introduction

Actin filaments (F-actins) and microtubules (MTs) are major components of the cytoskeleton of eukaryotic cells. Each involved in fundamental cellular processes, such as cell division, directional cell expansion, organelle movement and signal transduction. Studies of cross-talk between F-actin and MT networks have led to the conclusion that actin-MT interactions are essential for the regulation of these cytoskeletal networks in eukaryotes [1]. In

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plants, actin has been postulated to play critical roles in MT-mediated cytomorphogenesis. Plant shape is determined by the spatial and temporal regulation of cell division and cell expansion. During interphase, directional cell expansion is controlled by interphase cortical MTs, whereas during cell division, arrays of MTs, such as preprophase bands (PPBs), spindles and phragmoplasts contribute to division site determination, chromosome movement and cell plate formation, respectively. Although electron microscope (EM) observations, studies with fluorescence probes, pharmacological studies and molecular genetic studies have provided insights into the possible cross-talk between actin and MT-systems, little is known about how actin controls MT organization during morphogenesis [2]. The PPB is a plant-specific cytokinetic apparatus that establishes the future site of cell division, and the evidence for actin-MT cross-talk has been obtained from fluorescent microscope studies [3]. Recently, we have succeeded in observing how microfilaments (MFs) interact with MTs and thereby cause MT bundling using electron tomography analysis of cryofixed cells [4]. In this chapter, we review how interactions between F-actins and MTs control plant cell expansion and cell division. We then explain why electron tomography is an excellent tool for the analysis of the 3D architecture of MF-MT interactions during PPB development. Finally, we discuss candidate molecules that might mediate these actin-MT interactions.

2. Evidence for the involvement of actin-MT interactions in plants

A list of studies supporting the hypothesis that actin-MT interactions play important roles in plant morphogenesis is shown in **Tables 1** and **2**. In electron micrographs, F-actins give rise to ~6 nm in diameter MFs and are seen in close proximity to and aligned with MTs. Such MFs are linked to the MTs through cross-bridging structures (see papers marked EM in **Tables 1** and **2**). Fluorescent microscopy provides several methods for studying F-actins and MTs. Most frequently, F-actins and MTs are visualized by means of specific antibodies with attached fluorescent tags (see papers marked IF in **Tables 1** and **2**). Fluorescent-labeled phalloidins are also used to detect F-actins (see papers marked Ph in **Tables 1** and **2**). Microinjection of fluorescent-labeled cytoskeletal proteins or phalloidin has been used to examine cytoskeletal dynamics in living cells (see papers marked Inj (Ph/) in **Tables 1** and **2**). Live cell studies of expressed chimera peptides with fluorescent protein-tags have also yielded important results (see papers marked e.g. live GFP-AtFim1/ in **Tables 1** and **2**). An alternative is to use cytoskeleton-modifying drugs to investigate cytoskeletal involvement in cellular processes in different types of cells (**Tables 1B**, **C** and **2B**). For example, to determine whether actins exert a role in a MF-mediated function of a cell, the disruption or stabilization on the organization of MTs, and *vice versa* can be determined. Genetic modifications in combination with cytoskeleton-altering drugs provide yet another tool for investigating MF-MT interactions (**Tables 1B**, **C** and **2B**)

2.1. Directional cell expansion

The shape of plant cells is defined by the mechanical properties of their cell walls, and cortical MTs help determine plant cell shape by controlling the direction of deposition of cell

References	Plant	Cell type	Imaging method
(A) Colocalization of actins with MTs/ MFs associated with MTs			
Franke et al. [5]	*Liliumlongiflorum*	Pollen tube	EM (chem)
Seagull and Heath [6]	*Raphanussativus*	Root hair	EM (chem)
Hardham et al. [7]	*Graptopetalumparaguayense*	Leaf	EM (chem)
Tiwari et al. [8]	*Triticum* *Agrostis* *Phleum*	Leaf trichome Leaf epidermal cell Root tip cell	EM (cryo) EM (chem)
Emons [9]	*Equisetum hyemale* *Limnobiumstoloniferum*	Root hair Root hair	EM (cryo)
Lancelle et al. [10]	*Nicotianaalata*	Pollen tube	EM (cryo)
Kobayashi et al. [11]	*Zinnia elegans*	Suspension culture cell (tracheary element)	Ph/ IF
Pierson et al. [12]	*Nicotianatabacum* *Liliumlongiflorum*	Pollen tube Pollen tube	Ph/IF
Sonobe and Shibaoka [13]	*Nicotianatabacum*	Suspension culture cell	Ph/IF
Ding et al. [14]	*Nicotianatabacum*	Root tip cell	EM (cryo)
Kengen and Derksen [15]	*Nicotianaplumbaginifolia*	Protoplast	EM (DC)
Lancelle and Hepler [16]	*Nicotianaalata*	Pollen tube	EM (cryo)
Cleary et al. [17]	*Selaginellakraussiana*	Differentiating guard cell	Ph/MT
Tominaga et al. [18]	*Hydrocharisdubia*	Root hair	Ph/IF, EM (chem)
Murata et al. [19]	*Nicotianatabacum* *Allium cepa*	Root tip cell Cotyledon epidermal cell	EM (cryo)
Sampathkumar et al. [20]	*Arabidopsis thaliana*	Epidermal cell of root	Live (GFP-FABD/ mCherry-TUA5)
(B) F-actin disruption/stabilization causes alternation in MT organization			
Kobayashi et al. [11]	*Zinnia elegans*	Suspension culture cell (tracheary element)	Ph/IF
Seagull [21]	*Gossypiumhirsutum*	Cotton fiber	IF/IF
Kadota and Wada [22]	*Adiantumcapillus-veneris*	Protonemal cell	Ph/IF
Hasezawa et al. [23]	*Nicotianatabacum*	Suspension culture cell	Ph/IF
Takesue and Shibaoka [24]	*Vignaangularis*	Epidermal cell of epicotyl	Ph/IF
Blancaflor [25]	*Zea mays*	Cortical cell of root	Ph/IF
Schwab et al. [26]	*Arabidopsis thaliana (dis2)*	Trichome	Live (-/GFP-MAP4)
Saedler et al. [27]	*Arabidopsis thaliana (dis2)*	Trichome	Live (CFP-mTalin/ YFP-MAP4)
Timmers et al. [28]	*Medicagotruncatula*	Root hair	Live (GFP-FABD2/ GFP-MBD,YFP-EB1)
Sainsbury et al. [29]	*Allium porrum*	Leaf epidermall cell	Live (-/GFP-MBD)

