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Understanding Cytoskeletal Dynamics During the Plant Immune Response

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Abstract

The plant cytoskeleton is a dynamic framework of cytoplasmic filaments that rearranges as the needs of the cell change during growth and development. Incessant turnover mechanisms allow these networks to be rapidly redeployed in defense of host cytoplasm against microbial invaders. Both chemical and mechanical stimuli are recognized as danger signals to the plant, and these are perceived and transduced into cytoskeletal dynamics and architecture changes through a collection of well-recognized, previously characterized players. Recent advances in quantitative cell biology approaches, along with the powerful molecular genetics techniques associated with *Arabidopsis*, have uncovered two actin-binding proteins as key intermediaries in the immune response to phytopathogens and defense signaling. Certain bacterial phytopathogens have adapted to the cytoskeletal-based defense mechanism during the basal immune response and have evolved effector proteins that target actin filaments and microtubules to subvert transcriptional reprogramming, secretion of defense-related proteins, and cell wall–based defenses. In this review, we describe current knowledge about host cytoskeletal dynamics operating at the crossroads of the molecular and cellular arms race between microbes and plants.

OVERVIEW

Recent advances in quantitative fluorescence microscope imaging, molecular genetic approaches, and model systems for studying plant-microbial interaction have illuminated a myriad of intracellular responses that allow host plants to defend themselves against microbial invaders. In particular, new evidence for polar transport of defense molecules to the plasma membrane (PM), vesicle trafficking, and cytoskeletal rearrangements demonstrate the multifaceted and rapid nature of intracellular and apoplastic defenses in host plants (4, 24). Much of this evidence comes from the *Arabidopsis-Pseudomonas* pathosystem, but exploration of other systems is extending the list of conserved defense signaling components to agronomic crops. One noteworthy advance is the identification of several actin-binding proteins (ABPs) that perceive early hallmarks of defense signaling and alter actin cytoskeletal dynamics to markedly increase the overall abundance of filaments during the innate immune response. The importance of cytoskeletal remodeling in host cells is further emphasized by the elucidation of bacterial effector proteins that are deployed to target microtubule and actin filament organization or turnover. Here, we review current understanding of the cytoskeleton as a platform for sensing and transducing signals during plant defense responses.

A MULTILAYERED DEFENSE SYSTEM RECOGNIZES MICROBIAL INVADERS

Unlike animals, plants lack specialized immune cells and rely on innate immunity exclusively to defend against microbial invaders. Through evolution, plants have developed a multilayered immune system to protect individual cells against pathogen attack and infection (7, 18). The first layer of defense involves the perception of danger signals by PM-localized pattern recognition receptors (PRRs). These danger signals can be either nonself molecules, known as microbe-associated molecular patterns (MAMPs), or host-derived damage-associated molecular patterns (DAMPs) that are released upon pathogen perception or pathogen-induced cell damage (7, 107). The best-characterized class of PRRs comprises an extracellular domain with leucine-rich repeats (LRRs), a single transmembrane spanning motif, and a cytoplasmic kinase domain, referred to as an LRR-RK (receptor kinase) (77, 107). One example, FLAGELLIN-SENSING2 (FLS2), binds to bacterial flagellin or N-terminal peptide mimics (e.g., flg22) (27). Another example, the EF-Tu RECEPTOR (EFR), recognizes bacterial elongation factor Tu or the peptide mimics elf18 and elf26 (128). Fungal chitin, β-1,4-linked polysaccharides of GlcNAc, and bacterial peptidoglycans are perceived by receptors containing extracellular lysin motifs (LysM) and an intracellular protein kinase domain (12, 83, 116, 117). Upon ligand binding, PRRs form complexes with additional integral membrane and cytoplasmic protein kinases to further activate a basal resistance response called pattern-triggered immunity (PTI). One coreceptor shared by many PRRs as well as phytohormone receptors, BRI1-ASSOCIATED KINASE (BAK1), is also an LRR-RK (11, 15, 34, 97). Early hallmarks of PTI include cytosolic acidification, changes in cytoplasmic streaming patterns, rapid generation of reactive oxygen species (ROS), transient increases in cytosolic Ca^{2+}, and enhanced phospholipid turnover as well as activation of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) phosphorylation cascades. Long-term responses include transcriptional reprogramming, secretion of pathogenesis-related (PR) proteins, and callose deposition (77). PTI normally prevents nonadapted microbes from infecting plant cells and tissues and is an important barrier against disease.

Pathogenic microbes, in turn, have evolved to deliver a plethora of virulence factors and effector proteins into host cells to circumvent PTI and promote parasitism (22, 56, 77, 78). These effectors target host immune signaling components at seemingly every stage of plant immunity,

including PRRs, MAPK pathways, transcriptional machinery, and vesicle trafficking (22, 56, 76–78, 95). To counter pathogen effectors, plants have evolved a second layer of immunity consisting of intracellular resistance proteins that recognize specific effectors. Many of these cytoplasmic immune receptors belong to a family of nucleotide-binding LRR proteins, or NLRs (17). The recognition of microbial effectors by host receptors induces effector-triggered immunity (ETI). ETI reinstates and amplifies PTI transcriptional programs and antimicrobial defenses and is often associated with localized cell death, also referred to as the hypersensitive response (HR), which ultimately restricts the proliferation and spread of pathogens (18, 52).

CYTOSKELETAL REMODELING IS ASSOCIATED WITH PLANT DEFENSE

The Focal Response to Attack by Fungi and Oomycetes

The plant cytoskeleton comprises a dynamic cellular framework that supports a myriad of processes, including cell expansion, mitosis and cytokinesis, organelle transport, and cell wall deposition. Networks of actin filaments and microtubules are continuously remodeled and respond rapidly to hormones, developmental cues, and biotic and abiotic stimuli. It has long been appreciated that the actin cytoskeleton plays an essential role during plant immunity, mainly as tracks for long-distance and polar transport of materials to the PM and cell wall (32, 99, 104). Historically, rearrangements of the actin cytoskeleton have been observed in a broad range of plant-microbe interactions (19, 32, 39, 41, 71, 89). For example, radial actin bundles focus toward the attempted entry site of fungi and oomycetes, and actin remodeling is necessary for defense against pathogen penetration (62, 84, 99, 106). When plants are treated with cytochalasin E, a drug that perturbs host cytoskeletal rearrangements but not the microbial cytoskeleton, the incidence of penetration during nonhost interactions is markedly increased (58, 62, 82), suggesting this focal response is part of basal immunity. In barley epidermal cells responding to the biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*), actin filaments focus prominently on the attack site in a resistant mutant, *mlo5*, whereas the response is subtler in susceptible wild-type barley (84). The actin focal response can be recapitulated in plant cells that are prodded with a tungsten microneedle or nanoindentation technique, leading to the formation of an extensively bundled filament array directly under the site of mechanical stimulation (10, 33). This is thought to mimic the physical force exerted by pathogen invasion, and it has been commonly assumed that the mechanical force of penetration is responsible for actin reorganization in host cells. However, the penetration-deficient *Mps1* mutant of the rice blast fungus *Magnaporthe oryzae* elicits rearrangement of actin arrays, leading to speculation that this is due to chemical signaling from the pathogen rather than mechanical stress elicited by the penetration peg (120).

