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Critical role of actin bundling in plant cell morphogenesis

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ABD2, actin binding domain 2 of fimbrin; ADF, actin depolymerization factor; FP, fluorescent protein; GFP, green fluorescent protein; IAA, indole-3-acetic acid; mTn, actin binding domain of mouse talin; NAA, 1-naphthalene acetic acid; TIBA, 2,3,5-triiodobenzonic acid; YFP, yellow fluorescent protein

Actin microfilaments form highly-organized structures, such as arrays, meshwork and bundles, and are involved in cell division, organelle motility and intracellular transport in plants. Such organization is indicative of their vital role in directing plant cell morphogenesis. However, the physiological impact of changes in actin bundling level has been unclear until recently, despite progress in our understanding of their roles following actin microfilament disruption. Recent advances have shown that actin bundling levels can be modified through expression of actin binding proteins, or by using auxin transport inhibitors or auxin itself. The technical aspects of these findings have underscored the importance of actin bundling levels in plant cell morphogenesis, including cell division, cell elongation/expansion and stomatal movements. Based on such progress, we discuss the possible mechanisms that connect the actin microfilaments status with cellular dynamics.

Introduction

Actin microfilaments are crucial cellular components of eukaryotic cells and function in multiple cellular processes, most notably cell morphogenesis, cell motility and intracellular transport. Such functions are closely related to their highly-ordered organization, such as in arrays, meshwork and bundles. Classically, actin microfilaments have been visualized by electron microscopy or fluorescent labelling of fixed or microinjected cells. While these classical approaches are still useful, especially in organisms that are unamenable to genetic transformation, the actin microfilaments are highly sensitive to chemical and physical fixation protocols and so the final appearance of the microfilaments may vary significantly between expert individuals and laboratories. Actin bundling, in particular, is difficult to address by classical visualization techniques, as artificial bundles are often induced by chemical fixation or phalloidin labelling. Labelling of actin microfilaments with fluorescent proteins (FPs), including the green fluorescent protein (GFP) and yellow fluorescent protein (YFP), have revealed the more intact organization and dynamic behavior of actin microfilaments. Importantly, visualization by such live imaging techniques has promoted an almost global consensus about actin organization, and has provided the opportunity to critically examine their detailed organization and bundling. Recent improvements in actin microfilament reporter systems and progress in the study of auxin transport carriers have enabled us to artificially induce actin bundling in living cells. These technical advances are providing new insights into the importance of actin bundling in plant cell morphogenesis.

In this article, we first introduce recent actin bundling techniques in the context of actin reporters and auxin signaling. Thereafter, the significant roles of actin bundling in plant cell morphogenesis, including cell division, cell elongation/expansion and stomatal movements, are considered. Due to space limitations, recent advances in the actin bundling proteins are not addressed, but the reader is referred to our previous review[1] or to a more recent and specific review by Thomas et al. (2009).[2]

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Induction and Visualization of Actin Bundling through Expression of FP-mTn

FP fused to the actin binding domain of mouse talin (FP-mTn) is a popular actin reporter in plants,[3] although recent studies have shown that FP-mTn induces extra-bundling of actin microfilaments.[4-8] Ketelaar et al. (2004), using in vitro actin microfilament co-sedimentation assays, demonstrated that FP-mTn inhibits the actin depolymerization activity of actin depolymerization factor (ADF).[4] As ADF disfunction does not directly result in actin bundling, as shown by real-time in vitro visualization systems,[9] the molecular mechanisms of FP-mTn-mediated actin bundling appear to be more complex and related to other components, such as actin bundling proteins.[1,2] It has been recognized that FP-mTn overexpression tends to perturb cellular functions, including root hair tip growth,[4] organelle motility,[5] synchrony of cell division,[6] gravitropism[7] and stomatal opening.[8] Consequently, FP-mTn expression techniques have helped identify the relationships between actin bundling and various plant cellular events