References	Plant	Cell type	Imaging method
(C) MT disruption/stabilization causes alteration in actin organization			
Kobayashi et al. [11]	*Zinnia elegans*	Suspension culture cell (tracheary element)	Ph/ IF
Chu et al. [30]	*Secalecereale*	Root tip cell	Ph/ IF
Tominaga et al. [18]	*Hydrocharisdubia*	Root hair	Ph/ IF
Collings et al. [31]	*Nicotianatabacum*	Suspension culture cell	IF/ IF
Collings and Wasterneys [32]	*Arabidopsis thaliana*	Root epidermal cell	IF/ IF
Collings et al. [33]	*Arabidopsis thaliana (mor1)*	Root epidermal cell	IF/ IF
Smertenko et al. [34]	*Nicotianatabacum* *Arabidopsis thaliana*	Suspension culture cell Root	Live (GFP-Lifeact/-)
Shevchenko [35]	*Arabidopsis thaliana*	Root transition zone	Liv (GFP-ABD2/-)

EM (chem), electron microscopy with chemical fixed materials;EM (cryo), electron microscopy with cryo-fixed materials; EM (DC), electron microscopy of dry cleaved sample; IF, immunofluorescence method; Ph, phalloidin labelled with fluorescent dyes; Live, live imaging

Table 1. Actin-MT cross-talks in cell expansion.

References	Plant	Cell type	Imaging method
(A) F-actins (or MFs) in the PPB			
Kakimoto and Shibaoka [36]	*Nicotianatabacum*	Suspension culture cell	Ph/IF
Palevitz [37]	*Allium cepa*	Root tip cell	Ph/IF
Traas et al. [38]	*Daucuscarota*	Suspension culture cell	Ph/-
Lloyd and Traas [39]	*Daucuscarota*	Suspension culture cell	Ph/-
McCurdy et al. [40]	*Triticumaestivum*	Root tip cell	IF/-
Palevitz[41]	*Allium cepa*	Root tip cell	Ph/IF
Katsuta et al. [42]	*Nicotianatabacum*	Suspension culture cell	Ph/IF
McCurdy and Gunning [43]	*Triricumaesrivum*	Root tip cell	IF/IF
Mineyuki and Palevitz [44]	*Allium cepa*	Cotyledon epidermal cell	Ph/IF
Ding et al. [45]	*Nicotianatabacum*	Root tip cell	EM (cryo)
Cleary et al. [46]	*Tradescantiavirginiana*	Stamen hair cell	Inj (Ph/tubulin)
Eleftheriou and Palevitz [47]	*Allium cepa*	Root tip cell	IF/IF
Liu and Palevitz [48]	*Allium cepa* *Tradescantiavirginiana*	Root tip cell	IF/IF
Panteris et al. [49]	*Adiantumcapillus-veneris*	Root tip cell	Ph/IF
Cleary [50]	*Tradescantiavirginiana*	Stomatal complexes	Inj (Ph/-)
Cleary and Mathesius [51]	*Tradescantiavirginiana*	Leaf epidermal cell	Ph/IF
Baluska et al. [52]	*Zea mays*	Root tip cell	IF/-
Zachariadis et al. [53]	*Pinusbrutia*	Root tip cell	Ph/IF
Sano et al. [54]	*Nicotianatabacum*	Suspension culture cell	Live (GFP-ABD2/-)