The focal response also requires myosin motor activity to achieve polarized actin bundles and local cell wall deposition at the site of penetration (123). Further, delivery of the defense-related protein PENETRATION RESISTANT3 (PEN3), a PM-localized ATP-binding cassette transporter in *Arabidopsis*, requires a functional actin cytoskeleton, whereas the syntaxin vesicle fusion protein PEN1 localization is actin independent (79, 112, 113). Cytoskeletal highways may serve to locally fortify the wall or direct defense machinery to specialized regions of the PM. Similarly, the formation of a specialized interface called the extrahaustorial membrane (EHM) during invasive growth of powdery mildew hyphae requires cytoskeletal delivery of specific proteins like RPW8.2 in *Arabidopsis* epidermal cells (63, 118). Thus, polarized vesicle trafficking, secretion, and PM domain generation appear to be conserved functions of actin cytoskeletal remodeling during the response to both beneficial and detrimental fungi and oomycetes (19, 32, 82, 112).

Global Cytoskeletal Remodeling in Response to Bacterial Phytopathogens and Diverse MAMPs

Whether similar cytoskeletal responses occur when plant cells encounter bacterial pathogens remained obscure until recently. To test this, Henty-Ridilla et al. (38) challenged plant cells with biotrophic bacterial phytopathogens, which lack a strategy to gain entry into the cytoplasmic volume of host cells and thus colonize the surface of leaves or proliferate in intracellular spaces between mesophyll cells (55, 69). To combine powerful genetic approaches with advanced light microscopy and quantitative image analyses, these authors exploited the *Arabidopsis–Pseudomonas* pathosystem (55). Inoculation with pathogenic *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) triggers a biphasic actin response in *Arabidopsis* leaf epidermal cells expressing the actin reporter GFP-fABD2 (**Figure 1**). Approximately 6–9 hours post-inoculation (hpi), a transient increase in actin filament abundance throughout the cytoplasm of epidermal cells is observed (38). Later, ~24 hpi, a marked increase in the extent of actin filament bundling or a reduction in the number of individual filaments is obvious (38). The initial actin response correlates with PTI because actin filament density increases in response to several PTI-eliciting microbes (38). In contrast, the enhanced

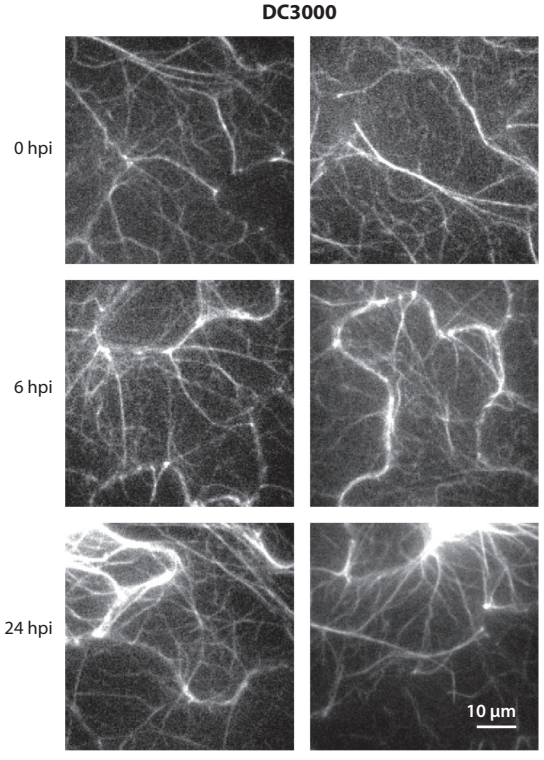

Figure 1

The actin cytoskeleton rearranges in response to inoculation with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000). Representative images of epidermal pavement cells from *Arabidopsis thaliana* cotyledons expressing the actin reporter GFP-fABD2. Treatment with *Pst* DC3000 elicits two distinct changes in the cortical actin array. At 6 hours post-inoculation (hpi), cells show an increase in the overall density of actin filament network compared with cells at 0 hpi or mock-treated control cells (*not shown*). At 24 hpi, the extent of actin filament bundling is markedly enhanced by bacterial infection. Epidermal cells from 10-day-old light-grown seedlings were imaged with spinning-disk confocal microscopy.

bundling at late time points correlates with effector-triggered susceptibility and depends on both a functional type III secretion system (T3SS) and bacterial effector proteins (38). Specifically, effector-less (D28E) and T3SS-deficient (*hrpH* or *hrcC*) bacterial strains fail to elicit actin filament bundling at late time points but exhibit a normal early proliferation of actin filaments (38).

Conserved bacterial and fungal signals are sufficient to elicit actin remodeling in epidermal cells from various *Arabidopsis* tissues (**Figure 2**). The increase in actin filament abundance could

