Capping Protein Modulates Actin Remodeling in Response to Reactive Oxygen Species during Plant Innate Immunity

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Plants perceive microbe-associated molecular patterns and damage-associated molecular patterns to activate innate immune signaling events, such as bursts of reactive oxygen species (ROS). The actin cytoskeleton remodels during the first 5 min of innate immune signaling in Arabidopsis (Arabidopsis thaliana) epidermal cells; however, the immune signals that impinge on actin cytoskeleton and its response regulators remain largely unknown. Here, we demonstrate that rapid actin remodeling upon elicitation with diverse microbe-associated molecular patterns and damage-associated molecular patterns represent a conserved plant immune response. Actin remodeling requires ROS generated by the defense-associated NADPH oxidase, RBOHD. Moreover, perception of flg22 by its cognate receptor complex triggers actin remodeling through the activation of RBOHD-dependent ROS production. Our genetic studies reveal that the ubiquitous heterodimeric capping protein transduces ROS signaling to the actin cytoskeleton during innate immunity. Additionally, we uncover a negative feedback loop between actin remodeling and flg22-induced ROS production.

In plant cells, the first layer of innate immunity is initiated by the detection of various danger signals by pattern recognition receptors (PRRs; Boller and Felix, 2009; Dodds and Rathjen, 2010). PRRs directly sense conserved microbe-associated molecular patterns (MAMPs), such as bacterial flagellin and elongation factor-Tu, as well as chitin from fungal cell walls. In addition to MAMPs, PRRs also perceive endogenous damage-associated molecular patterns (DAMPs), including plant cell wall fragments released by pathogen lytic enzymes or peptides synthesized de novo during pathogen infection (Boller and Felix, 2009). Activation of PRRs leads to pattern-triggered immunity (PTI), including initiation of mitogen-associated and calcium-dependent protein kinase cascades; bursts of calcium and reactive oxygen species (ROS); fluxes in phospholipid production and turnover; transcriptional reprogramming; and cellular responses such as cell wall fortification by callose deposition (Boller and Felix, 2009; Henty-Ridilla et al., 2013b, 2014; Li et al., 2015b). This actin remodeling is required for defense responses such as callose deposition in the cell wall, transcriptional reprogramming of defense genes, and subsequently contributes to plant resistance against virulent and avirulent microbes (Henty-Ridilla et al., 2013b, 2014; Li et al., 2015b). This actin remodeling is required for defense responses such as callose deposition in the cell wall, transcriptional reprogramming of defense genes, and subsequently contributes to plant resistance against virulent and avirulent microbes (Henty-Ridilla et al., 2013b, 2014; Li et al., 2015b). Quantitative analyses of actin dynamics in live cells have uncovered the potential molecular mechanisms that contribute to increased filament abundance. These include reduction in filament disassembly via inhibition of actin depolymerizing factors (ADF4 or ADF1; Henty-Ridilla et al., 2014) and increases in free barbed ends for filament

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assembly by negative regulation of the heterodimeric capping protein (CP; Li et al., 2015b). CP is a ubiquitous regulator of actin filaments (Cooper and Sept, 2008). It binds the barbed end of actin filaments to inhibit actin polymerization and filament-filament annealing in vitro (Huang et al., 2003; Cooper and Sept, 2008). Genetic evidence from various organisms demonstrates that the availability of barbed ends for actin assembly is inversely correlated with cellular CP levels (Kim et al., 2004; Li et al., 2012, 2014a). Moreover, CP has been implicated in the crosstalk of signaling events from membrane to actin cytoskeleton via phospholipid binding (Pleskot et al., 2013). Plant CP is unique in the ability to bind the stress signaling phospholipid, phosphatidic acid (PA), which inhibits capping activity and uncaps filament ends in vitro and in vivo (Huang et al., 2006; Li et al., 2012; Pleskot et al., 2013). The pathways for actin remodeling during innate immune signaling are both parallel and convergent, and potentially tissue-specific. In hypocotyl epidermal cells, for example, ADF4 regulates dynamics in response to elf26 (a peptide mimic of bacterial elongation factor-Tu) but is not required for chitin signaling, whereas CP and ADF1 are implicated in both pathways (Henty-Ridilla et al., 2014; Li et al., 2015b). These data reveal the complexity of molecular machinery that senses and transduces immune signaling to actin rearrangements.

In Arabidopsis, actin remodeling in response to MAMPs is, to our knowledge, a new early hallmark of signaling that requires cognate PRR complexes (Henty-Ridilla et al., 2013b, 2014; Li et al., 2015b). Upon perception, other rapid signaling events, such as increased cytosolic Ca\(^{2+}\), mitogen-associated protein kinase phosphorylation, accumulation of ROS, and fluxes in certain signaling phospholipids, occur within seconds to minutes (Boller and Felix, 2009; Canonne et al., 2011). Data from a recent study suggest that phospholipase D (PLD)-dependent PA signaling is required for actin remodeling upon perception of MAMPs (Li et al., 2015b). Moreover, PA mediates actin remodeling via its inhibitory effect on CP (Li et al., 2012, 2015b). In addition to PA, the entire complement of immune signals that impinge on actin cytoskeleton remodeling and its response regulators need to be further investigated.