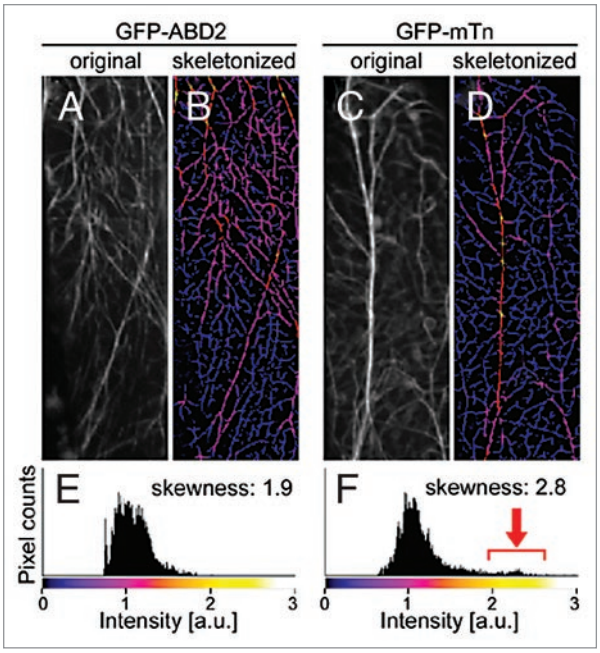

Figure 1. Differences in actin organization between Arabidopsis hypocotyls cells expressing GFP-ABD2 and GFP-mTn. GFP-mTn-labelled actin microfilaments (C) have more bundled forms than GFP-ABD2-labelled microfilaments (A). The bundling was quantitatively evaluated by the image processing techniques reported by Higaki et al.[8] First, we obtained skeletonized images (B and D) in order to collect pixels constituting the actin microfilaments. Intensities of the skeletonized actin microfilament pixels are shown by the pseudo colors. As visualized by the intensity histograms, the number of high intensity pixels in the skeletonized image of GFP-mTn-labelled actin microfilament bundles (F, red arrow) is greater than that in the image of the GFP-ABD2-labelled actin microfilament array (E). As the high intensity pixel numbers increase, the skewness of the intensity distribution also increases.

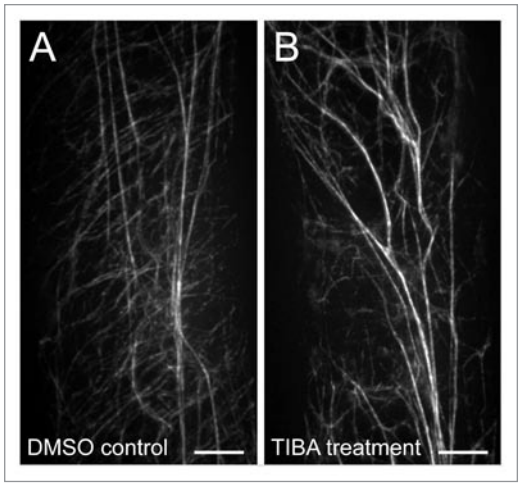

Figure 2. Effects of TIBA treatment on actin organization in tobacco BY-2 cells. Treatment with 10 μM TIBA for 180 min induced extra-bundling of GFP-ABD2-labelled actin microfilaments (B) compared with the DMSO control (A). Scale bars indicate 5 μm.

as described below. Caution, however, is required when interpreting data in cases where high FP-mTn expression levels are achieved throughout the plant using constitutive promoters and the resulting secondary effects on various tissues. Clearly, therefore, cell/tissue-specific expression techniques would be a preferable approach.