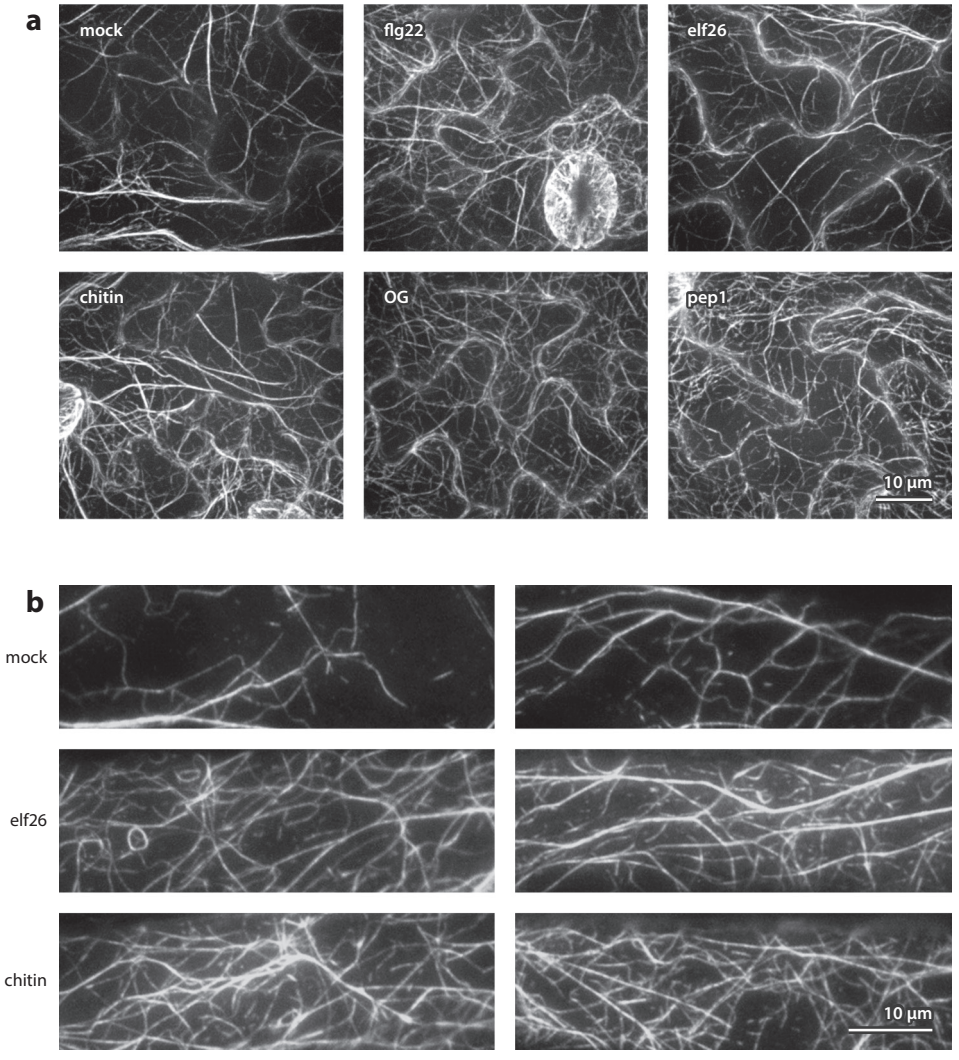

Figure 2

The density of actin filament arrays increases in response to microbe-associated molecular pattern (MAMP) or damage-associated molecular pattern (DAMP) treatment. (*a*) Representative images of epidermal pavement cells from *A. thaliana* rosette leaves treated with diverse MAMPs or DAMPs for 5 to 15 min. Treatments led to an increased abundance of actin filaments within minutes of elicitation. Images were taken from 3 to 4-week-old rosette leaves with spinning-disk confocal microscopy. (*b*) Variable-angle epifluorescence microscope images of epidermal cells from 5-day-old dark-grown hypocotyls treated with mock, elf26, or chitin for 5 min.

be mimicked by treating with diverse MAMPs and DAMPs and is required for plant resistance against virulent and avirulent microbes (37, 38, 54, 74), further demonstrating that actin remodeling represents a conserved hallmark of the innate immune response. By marking homozygous *Arabidopsis* mutants that are deficient for signal perception or transduction components with the GFP-fABD2 actin reporter, it is possible to test the contribution of known players to actin remodeling. Knockouts for FLS2, EFR, and LYK1/4 fail to perceive the cognate MAMPs (flg22, elf26, and chitin, respectively) and do not increase the density of actin filament arrays (37, 38, 74). Similarly, the contributions of coreceptor BAK1 and cytoplasmic kinase BOTRYTIS-INDUCED KINASE1 (BIK1) (65) to signaling to actin rearrangements were demonstrated by combining genetics and quantitative cell biology (38, 72).

To further dissect the molecular mechanisms underlying actin remodeling during innate immune signaling with high spatial and temporal resolution, the etiolated *Arabidopsis* hypocotyl system has been established as a model system to monitor single-filament dynamics following treatment with MAMPs (**Figure 2*b***) (36, 37, 74). In epidermal cells from etiolated hypocotyls, actin arrays comprise two interspersed populations—single filaments and actin filament bundles—with distinct turnover mechanisms (19, 71, 105). Individual actin filaments are short, faint and ephemeral structures, with average lifetimes of 15–30 s and maximum lengths of 10–15 μm (19, 36, 71, 105). By comparison, bundles are bright, long, and long-lived, with average lifetimes of several minutes and average lengths of 35 μm (71, 105). Bundles assemble by a catch-and-zipper process from single filaments or small bundles at a frequency of 7×10^{-5} events/μm^2 s^{-1} and disassemble by either severing or filament unbundling (71). Single filaments, by contrast, are incessantly remodeled through dramatic growth and disassembly known as stochastic dynamics (19, 36, 71, 105). Elongation rates at filament plus ends approach 2 μm/s, and filaments are disassembled by prolific severing activity that breaks them into small fragments that depolymerize slowly (105). This continuous rearrangement is proposed to function as a surveillance mechanism to sense and respond to signals that alter actin dynamics, resulting in the construction of new actin arrays (19, 36, 105).

MAMP-induced actin remodeling in hypocotyl epidermal cells results in increased actin filament abundance but occurs much faster (i.e., within minutes) than the reorganization observed in response to bacteria (37, 38, 74). Furthermore, upon elicitation, quantitative analysis of actin filament dynamics reveals significant changes to actin turnover, including significantly reduced filament severing frequency, increased filament length and lifetime, and elevated filament-filament annealing. These data suggest that the increased filament abundance results from enhanced filament formation and decreased filament disassembly (37, 74). Moreover, cross-correlation analysis of pixel intensities reveals that altered actin dynamics is episodic and transient, with bouts of enhanced turnover occurring at 5 and 30 min post-treatment for chitin and 5 and 45 min post-treatment for elf26 (74). These precise and rapid effects on actin dynamics parameters help guide the search for specific ABPs that sense and transduce intracellular hallmarks of PTI into increased filament abundance (see below).

Stomata are composed of a pair of specialized epidermal cells referred to as guard cells, which control the size of the stomatal aperture to regulate gas exchange and water transpiration between plant interior and the environment. As natural surface openings on leaves, stomata are considered passive ports of bacterial entry during infection. However, it was found that stomata close rapidly in response to bacterial inoculation or MAMP stimulation (80), suggesting that stomatal closure is part of the innate immune response (81). Stomatal movements are closely correlated with actin rearrangement of guard cells in response to various stimuli (71). Higaki et al. (42) developed advanced image analysis metrics to describe remodeling of actin arrays during stomatal movement in *Arabidopsis*. They found that actin cytoskeleton in guard cells undergoes dramatic spatial

reorientation, which parallels the response to circadian rhythms (42). Recently, Shimono et al. (100) utilized these tools to examine actin architecture during bacteria- and MAMP-induced stomatal movement. When treated with bacterial pathogens, the actin cytoskeleton reorients from radial to longitudinal arrays of actin bundles coinciding with stomatal closure. In cells treated with MAMPs, however, actin rearrangement occurs differently, with the frequency of radial actin bundles increasing over the course of treatment (100). In both cases, the authors fail to detect increases in actin filament density such as observed in epidermal cells from leaves and hypocotyls (37, 38, 72, 74). Nevertheless, the mechanisms that control actin dynamics during innate immunity in guard cells should be examined further, and the dependence of stomatal closure on bacterial effector proteins evaluated.