References	Plant	Cell type	Imaging method
Li et al. [55]	*Arabidopsis thaliana*	Suspension culture cell	Ph/IF
Higaki et al. [56]	*Nicotianatabacum*	Suspension culture cell	Live (GFP-ABD2/ -)
Panteris et al. [57]	*Zea mays, Triticumturgidum*	Leaf stomatal complexes	Ph/ IF
Takeuchi et al. [58]	*Allium cepa*	Root tip cell	IF/ IF
Takeuchi et al. [4]	*Allium cepa*	Cotyledon epidermal cell	EM (cryo)
(B) F-actin disruption causes alternation in MT organization in the PPB			
Mineyuki and Palevitz [44]	*Allium cepa*	Cotyledon epidermal cell	Ph/ IF
Eleftheriou and Palevitz [47]	*Allium cepa*	Root tip cell	IF/ IF
Granger and Cyr [59]	*Nicotianatabacum*	Suspension culture cell	Live (-/ GFP-MBD)
Li et al., [55]	*Arabidopsis thaliana*	Suspension culture	IF/ IF
Kojo et al. [60]	*Nicotianatabacum*	Suspension culture cell	Live (-/GFP-tubulin)
Takeuchi et al. [58]	*Allium cepa*	Root tip cell	IF/ IF
(C) Actins in spindle			
Forer and Jackson, Forer et al. [61–63]	*Haemanthuskatherinae*	Endosperm	EM (chem)
Seagull et al. [64]	*Medicago sativa*	Suspension culture cell	Ph/IF
Traas et al. [38]	*Daucuscarota*	Suspension culture cell	Ph/-
Traas et al. [65]	*Splanummelongena*	Miotic cell	Ph/IF
Czaban and Forer [66]	*Haemanthuskatherinae*	Endosperm	Ph/IF
(D) Actins in phragmoplast			
Clayton and Lloyd [67]	*Allium cepa*	Root tip cell	Ph/IF
Gunning and Wick [68]	*Tradescantiavirginiana*	Stamen hair cell	Ph/IF
Schmit et al. [69]	*Haemanthuskatherinae*	Endosperm	Ph/IF
Kakimoto and Shibaoka [36]	*Nicotianatabacum*	Suspension culture cell	Ph/IF
Traas et al. [38]	*Daucuscarota*	Suspension culture cell	Ph/-
Palevitz [70]	*Allium cepa*	Root tip cell	Ph
Schmit and Lambert [71]	*Haemanthuskatherinae*	Endosperm	Ph, IF/ IF, EM (chem)
Lloyd and Traas [39]	*Daucuscarota*	Suspension culture cell	Ph/IF
Kakimoto and Shibaoka [72]	*Nicotianatabacum*	Suspension culture cell	EM (chem)
Traas et al. [65]	*Splanummelongena*	Miotic cell	Ph/ IF
Schmit and Lambert [73]	*Haemanthuskatherinae*	Endosperm	Inj (Ph/-)
Zhang et al. [74]	*Tradescantiavirginiana*	Stamen hair cell	Inj (Ph/tubulin)
Collings et al. [75]	*Allium cepa*	Root tip cell	IF/IF
Collings and Wasteneys [32]	*Arabidopsis thaliana*	Root tip cell	IF/IF

EM(chem), electron microscopy with chemical fixed materials; EM(cryo), electron microscopy with cryo-fixed materials;Live, live imaging; IF, immunofluorescence method; Ph, phalloidin labeled with fluorescent dyes; Inj, microinjection of fluorescent-labeled probes

Table 2. Actin-MT cross-talks in cell division.

wall fibrils [76–78]. In pollen tubes and root hair cells, cortical MTs run parallel to the cell axis and a similar F-actin pattern has been observed by EM images (see papers marked EM in **Table 1A**). Transversely aligned cortical MTs are observed in the elongating cells of roots, suspension culture cells and fern protonemata. Transversely arranged F-actins have also been observed in these cells (**Table 1A**) as well as MFs associated with cortical MTs (see papers marked EM in **Table 1A**). In dry-cleaved preparations of tobacco protoplasts, Kengen and Derksen [15] were able to follow MT-associated MFs over distances up to 1.46 μm. The presence of MTs connected to MFs via cross-bridging molecules [10, 14] suggests that the organization of such MTs is influenced by the direct interaction with F-actins. The fact that the short F-actins can slide along MTs as seen in live cell imaging studies using fluorescent-labeled MTs and F-actins suggests an involvement of motor proteins in this cross-bridging process [20]. Pharmacological studies suggest that F-actins either play a role in cortical MT organization, or that the MTs can regulate the organization of actin (**Table 1B**, **C**).

In addition to the direct physical actin-MT interactions described in the previous paragraph, there are other MT systems in which different types of actin-MT interactions have been observed. For example, during the formation of tracheary elements in cultured cells of *Zinnia*, F-actins are formed between reticulate arrays of MT bundles. The fact that the disruption of the F-actin aggregates by cytochalasin affects the MT organization in this system suggests that the F-actin aggregates are involved in MT organization [11, 79]. Coordination of MT and actin networks is also required for morphogenesis of cells showing complex expansion patterns such as leaf pavement cells and trichomes [80–83]. Actins and MTs have been shown to serve distinct roles during the formation of the intricately shaped leaf mesophyll and epidermal cells. Thus, the cortical MT bundles appear to determine the sites of deposition of cell wall molecules that prevent cell wall expansion, whereas actin patches regulate the formation of the cell lobes [80]. Formation of separate MT and actin domains appears to be coordinated by the activity of RopGTPase [82].