In contrast, FP fusion to the actin binding domain 2 of fimbrin (FP-ABD2) has less secondary effects on actin bundling than in a FP-mTn expression line (**Fig. 1**).[8] Although bundling can be evaluated qualitatively, it is also important that it be assessed quantitatively since manual evaluations have far less objectivity. Nick et al. (2009) quantitatively evaluated the thickness of actin filaments using a fluorescent intensity profile across the filaments.[7] Furthermore, we recently reported that the skewness of the fluorescent intensity distribution of the cytoskeletal signals extracted from a microscopic image is useful for evaluating the cytoskeletal bundling level, and that this approach has confirmed that GFP-ABD2 has less effect on actin bundling (**Fig. 1**).[8] However, as organelle motility also appears to be slightly suppressed in an FP-ABD2 expression line,[5] overexpression of FP-ABD2 also entails some risk of inhibiting actin functions. Clearly, careful attention must be paid to the level of expression and the phenotypes of the overexpression lines. FP-fusion with Lifeact, which has recently been reported as a new actin microfilament reporter,[10,11] also should be investigated for its secondary effects in the case of overexpression.

Effect of an Auxin Transport Inhibitor and Auxin on Actin Bundling

Although the mechanism of action of 2,3,5-triiodobenzonic acid (TIBA), which is widely known as an auxin transport inhibitor, had been unclear for many decades, recent live imaging studies on auxin transport carriers has revealed its actin-mediated action. The dynamic cycling of the auxin efflux carrier, PIN1, between the plasma membrane and endosomes is sensitive to TIBA,[12] whereas its plasma membrane localization is less sensitive.[12-14] On the other hand, TIBA is more effective on both the cycling and plasma membrane residence of the auxin influx carrier, AUX1.[13] Through the use of the actin depolymerization inhibitor, cytochalasin D, it was found that PIN1 cycling is sensitive to actin,[12] suggesting that TIBA could target the rearrangement of actin microfilaments. Dhonukshe et al. (2008) clearly demonstrated that TIBA effectively induces actin bundling and actin microfilament dynamics (**Fig. 2**).[15] Importantly, the bundling effect of TIBA has been confirmed not only in plant cells but also in yeast and mammalian cells, suggesting that TIBA primarily influences actin microfilaments.[15] Differences in the sensitivity of PIN1 and AUX1 localization to TIBA suggest the existence of multiple membrane trafficking mechanisms that have different actin contributions. The effects of TIBA on the cycling and localization of other plasma membrane proteins would be an attractive subject for future studies.

Auxin itself has also been proposed as an effector of the actin bundling level. Exogenous addition of auxin, including indole-3-acetic acid (IAA) and 1-naphthalene acetic acid (NAA), but not of 2,4-dichlorophenoxyacetic acid (2,4-D), has been shown

to induce dissociation of FP-mTn-labelled actin bundles in rice coleoptiles[7,16] and tobacco BY-2 cells.[6] Fluorescent-labelled phalloidin staining has also demonstrated that IAA treatment did not cause prominent debundling in epidermal cells of wild type tobacco petioles, but did so in an actin-bundled tobacco mutant that had been isolated as an activation tag line showing aluminum tolerance.[17] Therefore, IAA and NAA could cancel out the extra-bundling, such as in FP-mTn expression lines, but not in cells in which the actin filaments were fully arranged in fine arrays. In contrast, IAA and NAA, as well as TIBA, induced actin bundling in Arabidopsis root cells.[14] Interestingly, 2,4-D, which is less effective in rice coleoptile and tobacco BY-2 cells,[6,7] induces fragmentation of actin microfilaments similar to the actin polymerization inhibitor, latrunculin B.[14] Such differences in auxin effects on actin microfilaments may be due to variation in auxin sensitivity between the shoot and root. Clearly, however, further studies using various tissues and auxin-signaling mutants are needed to clarify the relationship between auxin and actin.