Similar to the actin cytoskeleton, microtubule rearrangements also occur in response to invading pathogens (31, 41, 59). During infection by fungi and oomycetes, localized microtubule depolymerization is often observed at the contact site (31, 59, 93, 106). Pharmacological disruption of host microtubules results in significantly increased penetration efficiency (31). Microtubules also contribute to plant immunity against bacterial pathogens. Chemical and genetic perturbation of microtubule cytoskeleton enhances plant susceptibility to virulent and avirulent bacteria (14, 28, 68). However, in contrast to the increased density of actin filaments, no significant changes in microtubule organization are observed during early stages of bacterial interaction (28, 68). Additionally, treatments with MAMPs/DAMPs fail to elicit microtubule remodeling in host cells (5, 13), indicating that the microtubule cytoskeleton may not respond to innate immune signaling. Nevertheless, several bacterial effectors have been found to remodel microtubules by targeting host cytoskeletal regulators (14, 28, 68). This is discussed further below.

HALLMARKS OF PATTERN-TRIGGERED IMMUNITY SIGNALING TO THE CYTOSKELETON THROUGH ACTIN-BINDING PROTEINS

A plethora of ABPs modulate cytoskeletal organization and dynamics in eukaryotic cells (36, 71). These have various functions, including regulating filament nucleation, governing the size and function of the monomeric actin pool, controlling the availability of filament ends for assembly, severing the filament backbone, and generating cross-linked meshworks or higher-order actin filament bundles (19, 71). Because many of these conserved proteins sense intracellular secondary messengers such as Ca^{2+}, phospholipids, and pH and are modulated through posttranslational modifications (PTMs) such as phosphorylation or oxidation, ABPs are excellent candidates for transducing signals into cytoskeletal remodeling. The search for sensors can be focused toward a subset of the hundreds of potential players, because MAMP treatment phenocopies the effects on single actin filament dynamics in homozygous mutants for several key ABPs (71). In particular, the marked reduction of filament severing and significant increase in filament-filament annealing (37, 74) suggest that actin depolymerizing factor (ADF) and capping protein (CP) function at the crossroads of signaling to the cytoskeleton and are inhibited by MAMP-elicited second messengers. On the basis of data from the genetic and pharmacological dissection of MAMP signaling in *Arabidopsis*, we propose a simple model explaining how early signaling events are transduced into actin remodeling (**Figure 3**). Details are described below.

Reactive Oxygen Species and Cytoskeletal Remodeling

Production of apoplastic ROS is one early hallmark of PTI, with a transient increase at 5–10 min post-MAMP treatment of *Arabidopsis* leaf disks (34, 72). Reactive oxygen species serve as antimicrobials, cross-linkers of the plant cell wall to block pathogen ingress, and important signaling