During trichome morphogenesis, control of MT dynamics by actin has been inferred from studies of *distorted2* (*dis2*) mutants defective in the actin-related ARP2/3 complex. The ARP2/3 complex regulates actin polymerization and the *dis2* mutation gives rise to distorted trichomes. The mutant trichomes exhibit changes in MT organization that is similar to those seen in cytochalasin-treated cells [26, 27]. The *tortifolia2* (*tor2*) mutant has a mutation in α-tubulin4 that causes aberrant cortical MT dynamics and overbranching of the trichomes. The double mutant of *tor2* with *dis1*, another ARP2/3 complex mutant, shows complete loss of anisotropic growth, and MT organization in the mutant is severely disturbed in comparison with the respective single mutants. Based on these observations, Sambade [84] proposed that cortical MTs have two different functions, actin-dependent and actin-independent functions during trichome differentiation.

2.2. Cell division

Spindle: Forer's group has suggested that F-actins are present in the spindles of *Haemanthus* endosperm based on EM observations of chemically fixed cells decorated with heavy meromyosin as well as on fluorescent microscope observations using rhodamine phalloidin [61–63, 66]. In contrast, Schmit and co-workers [69, 71, 73] using live *Hemanthus* endosperms injected with fluorescent phalloidin probes or fixed *Hemanthus* endosperms stained with fluorescent phalloidin, reported that the concentration of F-actins is low inside spindles and that instead they form

a cage-like structure around the spindle. The actin cage has also been observed in *Tradescantia* stamen hair cells [46]. Disruption of the actin cages around spindles alters both spindle positioning and spindle orientation [60, 85].

Phragmoplast: Actins have been shown to be present in phragmoplasts by means of fluorescent dye-labeled phalloidin [36–38, 67, 68]. However, both EM and fluorescent microscope observations have shown that the organization of phragmoplast actin differs from that of the MTs [32, 71, 75, 85]. A high density of short F-actins is seen in the equatorial plane oriented parallel to the MTs. F-actins identified by heavy meromyosin decoration have also been observed near the forming cell plate, and the heavy meromyosin arrowheads on the F-actins point away from the cell plate [72].

PPB: The presence of actins in PPBs has been documented in a variety of tissues through fluorescence microscopy techniques (**Table 2A**). Single MFs associated with PPBs in high-pressure frozen cells have also been visualized by EM [4, 45]. The development of PPBs is an interesting process in terms of actin-MT interactions, because actins are associated with MTs during the early developmental stages but are absent from later stages. This is illustrated in **Figure 1**. PPBs develop from broad bands of MTs in the G2 phase of the cell cycle. Cells exhibiting an early broad PPB also possess a broad cortical F-actin array (Interphase in **Figure 1**),

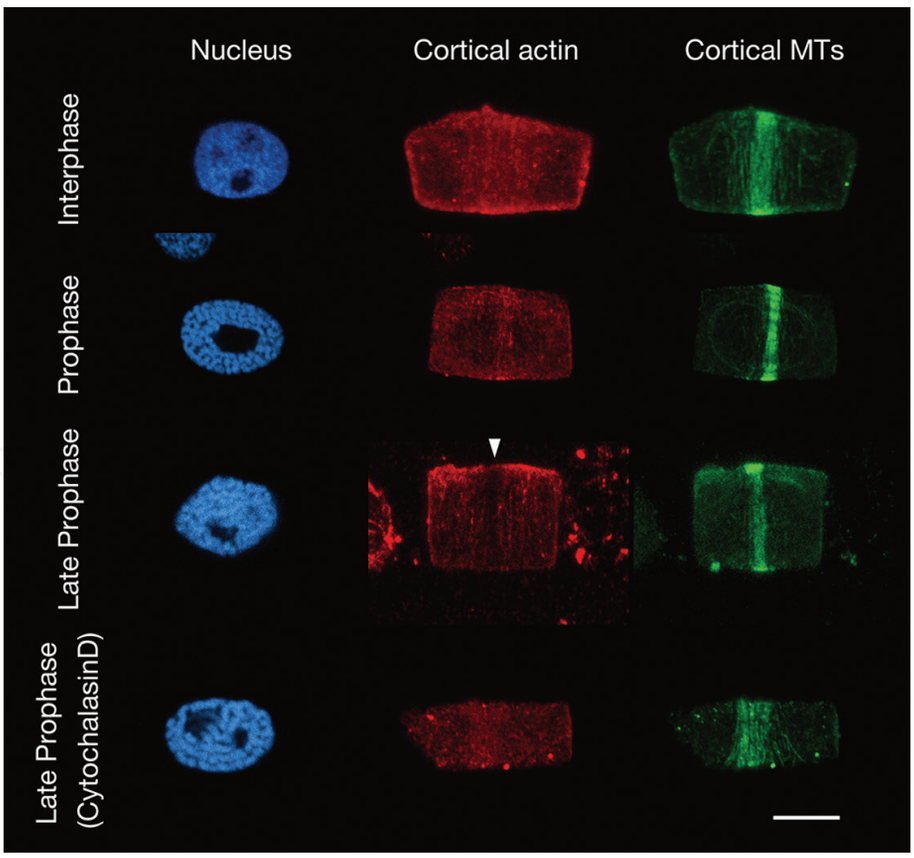

Figure 1. Actin-MT cross-talk during PPB development in root tip cells of onion seedlings. Cells were triple labeled for actin (red), tubulin (green) and DNA (blue). Interphase, the broad PPB MT and actin. Prophase, the narrow PPB MT and actin. Late prophase, a narrow PPB with ADZ (arrowhead). Late prophase (Cytochalasin D), a late prophase cell treated with 20 μM cytochalasin D for 30 min. Scale bar: 10 μm. Modified from Ref. [58].