Cell Division, Cell Elongation/Expansion and Actin Bundling

Although the involvement of actin microfilaments in cell division has been energetically pursued using classical as well as more recent live imaging techniques and pharmacological approaches that disrupt the actin microfilaments, our understanding of the relationship between actin bundling and cell division is fairly recent. YFP-mTn-induced actin bundling impairs the synchrony of tobacco BY-2 cell division, and exogenous addition of IAA or NAA cancels this actin bundling and cell division abnormality,[6] suggesting that actin bundling is involved in cell division. However, actin bundling induced in Arabidopsis roots by IAA, NAA or TIBA treatment does not affect the cell division rate, whereas disruption of actin microfilaments decreases the cell division rate,[14] and this implies that actin bundling is not directly linked with the cessation of cell division. On the other hand, cell cycle-dependent rearrangements of actin microfilaments have been investigated by GFP-ABD2 expression in tobacco BY-2 cells.[1] Actin bundles running in the transvacuolar strands generally appear at the G_1 phase[18] but then disappear in mitosis. Despite the bundles, fine meshworks form in the cell cortex at metaphase[19] and fine short filaments accumulate around the forming cell plate at telophase.[20] Thus, the actin bundling level appears to be strictly regulated by the cell cycle, although the significance of actin debundling in mitosis is still currently unclear. These questions will certainly be addressed by future imaging studies that focus on the effects of artificial actin bundling on cell division determination, mitotic spindle formation and phragmoplast dynamics.

Cell elongation/expansion is also thought to involve actin bundling. TIBA-, IAA- or NAA-induced actin bundling inhibits the cell elongation rate in Arabidopsis roots.[14] YFP-mTn-induced actin bundling also suppresses cell elongation in relation to the gravitropism of rice coleoptiles.[7] On the other hand, actin bundles have been observed near the cell cortex and large vacuolar surface in elongated BY-2 cells,[18,21] Arabidopsis elongated hypocotyls cells and in large leaf epidermal cells,[1,22,23] and display a very dynamic behavior.[1,18,22] These observations suggest that the dynamic rearrangement of actin bundles is important for cell elongation and also for the maintenance of intracellular trafficking in elongated cells. This assumption is supported by the finding that actin bundles become looser during the rapid turgor movements, seismonastic movements, of *Mimosa pudica*.[24] In BY-2 cells treated with the proteinacious elicitor, cryptogein, the vacuoles become large and form simple shapes prior to vacuolar rupture and induction of programmed cell death.[25] At vacuolation, the heavily-bundled actin microfilaments transiently appear in the cell cortex despite the actin bundles that run in the transvacuolar strands. While it might appear difficult to simply compare cell elongation with vacuolation in the execution in programmed cell death, these observations implicate the actin bundling levels in turgor pressure regulation.

Actin Bundling and Stomatal Opening: Our Hypotheses

We recently reported that through the quantitative evaluation of GFP-ABD2-labelled actin microfilament organization in Arabidopsis guard cells, transient actin bundling was found to occur in stomatal opening.[8] Furthermore, GFP-mTn-expressing guard cells displayed heavily bundled actin microfilaments and their stomatal opening was suppressed during the diurnal cycles.[8] Three-dimensional morphometrical analysis further showed that guard cells elongated when stomata opened,[26] thus also clearly demonstrating the importance of dynamic actin bundling in guard cell elongation. Indeed, the involvement of actin dynamics during guard cell movement has been suggested through various pharmacological, imaging and molecular genetic studies (for details see the excellent review by Galatis and Apostolakos (2004)).[27] Notably, Liu and Lian (1998) proposed a potassium channel-based positive feedback model in which actin microfilaments function as osmosensors, by self-disruption in response to guard cell swelling, so that potassium influx is consequently promoted and stomatal opening accelerated.[28] Stretch-activated calcium channels that are activated by actin microfilament disruption have been proposed as a brake of the positive feedback mechanism.[29] The positive feedback model is based on the following experimental observations; (1) the actin polymerization inhibitor, cytochalasin D, promotes potassium influx in guard cell protoplasts under hyperosmotic conditions but has no such effect in hypoosmotic conditions; and (2) hypoosmotic stress induces actin microfilament disruption in guard cell protoplasts. These experimental observations are basically supported by pharmacological studies in different experimental systems,[30-32] but actin disruption has not been observed in stomatal opening.[8,30,31] We therefore proposed a revised positive feedback model of Liu and Lian (1998),[29] in which actin debundling occurs in response to increased cell volume and accelerates stomatal opening.[8] Cytochalasin occasionally induces a reduction in actin bundles, and this supports our revised model.[33]