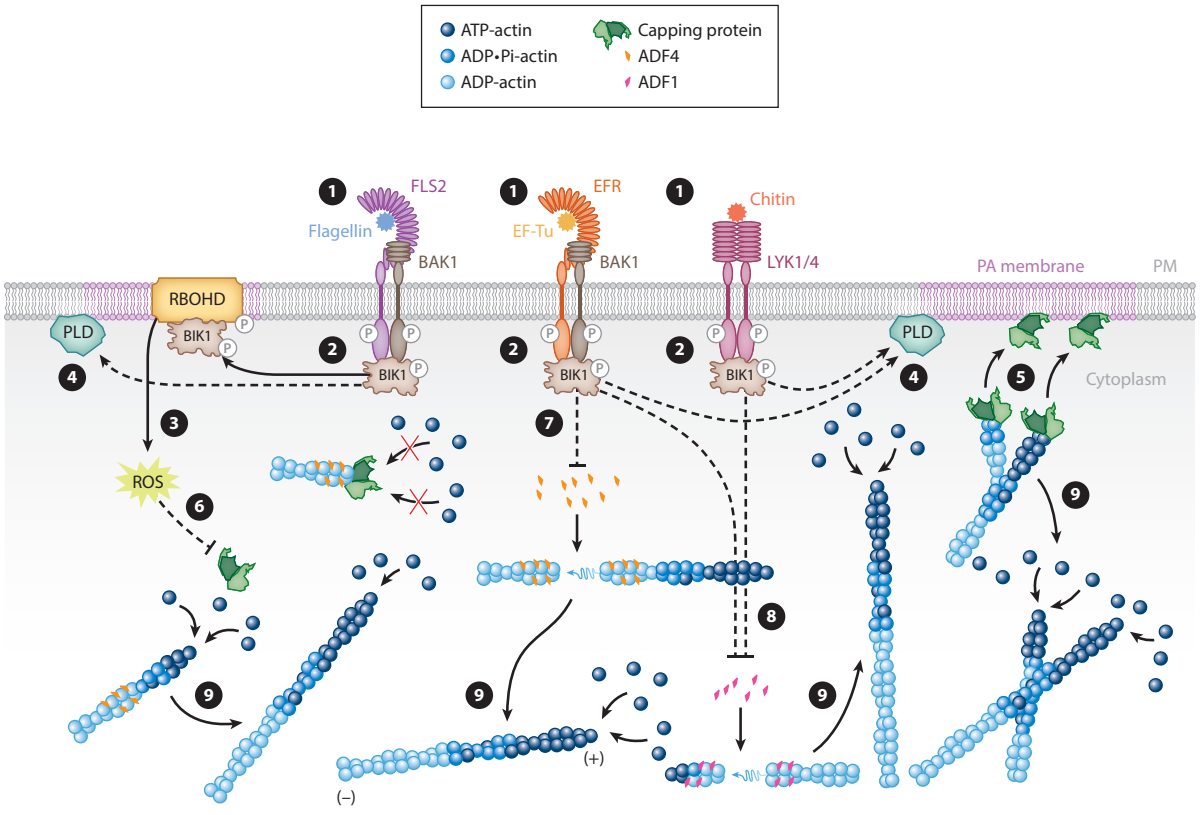

Figure 3

A model for actin remodeling during innate immunity. This graphic displays the major microbe-associated molecular pattern (MAMP) signaling components and key actin-binding proteins required for actin rearrangements during innate immune responses. ❶ The first layer of plant innate immunity is initiated by recognition of MAMPs by cognate pattern-recognition receptors (PRRs). FLS2, EFR, and CERK1/LYK1 and LYK4 are well-characterized PRRs in *Arabidopsis thaliana*, which perceive bacterial flagellin, elongation factor Tu (EF-Tu), and the β-1,4-linked GlcNAc chitooligosaccharide (chitin), respectively. ❷ Upon ligand binding, PRRs recruit coreceptor BAK1 and/or cytoplasmic kinase BIK1 to form PRR complexes, resulting in rapid phosphorylation of these components. BIK1 then dissociates from PRR complexes to further activate downstream signaling, such as ❸ reactive oxygen species (ROS) production upon RBOHD phosphorylation by BIK1; and ❹ fluxes in phospholipids, such as phosphatidic acid (PA) generated through activation of membrane-associated phospholipase D (PLD). The heterodimeric actin filament capping protein (CP) operates downstream of multiple MAMP signaling pathways by responding to both PA and ROS signals. ❺ CP binds to PA and is released from the barbed end of actin filaments, and/or ❻ elevated ROS levels stimulate actin filament uncapping through CP inactivation; both processes result in new filament assembly. Another hallmark feature of MAMP-induced actin abundance changes is reduced actin filament disassembly. This requires inhibition of actin depolymerizing factors (ADFs) to decrease the frequency of filament severing ❼ and ❽. However, different MAMP signaling requires specific ADF isoforms in different tissues. For example, ❼ ADF4 is implicated in the EFR signaling pathway in dark-grown hypocotyls but does not play a role in the immune responses triggered by fungal MAMP chitin. In contrast, ❽ ADF1 appears to be involved in a general response to multiple MAMPs. Consequently, ❾ the reduction in filament disassembly and increases in free barbed ends for filament assembly led to increased density of actin filaments in the cytoplasm.

molecules to activate additional defenses (91, 111). In *Arabidopsis*, MAMP-induced ROS production is mediated by the PM-localized NADPH oxidase, RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) (110, 111). RBOHD forms a constitutive association with PRR complexes at the PM. Upon MAMP perception, BIK1 directly binds and rapidly phosphorylates RBOHD (91). Moreover, RBOHD phosphorylation by BIK1 is critical for ROS production during innate immunity (53, 75). Recently, Li et al. (72) demonstrated that RBOHD-dependent ROS production is upstream of actin remodeling during innate immunity. Genetic and chemical disruption of RBOHD completely abrogates cytoskeletal remodeling in leaf epidermal pavement cells following perception of multiple MAMPs and DAMPs (72). Moreover, exogenous H_2O_2 treatments recapitulate MAMP-induced actin remodeling in epidermal cells from homozygous mutants of *rbohD* or PRR complex (e.g., *fls2*, *bik1*, and *bak1*) in the absence of flg22. These data suggest that perception of MAMPs by cognate receptor complexes triggers actin remodeling through activation of RBOHD-dependent ROS signaling (72, 91).

Phospholipase D/Phosphatidic Acid and Defense Signaling

Phosphatidic acid (PA) has emerged as a pleiotropic signaling phospholipid during plant defense responses (44, 125). It acts as a prolific membrane-localized signal, affecting downstream responses by binding to specific protein targets (44, 125). PA is generated by two enzymatic pathways: hydrolysis of structural lipids such as phosphatidylcholine by phospholipase D (PLD), and phosphorylation of diacylglycerol (DAG) by DAG kinase, which in turn is produced by the cleavage of PtdIns(4,5)P_2 by phospholipase C (44, 125). PA levels in plant cells increase rapidly upon elicitation with various immune signals, such as MAMPs, Nod factors, and pathogen effectors (1, 2, 20, 21, 57, 66, 67, 94, 114, 121, 122). PA fluxes might function upstream of ROS generation during the innate immune response. In *Arabidopsis* leaf cells, exogenously applied PA is sufficient to induce ROS production and activation of defense-responsive genes (1, 85). Similarly, pharmacological or genetic inhibition of PA production results in impaired production of ROS as well as a reduction in antimicrobial molecules stimulated by elicitors (2, 122). In addition, genetic disruption of certain PLD isoforms leads to altered host resistance to microbial pathogens (44, 125). For example, loss of a single isoform, PLDβ1, renders *Arabidopsis* more resistant to *Pst* DC3000 but more susceptible to the necrotrophic fungal pathogen *Botrytis cinerea* (126). Surprisingly, the *pldβ1* mutant has an elevated ROS response 12 h after challenge with pathogens (126), suggesting that PLDβ1 might be a negative regulator of ROS production. In a systematic approach, single and double knockouts for all 12 *Arabidopsis PLD* genes were challenged with virulent and avirulent *Pst* DC3000 strains; however, no single isoform could be linked to ETI (51). Loss of *PLDδ* did compromise cell wall–based defense against the nonhost powdery mildew fungus, *Erysiphe pisi*, as well as nonhost *Bgh* (51, 86), suggesting a role in PTI. These studies demonstrate the importance of PLD-dependent PA signaling during innate immunity (44, 125) but ask which, if any, isoforms function specifically during PTI, effector-triggered susceptibility (ETS), or ETI in different pathosystems.

PLD and PA fluxes are known to regulate actin organization and dynamics, both through direct interaction of PLDβ with monomeric and filamentous actin and through inhibition of CP activity by PA (87). A study by Li et al. (74) suggests that rapid actin remodeling elicited by MAMPs requires PLD-dependent PA signaling. Exogenous PA treatment mimics the actin remodeling that occurs following MAMP perception in hypocotyl epidermal cells, including an increase in actin filament density and enhanced filament-filament annealing (73, 87). Further, treatment with the alcohol isomer 1-butanol to inhibit PLD-dependent PA production completely suppresses actin remodeling following elicitation with elf26 or chitin (74). Similarly, chemical inhibition

of PLD with 5-fluoro-2-indolyl des-chlorohalopemide (FIPI) ameliorates the MAMP-induced actin responses (74). Collectively, these data indicate that PA production via the PLD pathway is essential for actin remodeling during MAMP signaling, but this remains to be tested genetically.

Actin-Binding Proteins Respond to Signals During Pattern-Triggered Immunity and Effector-Triggered Immunity

The transient increase in actin filaments during PTI could occur through multiple mechanisms, including enhanced filament nucleation, decreased turnover, increased filament stability, or all of the above. The parameters of single-filament turnover that change in epidermal cells following MAMP elicitation can help narrow the search, however. Two targets of MAMP signaling, ADF and CP, have been implicated in actin remodeling during innate immunity (37, 72, 74, 109). Their involvement in PTI (and ETI) and the signals that modulate their activity are discussed below.