and in the subsequent period, the actin band narrows in parallel with the narrowing of the PPB MT band (Prophase in **Figure 1**). However, when the PPB MTs reach their narrowest configuration in late prophase (Late prophase in **Figure 1**), the fluorescent actin signals start to disappear from the cell cortex region occupied by the MT band, giving rise to the actin-depleted zone (ADZ) [46, 48]. The ADZ forms prior to the breakdown of the nuclear envelope and persists throughout mitosis [46, 48]. It is known that F-actin disrupters, such as cytochalasins and latrunculins, not only prevent narrowing of the PPB MT but also rewidening of narrowed MT band (see references in **Table 2B**). The effect of cytochalasin D on PPB actin is unique. When cytochalasin D is applied to late prophase cells, PPB MT widening occurs and actins stay in the broadened PPB instead of disappearing from the PPB region (Late prophase (cytochalasin D) in **Figure 1**).

3. Evidences for MF-MT association revealed by electron tomography

3.1. Visualization of single F-actins in electron micrographs

Bundled MFs in plant cells were first observed in streaming *Nitella* cells by Nagai and Rebhun [86]. Subsequently, the MFs were identified as F-actins by heavy meromyosin decoration [87]. Since then, MFs have been observed in a variety of plant cells [6–8, 10, 14, 16, 45, 88–91]. Murata et al. [19] optimized the condition to preserve and visualize MFs in the cortical cytoplasm of plant cells. They applied high-pressure freezing/freeze-substitution followed by post-fixation with OsO_4 and uranyl acetate to achieved stable preservation and high contrast of the fine MFs in both tobacco root tips and onion cotyledons (**Figure 2**). A single, ~1-μm-long MF preserved by this method is illustrated in **Figure 2B**.

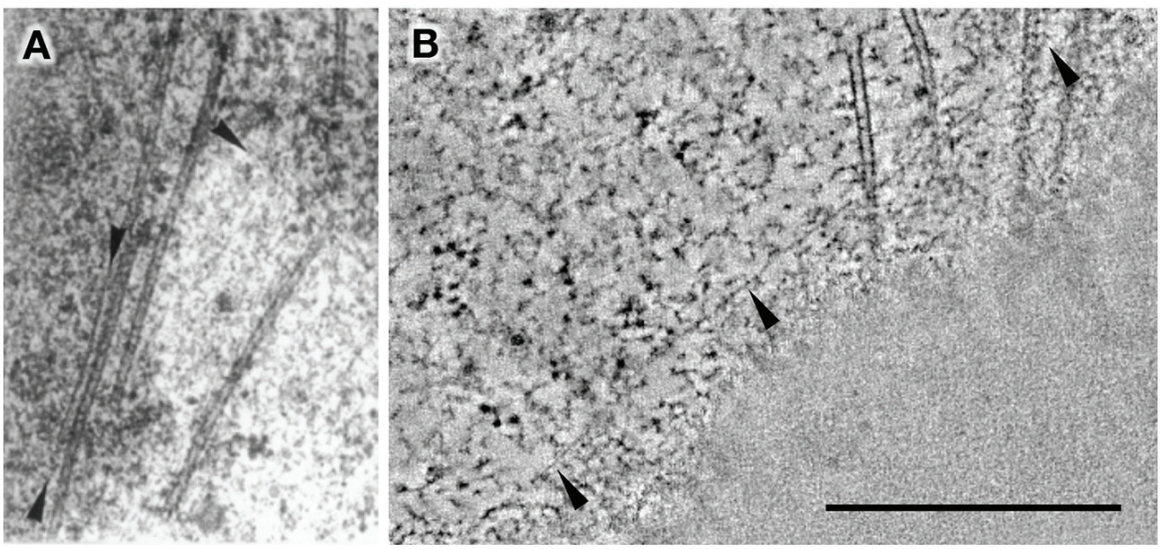

Figure 2. MFs in the high pressure frozen/freeze-substituted plant cell cortex. (A) A tobacco root tip cell. (B) An epidermal cell of an onion cotyledon. Arrowheads, MFs. Bar = 500 nm. (A) Modified from Ref. [19], (B) modified from Ref. [4].

3.2. Spatial relationship between MFs and MTs during PPB development

Electron tomography is a powerful method for visualizing and quantitatively analyzing the ultrastructural features of cells in 3D. The 3D volume is reconstructed from a series of 2D-EM images of successively tilted samples. It enables researchers to analyze the 3D organization of filamentous structures in cells and has been applied to study of F-actin networks in lamellipodia [92] and MTs in hyphae [93]. Electron tomography has also been applied to cell division studies in plants, most notably to obtain quantitative information on the organization of cortical MTs as well as cell plate forming structures [94–96].