How then does dissolution of actin bundling promote stomatal opening? Although at present we have no direct experimental

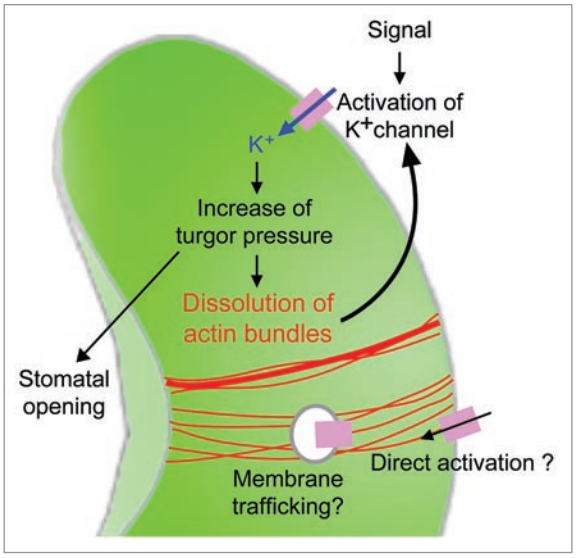

Figure 3. Model for pathway by which actin bundle dissolution promotes stomatal opening. This model is based primarily on the original model proposed by Liu and Luan (1998)[28] and as revised on the basis of our recent observations that actin microfilaments are transiently bundled in the process of stomatal opening and in suppression of stomatal opening in the GFP-mTn expression line.[8] Actin bundle dissolution accelerates the increase in guard cell volume and stomatal opening by activation of potassium channels via direct binding to the channels or modification of membrane trafficking of the channels.

evidence, we would like to propose two possible mechanisms that may explain how actin bundle dissolution induces activation of potassium channels in guard cells (**Fig. 3**). One possible mechanism is through the modulation of potassium channel activity by the dissolved actin bundles. In animal cells, actin microfilaments have been proposed to directly interact and modulate ion channels, including sodium,[34-36] chloride[37] and potassium[38] channels. Therefore, the dissolved actin microfilament bundles may actually attach to the channels in guard cells and modulate their activity. This possibility is supported by the radially-striped localization pattern of a potassium channel KAT1-GFP fusion[39] that resembles the radial patterns of dissolved actin microfilament bundles in guard cells of open stomata. The other possible mechanism of potassium channel activation in guard cells is through trafficking of the channels, as proposed in the case of auxin transport carriers, as previously described. The involvement of actin microfilaments in the translocation of water channel aquaporins between the plasma membrane and endocytic vesicles in animal cells is well documented.[40-42] In plant guard cells, KAT1 localizes to both the plasma membrane and endosomes,[43,44] indicative of the importance of membrane trafficking in guard cell ion homeostasis. The attachment of endosomes to actin microfilaments has been observed in root hair cells and cytokinetic BY-2 cells,[20,45] while myosin VIII also co-localizes to endosomes in root and leaf cells.[46] These results suggest an actin-based mechanism of endosome movement in plants. Taken together, these results imply that the dissolution of actin bundles stimulates membrane trafficking and increases the number of activated potassium channels in the plasma membrane of guard cells. Future studies on the co-localization of actin microfilaments and channels and their dynamics will allow us to clarify the mechanism of actin microfilament dissolution-driven promotion of stomatal movement.

Perspective

Although recent progress has revealed the importance of the actin bundling level in cell division, cell elongation/expansion and stomatal movements in plants, there are still several missing links in our understanding of their detailed mechanisms. Endomembrane dynamics, including the cycling of plasma membrane proteins, would be a promising area of study to resolve these issues. On the other hand, actin microfilaments are known to be involved in other cellular components, such as organelles, microtubules and cell wall.[47] Thus, extensive studies on the dynamics of various cellular components would undoubtedly contribute to our further understanding of actin bundling roles in plants.

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