ADFs are a family of depolymerizing and severing factors that destabilize actin filaments in vitro and in vivo (36, 47, 71). *Arabidopsis* has 11 *ADF* genes belonging to 4 subclasses, with differential expression throughout the plant (98). An *adf4* mutant has 2.5-fold lower severing frequency in hypocotyl epidermal cells compared to wild type as well as increased filament bundling (35). The former phenotype is recapitulated by elf26 treatment of wild-type hypocotyls and leads to the model that ADF4 is a target for inhibition by MAMP signaling, resulting in longer and longer-lived actin filaments (**Figure 3**). This hypothesis is confirmed by demonstrating that actin remodeling and single-filament dynamics in *adf4* are insensitive to elf26 treatment (37). Surprisingly, ADF4 appears to function specifically in EFR signaling, as chitin elicits an increase in actin filament density in *adf4* epidermal cells (37). However, disruption of another subclass I ADF, *adf1*, leads to an unresponsive actin array to all MAMPs tested, suggesting that different ADFs function in overlapping or convergent innate immune responses (37, 74). ADFs can be phosphorylated in vitro and in vivo and are responsive to changes in cytosolic pH. Which signals lead to inhibition of ADF activity during PTI remains unanswered.

ADF4 also plays a role during ETI and activation of gene-for-gene resistance (47, 89). The *adf4* mutant, but not other subclass I mutants, is more susceptible to avirulent *Pst* DC3000 harboring the type III effector (T3E) AvrPphB but not to virulent DC3000 or strains harboring AvrRpt2 or AvrB (109). Given that AvrPphB targets PBS1 kinase activation by proteolytic cleavage, the effects of ADF4 are probably an indirect consequence of inhibition of defense signaling. Nevertheless, cytoskeletal turnover is implicated in ETI, as treatment with cytochalasin D partially rescues HR elicited by AvrPphB in *Arabidopsis* leaves (109). This might be due to nuclear actin or ADF operating through transcriptional reprogramming. The *adf4* mutant has dramatically reduced expression of *RPS5*, the cognate R gene for AvrPphB (90). Moreover, ADF4 phosphorylation, which negatively regulates actin binding, appears to be important during ETI because a phosphomimic mutant (ADF4-S3E) complements the disease symptoms and HR phenotype, whereas a phosphonull (ADF-S3A) does not (90). However, the identity of a kinase that phosphorylates ADF during defense remains to be elucidated.

Conversely, subclass I ADFs are implicated in the susceptibility of *Arabidopsis* to a pathogenic powdery mildew fungus (48, 49). The *adf4* mutant as well as *ADF1-4RNAi* lines show increased resistance to adapted *Golovinomyces orontii* (48). Reduction of ADFs correlates with increased ROS production and cell death. In part, this might be due to altered actin organization in knockdown lines, which show modestly increased filament density around haustoria at early time points during infection (48). More likely, susceptibility is a nuclear function for ADF because neither phosphomimic (S6D) nor phosphonull (S6A) complement increased resistance against *G. orontii* in the *adf4* mutant (48). Complementation studies demonstrated, however, that nuclear localization of

ADF4 is necessary for susceptibility to the powdery mildew fungus (48). These results suggest that the molecular mechanism and contribution of ADF4 to resistance against avirulent bacteria (e.g., *Pst* DC3000 AvrPphB) versus susceptibility to an adapted powdery mildew fungus have overlap as well as substantial differences.

Finally, ADFs appear to play a role in resistance of small grain crops against rust pathogens belonging to *Puccinia* spp. (47). Barley *HvADF3* is part of a three-gene resistance locus (including *HvRga1* and *HvRpg5*) necessary for protection against the incompatible stem rust *P. graminis* f. sp. *tritici* race QCCJ (119). Individual VIGS suppression of HvADF3 changed the reaction from incompatible to compatible, indicative of a cooperative role for ADF in pathogen recognition and resistance. In wheat, TaADF7 and TaADF4 contribute to resistance against the stripe rust pathogen *P. striiformis* f. sp. *tritici* (25, 124). Suppression of these ADF isoforms results in increased susceptibility to the avirulent *Pst* CYR23 strain. Moreover, knockdown of TaADF7 and TaADF4 correlates with greatly reduced ROS and HR during infection, and these could be overcome with cytochalasin B or latrunculin B (LatB) treatment (25, 124). Collectively, these data indicate that ADFs positively modulate immunity in wheat and barley via regulation of actin cytoskeletal organization.

Actin filament CP is a second ABP implicated in sensing signals and contributing to actin remodeling during PTI (71, 72). CP is an obligate heterodimer comprising α and β subunits (CPA and CPB), each encoded by a single gene in *Arabidopsis* (45). Recombinant CP binds to filament plus ends with high affinity and prevents assembly and disassembly of monomers (45). It also suppresses filament-filament end joining or annealing. Genetic disruption of either subunit leads to the reduction of CP heterodimers in cells and consequent defects in actin filament organization and dynamics (73). The *cpa* and *cpb* mutants have increased actin filament abundance, enhanced filament length and lifetime, and up to a sixfold increase in filament annealing (73). Because these phenotypes mimic the response of wild-type cells to MAMP treatment, the authors hypothesize that CP is inhibited during PTI, resulting in increased actin filament density (**Figure 3**). Indeed, epidermal cells from hypocotyls or mature rosette leaves of *cp* mutants fail to remodel their actin cytoskeleton following treatments with multiple MAMPs (72, 74). The importance of this ABP for perception and resistance against a myriad of pathogens is illustrated by the enhanced susceptibility of *cp* mutants to not only virulent and avirulent *Pst* DC3000 strains but also the necrotrophic fungus *Alternaria brassicicola* (74).

How CP activity is inhibited during PTI has also been determined. CP is the only known eukaryotic ABP to bind and be negatively regulated by PA in vitro (46, 88). The fact that PLD-generated PA is a hallmark of PTI leads to the hypothesis that CP activity is negatively regulated by PA during the innate immune response (74), resulting in enhanced availability of filament ends and increased actin assembly (**Figure 3**). Consistent with this model, *cp* mutants are completely unresponsive to exogenously applied PA and fail to remodel their actin cytoskeleton in the presence or absence of MAMPs (72–74). Moreover, blocking PA production with 1-BuOH or FIPI abrogates the ability of wild type to increase actin filament density following MAMP treatment but has no effect on *cp* mutants (74). CP is also a sensor of ROS during defense signaling; specifically, exogenous H_2O_2 treatment elicits an increase in actin filament abundance in wild-type leaf epidermal cells, whereas *cp* mutants are unresponsive to such treatments (72). Loss of *cp* abrogates cell wall–mediated defenses by reducing callose formation and also mitigates aspects of transcriptional reprogramming (74). Thus, CP appears to be a convergence point for multiple PRR pathways and several hallmark events (i.e., ROS and PA fluxes) during PTI in different tissues; inhibition of capping activity transduces these stimuli into increased accumulation of actin filaments (**Figure 3**). Whether ROS production is downstream of PLD/PA or a parallel pathway for inhibition of CP remains to be determined.