PPB development in epidermal cells of onion cotyledons is well-characterized [96, 97], and this experimental system has proven advantageous for elucidating the events associated with PPB formation. In particular, it has enabled us to characterize the organization of MFs in the PPBs of onion cotyledon epidermal cells by means of electron tomography of high pressure frozen/freeze-substituted tissues. For these studies, a basal part of the cotyledon was cut and immediately high-pressure frozen, then samples were freeze-substituted and embedded in Spurr's resin [19]. Tomograms were generated from the tilted image series of 250-nm-thick serial sections of PPB-containing cells, and the tomograms were analyzed with IMOD [98]. The tomograms obtained by these means covered a ~4 μm^2 large area of the cell cortex and enabled us to obtain quantitative information on the distribution of coated and non-coated vesicles in PPBs, the number and length of PPB MFs, as well as on the spatial relationship between individual fibers of MFs and MTs in the PPBs [4, 96]. During the course of these studies, we made approximately 50 serial sections for each cell, which enabled us to determine both the nuclear stage and the corresponding developmental stage of the PPB for each cell used for the electron tomography analysis. The results are reported in Ref. [4] and are briefly summarized in **Figure 3**.

Tomographic models of **Figure 3A–E** show changes in MT and MF organization during this process. As reported previously [44], most cortical MTs (magenta lines) are not arrange transversely in interphase cells of the basal region of the onion cotyledon epidermis and very few MT-MF interactions are seen (**Figure 3A**). At the onset of PPB formation, the MTs become organized into loose, irregular arrays transversely oriented to the longitudinal axis of the cells and the first MT-associated MFs (yellow lines) start to appear (**Figure 3B**). During this MT reorganization, groups of two or three MTs initiate the formation of MT bundles by moving closer together and becoming more aligned (**Figure 3C**). Then, pairs of closely aligned MTs serve as templates for the assembly of increasingly large MT clusters (**Figure 3D**). A majority of the MFs become aligned with and many become bound to the MTs. This spatial relationship is maintained until the MT band narrows. In late prophase, the density of the MFs declines within the PPB region (bracketed area) compared to the area outside of the PPB where the cortical MFs are randomly oriented (**Figure 3E**, **F**). This stage appears to correspond to late prophase during which the ADZ is formed (**Figure 1**). As the behavior of MFs is similar to those reported in F-actins by fluorescent microscopy, the MFs described here seem to correspond to F-actins. MFs in PPBs were single, relatively short (168 ± 14 nm) filaments. In contrast, the longer (>500 nm) MFs were seen only in the cortex of interphase cells and were not bound to MTs (**Figure 2B**). This suggests that single short F-actins play an essential role in the formation of actin-MT interactions during PPB formation.