BACTERIAL EFFECTORS MODULATE HOST CYTOSKELETAL ORGANIZATION

Several lines of evidence predict that microbial effectors target the host cytoskeleton to subvert defense responses (19). First, leaf epidermal cells exhibit a biphasic response to virulent bacterial infection, with late remodeling at ~24 hpi reducing the number of single filaments and increasing the extent of filament bundling (38, 101). Actin remodeling is abrogated in leaves infected with nonpathogenic bacterial strains that are deficient for effectors or lack a functional T3SS. Second, delivery of R genes and defense proteins to the PM depends on an intact actin cytoskeleton (79, 113, 118). Third, when defense breaks down in host plants perturbed for ADF, HR is restored by treatment with actin inhibitors (25, 109, 124). Fourth, bacterial pathogens that infect mammalian cells deploy a plethora of ABP-like effectors to hijack host cell vesicle trafficking or power intracellular bacterial locomotion (19, 29). Not surprisingly, recent studies have identified several phytopathogen effectors that modulate host cytoskeletal organization. One study, exploring the diversity of T3E subcellular localization in host cells, uncovered HopAV1 and HopAZ1 from *P. syringae* pv. *actinidiae*; GFP fusion proteins for these two effectors decorate filamentous structures in *Nicotiana benthamiana* leaf cells, suggesting cytoskeletal association (16). Clearly, more will be discovered as additional bacterial and fungal effectors are scrutinized.

HopW1, a T3E from *P. syringae* pv. *maculicola* strain ES4326, was the first bacterial effector to demonstrate direct interactions with the plant cytoskeleton (50, 54). When *Pst* DC3000 harboring HopW1 is used to infect *N. benthamiana* leaves, actin filament density is significantly reduced and this can be phenocopied by LatB treatment. Similarly, when ectopically expressed in *N. benthamiana*, HopW1-RFP markedly disrupts actin arrays and localizes to intense peripheral patches. A C-terminal truncated HopW1 disassembles nonmuscle actin filaments in vitro, but the molecular mechanism remains unclear. Further studies are necessary to establish whether HopW1 is a monomer-binding, filament-severing, or filament-capping protein. The virulence activity of HopW1 might be explained by its ability to perturb endocytosis and vesicle trafficking. One hypothesis is that HopW1 counteracts the initial transient increase in actin filament density, induced during early stages of bacterial infection, that is necessary for intense vesicle trafficking during defense (54).

Shimono et al. (101) took a systematic approach to identify *Pst* DC3000 T3Es that alter actin architecture during effector-triggered susceptibility. By infecting leaves of *Arabidopsis* plants expressing GFP-fABD2 with several polymutants that eliminated T3E gene clusters, they identified several suites of effectors necessary for the late increase in the extent of actin filament bundling or decrease in filament density. Among the polymutants that cause decreased bundling and increased filament density relative to DC3000 infection, the gene cluster deletion ΔIX is most potent. Another cluster polymutant, ΔCEL, has the opposite effects on actin organization compared to DC3000 (i.e., enhanced actin bundling and decreased filament density). This suggests that DC3000 T3Es might have opposing effects on actin architecture. Within the IX cluster, reintroduction of *HopG1* is sufficient to restore actin remodeling to levels consistent with those induced by DC3000 at 24 hpi. Conversely, the hopG1 single mutant altered the host actin architecture like the ΔIX polymutant did, with reduced extent of bundling and increased density compared to DC3000. Whether ectopic expression of HopG1 in plant cells is sufficient to alter actin architecture was not tested, nor was the ability of recombinant HopG1 to interact with actin in vitro. Nevertheless, the microtubule motor kinesin, a cytoskeletal-interacting partner, was identified. The significance of the association between HopG1 and a mitochondrial-localized motor protein on actin architecture or function remains obscure but is consistent with the previous localization of HopG1 to mitochondria (6). Nevertheless, a knockdown mutant for this particular kinesin shows reduced

susceptibility to *Pst* DC3000 and reduced chlorosis (101). The authors speculate that HopG1 targeting of an actin-associated kinesin indirectly induces filament bundling during infection and that altered actin architecture simulates host cell chlorosis and other disease symptoms (101).

The microtubule cytoskeleton is also a target for bacterial T3Es. HopZ1a from *P. syringae* pv. *syringae* interacts with and acetylates plant tubulin, resulting in disruption of microtubule arrays late in infection (68). Although exocytosis is primarily considered to be an actin-dependent process in plants (70), HopZ1a inhibits the secretion of secGFP and blocks cell wall–based defenses (68), perhaps leading to a new paradigm for trafficking of materials to the PM and extracellular matrix during biotic stress response. In a recent paper by Guo et al. (2016), microtubule-associated protein MAP65-1 is found to be a target of the bacterial effector HopE1 from *Pst* DC3000 (28). HopE1 binds to and dissociates MAP65-1 from the microtubule network, and requires the calcium sensor calmodulin in host cells. Interestingly, expressing HopE1 in planta does not significantly alter microtubule organization other than the dissociation of MAP65-1 from microtubules (28), leading to speculation that suppression of plant immunity by HopE1 occurs independently of microtubule rearrangements in host cells. Nonetheless, like HopZ1a, ectopic expression of HopE1 in plants perturbs immunity-related secretion and cell wall–based defenses (28). Specifically, HopE1 expression disrupts delivery of secGFP and the PR protein PR-1 to the apoplast. Indirect evidence that inhibiting protein secretion by HopE1 operates through microtubules comes from the observation that a *map65-1* mutant is more susceptible to *Pst* DC3000 and has defects in secretion of PR-1 (28). Finally, Cheong et al. (14) report that AvrBsT from *Xanthomonas euvesicatoria* interacts with *Arabidopsis* acetylated-interacting protein 1 (ACIP1), which is a microtubule-binding protein that colocalizes with microtubules in cells. Delivery of AvrBsT into host cells disrupts the localization of ACIP1 on microtubules (14); however, it remains unclear whether and how the interaction impacts microtubule function during plant immunity.