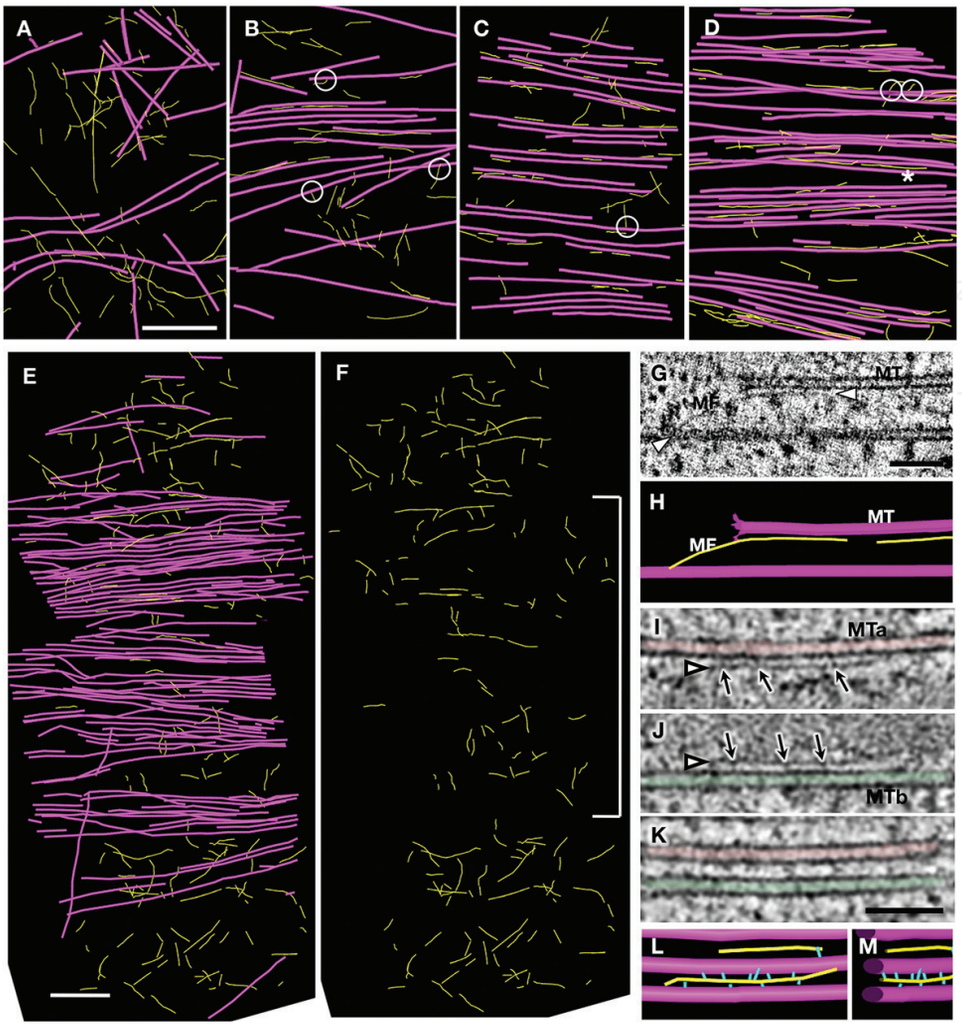

Figure 3. Changes in MT-MF interactions during PPB formation of onion cotyledon epidermal cells. All images are adapted from Figures 1, 3 and 4 in Ref. [4]. (A)–(F) Electron tomographic models showing the distribution of MFs (yellow lines) and MTs (magenta lines) in the cortical cytoplasm of tangentially sectioned cells. Circles, Contact sites between a MF end and a MT. Asterisk, A MF running along two MTs that is linked with these MTs. Bar = 500 nm. (A) Interphase cell with randomly oriented cortical MTs and MFs. (B) Interphase cell with some transversely oriented MTs. (C) Early prophase cell with a broad PPB. (D) Prophase cell containing a narrow PPB. (E) Late prophase cell with a narrow PPB. (F) Same model as shown in (E) but without the MT images. White bracket, the PPB region. (G)–(M) Images of MFs from tomograms of tangentially sectioned cell cortex regions. Bars = 100 nm. (G) A tomographic slice image showing a MF (arrowheads) associated with a cortical MT. (H) A tomographic model showing the MF and MTs illustrated in (G). (I)–(K) Three tomographic slices from a small volume of a PPB. (I) and (J) The single MF (arrowhead) is bound to two adjacent MTs, MTa (I) and MTb (J), by cross-bridges (arrows). (L) and (M) Tomographic models showing arrangement of the two MTs and one MF illustrated in (I)–(K).

Detailed analyses of the PPB tomograms revealed several interesting features of the MF-MT interactions. Single short MFs whose one end attaches to the surface of a MT are seen when PPB formation starts (circles in **Figure 3**). Subsequently, single MFs are seen running along MTs as they become attached to the MTs by cross-linkers. Two types of MF-MT cross-linkers can be distinguished. Short cross-bridges (~14 nm long) appear in early prophase and slightly longer ones (~17 nm long) during the later stages of PPB maturation [4]. However, the number

of MFs forming bridges between MTs is typically low during the early stages of PPB formation but increases as the development of the PPBs progresses. Similarly, during the earliest stages of PPB formation, the length of the majority of MFs is 50–100 nm. As the PPB matures, the length of the MFs increases suggesting that they are elongated while attached to the MTs. Two types of single MFs connecting adjacent MTs are seen. One type of MF forms bridging structures between MTs (**Figure 3G**, **H**), whereas the other consists of MFs running between two adjacent MTs, with close connections to both MTs. Interestingly, the linkers to the two MTs are not in the same plane (**Figure 3I–M**). When the PPBs mature, the number of MT-associated MFs decreases, whereas the number of MT-MT interactions increases. The MT-MT bridges form tight ladder-like connections between the MTs.

Based on these observations, we have developed a hypothesis for the role of short F-actins on MT bundling during PPB formation ([4], **Figure 4**). Actin-MT interactions in PPBs start when one end of a short F-actin fragment (~70 nm) attaches to a MT (**Figure 4A**). The F-actin fragment then becomes aligned along the MT by linkers (**Figure 4B**) and starts elongating (**Figure 4C**). Elongation of the MT-associated MFs enables them to connect two MTs that are separated by spaces that are too big to be bridged by MT-MT linker proteins (**Figure 4D**). As the zippering up of the MF-linked MTs continues, the MTs come closer together with the MFs becoming sandwiched between the two parallel MTs to which they are connected through alternating cross-links (**Figure 4E**). The MF mediated cross-links are replaced by direct MT-MT bridging structures (shown in Figure 7 in Ref. [4]) and the MFs disappear from the dense MT arrays. Based on their length, the MT-MT linkers can be divided into two groups. Long linkers (**Figure 4F**) are dominant during the early stages of PPB formation and are replaced by the short ones as the MT band narrows (**Figure 4G**). Although the molecules that form the linkers have yet to be positively identified, several MT associated proteins have been localized to PPBs [99, 100].

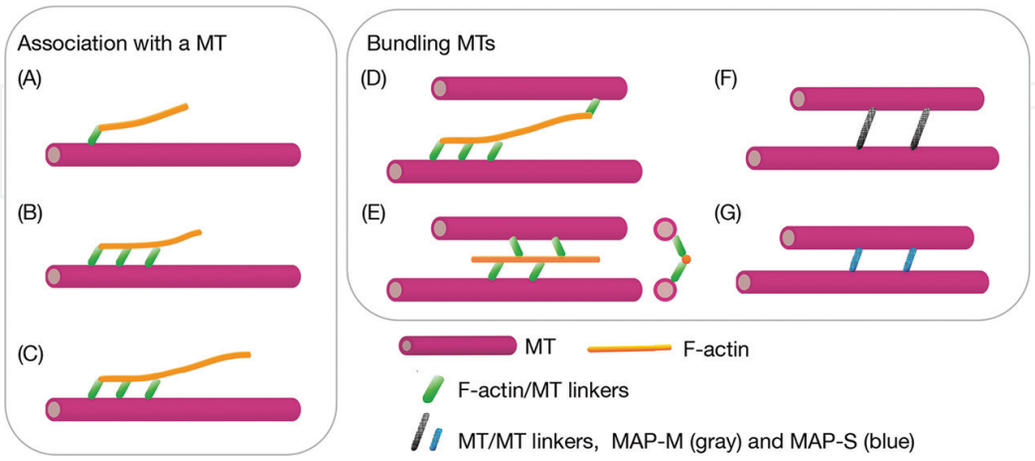

Figure 4. A model showing how single F-actins bind to MTs and contribute to MT bundling during the early stages of PPB formation. (A)–(C) Development of MT-associated MFs. (D) and (E) Two types of F-actin-MT connections between two adjacent MTs. (F) and (G) Two types of MT-MT connection by MAPs. Adapted from Ref. [4].