FUNCTIONS FOR CYTOSKELETAL REMODELING

Cytoskeletal rearrangements are necessary to choreograph robust resistance of host plants against microbial attackers. As discussed above, disrupting the actin cytoskeleton genetically or pharmacologically increases the penetration frequency of nonadapted fungi and oomycetes (58, 61, 62, 82, 84, 99). Further, disruption of host actin cytoskeleton by LatB treatment (38, 54) or mutations in key ABPs, such as ADFs, CP, or myosin XI, enhances plant susceptibility to bacterial and fungal pathogens (37, 74, 123). Given the manifold functions of the cytoskeleton during plant growth and development, it is perhaps not surprising that actin functions in multiple ways to assist plants in defense of their cells. These defense functions certainly include a combination of early and late events during PTI and ETI, such as vesicle trafficking and endo/exocytosis, attenuation of signaling and regulation of second messenger production, delivery of specialized proteins and defense molecules to the PM and apoplast, creation of specialized PM domains, fortification of the cell wall and deposition of callose, transcriptional reprogramming and expression of defense-related genes, and signaling to programmed cell death (PCD) during the HR.

Long-distance transport of vesicles as well as local control over secretion and endocytosis are conserved functions of the actin cytoskeleton important for plant defense (4, 95). One hallmark of PTI in *Arabidopsis* is ligand-induced endocytosis of FLS2 (96), which might attenuate signaling by removing active PRRs from the PM (102, 103). Pharmacological approaches indicate that myosin functions in internalizing FLS2 endosomes and actin are required for intracellular trafficking of this receptor (3). Advanced imaging of FLS2-GFP PM nanodomains along cortical actin filaments and altered membrane dynamics upon flg22 stimulation imply a mechanistic importance to this association (11); nevertheless, particular cytoskeletal configurations associated with PRR clustering

or internalization have yet to be described. Trafficking of defense proteins PEN1 and PEN3 to and from the PM depends on actomyosin function (79, 112, 113, 123). Surprisingly, immunity-related protein secretion and cell wall–based defenses also depend on microtubule-based transport (28, 68), and further investigations are necessary to uncover mechanistic details of the cross talk between microtubule and actin cytoskeleton during defense.

Several studies indicate that ROS production during innate immunity requires the function of both actin and microtubules. Li et al. (72) demonstrate that ROS signaling is upstream of actin remodeling elicited by MAMPs. However, perturbation of actin dynamics with LatB treatment or altered expression of CP enhances MAMP-induced ROS production, suggesting a negative feedback loop between actin remodeling and RBOHD activity or localization (72). Microtubules also play a role in the ROS burst triggered by flg22. Mutants with impaired microtubule dynamics, such as *map65* and *acip*, exhibit attenuated ROS production upon treatment with flg22 (14, 28). The mechanisms underlying how cytoskeletal remodeling regulates flg22-induced ROS production remain unclear. It has been suggested that perturbation of FLS2 endocytosis leads to impaired ROS production in response to flg22 (102, 103). It is also important to note that RBOHD PM dynamics are regulated by endocytosis (30), which could be associated with flg22-induced ROS production and affected by changes in host cytoskeleton function.

Deposition of the β-1,3-glucan polymer callose fortifies the cell wall hours after the host immune response to bacteria and fungi (115). Genetic and pharmacological data demonstrate a requirement for functional actomyosin cytoskeleton (37, 74, 123) and cortical microtubules (28, 68) in callose deposition. Whether this requires cytoskeleton-dependent exocytosis, endocytosis, or recycling of defense-associated callose synthases at the PM (23) needs further evaluation.

Disruption of the actin cytoskeleton also affects transcriptional activation of defense-responsive genes (37, 60, 74, 90, 92). For example, cytochalasin treatment of tobacco leaves induces expression of *PR1* and *PR2* genes in the absence of elicitation (60). Moreover, MAPK- and CDPK-dependent transcriptional reprogramming during innate immune activation (7–9, 108) require actin remodeling. Notably, Henty-Ridilla et al. (37) show that loss of ADF4 affects transcriptional changes in the elf26-activated CDPK pathway, whereas gene activation through the MAPK pathway, as well as chitin-induced transcriptional activation, is intact in the *adf4* mutant. CP-dependent actin remodeling is required for both MAPK- and CDPK-dependent transcriptional reprogramming activated by chitin signaling (74). These data suggest that different innate immune signaling networks require unique actin arrays or different ABPs. It is possible that nuclear actin and ABPs are involved in gene expression during plant immunity (26, 127). Porter et al. (90) suggest that nuclear-localized ADF4 participates in processes related to the expression of a resistance gene, *RPS5*, and subsequent defense activation. In contrast, nuclear localization of ADF4 is necessary for susceptibility, not resistance, to an adapted powdery mildew fungus (48).

Finally, the HR is a form of PCD that is crucial to prevent the spread of pathogens (64). Actin rearrangements are suggested to regulate elicitor-induced PCD by controlling vacuolar dynamics (39–41, 64). When challenged with elicitors from an oomycete or culture filtrates from the pathogenic bacteria *Erwinia carotovora*, PCD is stimulated in tobacco BY-2 cells; this process is accompanied by the reorganization of the actin cytoskeleton and vacuolar structures (39, 43). Following elicitation, bulb-like vacuoles are simplified into a large central vacuole with smaller spherical vacuoles prior to PCD (39, 43). These vacuolar dynamics are assumed to facilitate vacuolar rupture at the last step of PCD (39). Disruption of endoplasmic actin bundles enhances the disappearance of bulb-like vacuoles and the induction of PCD triggered by elicitors (39). These data suggest that the actin cytoskeleton negatively regulates elicitor-induced PCD by preventing vacuolar rupture (39–41).

SUMMARY POINTS

1. The plant actin and microtubule cytoskeletons participate in a myriad of fundamental intracellular processes, including vesicle trafficking, signal transduction, cell wall deposition, and membrane protein dynamics. Many of these processes are subverted, and the cytoskeleton repurposed, during defense of host cells against microbial invaders.
2. Remodeling of actin arrays features prominently during both early and late events associated with the innate immune response. In the focal response to nonadapted fungi and oomycetes, actin filament bundles focus toward the local site of attack, whereas the response to biotrophic bacterial pathogens is more general and leads to a significant increase in actin filament abundance in the cortical cytoplasm of host cells.
3. MAMPs and DAMPs are sufficient to trigger actin remodeling through cognate PRRs, and early signaling events such as ROS production and phospholipid fluxes play key roles in transmitting information to the cytoskeleton.
4. Two conserved ABPs, ADF and CP, are key intermediaries in transducing signals into increased actin filament density during PTI. Some ADF isoforms are associated with a gene-for-gene response and participate in ETI signaling, perhaps via their nuclear localization.
5. Several bacterial effector proteins that target, directly or indirectly, actin and microtubule cytoskeleton architecture and/or function during the infection process have been identified.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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