4. Molecular candidates involved in actin-MT interaction

The interaction between actins and MTs is mediated by linkers that connect the MFs to the PPB MTs. Here, we discuss several candidate proteins capable of binding to actins and to MTs and thereby control their organization and functions. The list includes several kinesins, formins and MT-associated proteins [101, 102].

Kinesins: One group of candidate proteins capable of forming actin-MT bridges belongs to the kinesin-14 family of proteins with actin-binding and calponin-homology domains (KCHs) reviewed in Ref. [103]. KCHs have been identified as plant-specific kinesins and belong to the minus end-directed kinesin subfamily. Members of the KCH protein family capable of forming actin-MT cross-bridges have been identified in a variety of plant species and tissues, in cotton fibers [104, 105], rice coleoptiles [106] and tobacco cells [106]. Two kinesin-14 family proteins, KingG [107] and NtKCH [108] localize to PPBs. Localization of the KingG also suggest its movement towards the minus-ends of MTs in vivo [107]. Although their function in the PPB is unknown, the KCHs may function to align F-actin to MTs and hereby contribute to the formation of MT bundles.

Formins: In plants, formins constitute a large family of proteins. Their primary function is to serve as regulators of the actin cytoskeleton. Since some plant formins have the ability to nucleate F-actins, they are considered candidate proteins for initiating the assembly of PPB F-actins [109–112]. Furthermore, considering that some formins have been shown to also possess MT-binding activity [113], they may play a role in generating MT-associated F-actins as described by Sampathkumar et al. [20]. The class II formin isoform AFH14 of *Arabidopsis* is a candidate protein for generating links between actin and MTs in PPBs, since it has been localized to PPBs. AFH14 can bind directly to and bundle actin and MTs via its FH1FH2 domain, which is a highly conserved site in formin family proteins. In addition, it shows cross-linking activity between F-actin and MT [114]. Another class II formin, the rice formin FH5 has been shown to interact with actin and MT [115, 116]. Binding of the *Arabidopsis* AtFH4 to MTs is mediated by the GOE domain, a conserved domain in the class I subfamily of *Arabidopsis* formins [117]. AtFH1, which is the main housekeeping formin in *Arabidopsis*, has also been suggested to participate in actin-MT cross-talk. Mutations of the *AtFH1* affect root cell expansion, root hair morphogenesis, and cytoskeleton structures and alter the dynamics of actin and MTs even as it lacks known MT-binding motifs [118].

Actin-related protein-2/3 (ARP2/3) complex: The ARP2/3 complex is another regulator of the F-actin in plants that can initiate actin polymerization as well as control F-actin organization and thereby cell shape. In contrast to formin that promotes the nucleation of unbranched filaments, ARP2/3 functions as an initiator of new F-actins that branch off of existing filaments [119–121]. Mutants defected in ARPC2, a subunit of ARP2/3 complex, displays aberrant trichomes, and the organization of both actin and cortical MTs is disturbed in the mutants [26, 27]. Control of the actin and MT cytoskeletal networks mediated by the ARP2/3 complex was also implied in a study of the SCAR2 mutant [122]. Recently, Havelkova et al. [123] reported that ARPC2 binds directly to MTs, suggesting a new mechanism of actin-MT interaction mediated by the ARP2/3 complex.

Other candidates: Phospholipase D (PLD) belongs to a superfamily of signaling enzymes that are associated with the plasma membrane. A PLD from tobacco has been postulated to mediate MT-plasma membrane linkages [124] and PLD activation correlated with MT reorganization [125]. PLDδ is detected in the periphery of *Arabidopsis* suspension culture cells, and it binds both to MTs and actin in vitro [126]. The PLDδ may play a role in initiating cytoskeleton remodeling. A pollen-specific MT-associated protein (MAP), SB401 in *Solanum* localizes to the cortical cytoplasm of pollen tubes where it binds to and bundles MTs and F-actins [127, 128] and they are possibly involved in F-actin and MT organization. The CLIP-associated protein (CLASP) is a MT-plus end directed motor protein. It is involved in both cell division and cell expansion, and in the organization of the cortical MTs [129, 130]. Although its interaction with actin in plants is unclear [129], human CLASP molecules have been reported to function as actin/ MT cross-linkers in interphase cells [131].

5. Conclusion

How actin-MT interactions contribute to cellular activities in plants is poorly understood. To address this question, we have employed electron tomography to examine the 3D relationship between MFs and MTs during PPB formation. At the onset, short MFs form bridges between adjacent MTs thereby starting the process of PPB narrowing. The narrowing process initially involves two types of MF-MT linkers. During later stages, the actin-mediated cross-links disappear and are replaced by two types of MT-MT linkers that act sequentially to complete MT bundling. The focus is now on identifying the different types of cross-linkers.

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