The rapid production of ROS is a conserved signaling output during immunity across kingdoms and species. ROS contributes to plant defense responses by reinforcing the cell wall and exerting cytotoxic effects to block pathogen invasion, or by acting as a second messenger to trigger additional immune responses, such as gene expression or stomatal closure (Suzuki et al., 2011; Nathan and Cunningham-Bussel, 2013). However, a role for ROS signaling to actin dynamics during innate immunity has not been established for either plant or animal cells. The interaction between ROS and actin cytoskeleton has long been recognized (Wilson and González-Billault, 2015). In mammalian cells, ROS regulate actin rearrangements associated with cellular processes such as cell adhesion, migration, wound repair, as well as neurite outgrowth (Chiarugi et al., 2003; Fiaschi et al., 2006; Munnamalai and Suter, 2009; Sakai et al., 2012; Taulet et al., 2012; Xu and Chisholm, 2014). In plant cells, ROS signaling is required for actin remodeling during the self-incompatibility response in pollen tubes and abscisic acid-induced stomatal closure (Wilkins et al., 2011; Li et al., 2014c). In Arabidopsis, flg22-induced stomatal closure also requires NADPH oxidase, RBOHD. This provides an indirect link between ROS and actin dynamics (Mersmann et al., 2010). Furthermore, several cytoskeletal targets for ROS have been found in both plant and animal cells. Actin itself is modified by ROS via glutathionylation during neutrophil migration (Sakai et al., 2012). Oxidant-induced apoptosis requires oxidation of coflin (Klamt et al., 2009). A recent study suggests that Arp2/3 complex participates in the response to ROS signaling during stomatal closure (Li et al., 2014c). Here, we demonstrate that, after elicitation by diverse MAMPs and DAMPs, ROS serves as a common upstream signal that mediates actin remodeling. Additionally, we provide genetic evidence that CP is an intermediary in ROS signaling to the actin cytoskeleton during innate immunity.

**RESULTS**

**Cortical Actin Arrays Respond Rapidly to a Diverse Array of Danger Signals**

Plant innate immunity is triggered by the detection of various danger signals, including MAMPs and DAMPs. Previously, we found that the cortical actin array in epidermal pavement cells from Arabidopsis rearrange after treatment with MAMPs, and this actin reorganization plays an important role during plant innate immunity (Henty-Ridilla et al., 2013b). Here, we extend our findings by applying DAMPs to Arabidopsis rosette leaves expressing the actin reporter comprising GFP fused with the second actin-binding domain of Arabidopsis FIM- BRIN1 (GFP-fABD2; Sheahan et al., 2004). The elicitors were syringe-infiltrated into rosette leaves and the cortical actin cytoskeleton of epidermal pavement cells was monitored with spinning disk confocal microscopy (SDCM).

Similar to our previous study, a more dense actin array was observed in wild-type cells treated with MAMPs (flg22 and chitin) compared to mock treated leaves (Fig. 1A). Moreover, we found that this actin rearrangement occurred faster than observed previously (Henty-Ridilla et al., 2013b); it could be detected as early as 5 min to 15 min after treatment (Fig. 1A). In addition, similar changes to actin filament arrays were observed when cells were treated with DAMPs such as Arabidopsis Pep1 (a 23-amino acid peptide processed from PROPEP1; Huffaker et al., 2006) and oligogalacturonides (OGs; D’Ovidio et al., 2004), whereas another bacterial MAMP, elf26, was indistinguishable from mock control (Fig. 1A). Organizational changes of the cortical actin array were quantified with a set of metrics called skewness and density to estimate the extent of actin filament bundling and the percentage of
occupancy of actin filaments, respectively (Higaki et al., 2010; Henty-Ridilla et al., 2013b). As shown in Figure 1B, actin filament abundance in wild-type cells was significantly elevated after treatment with all elicitors tested, except for elf26, when compared with mock-treated cells. No significant changes in the extent of filament bundling were detected among treatments (Fig. 1C). Taken together, these data suggest that actin filament abundance in epidermal pavement cells increases rapidly after treatment with MAMPs and DAMPs; this likely represents a broad-based cytoskeletal response during plant innate immunity.

Lack of the NADPH Oxidase, RBOHD, Abrogates the Actin Response to MAMPs and DAMPs

One of the common signaling events triggered by MAMPs and DAMPs is the production of apoplastic ROS; this occurs within minutes and is mediated by the Arabidopsis NADPH oxidase, RBOHD (Supplemental Fig. S1; Torres et al., 2006). Thus, we predict that elevated levels of ROS are required for the rapid changes to cortical actin arrays in response to MAMPs and DAMPs. To test this, we applied elicitors to the homozygous knockout mutant for rbohD expressing GFP-fABD2, and compared actin architecture quantitatively. As shown in Figure 1C, cortical actin arrays in rbohD mutant epidermal cells showed no significant differences compared with mock-treated, wild-type cells. Moreover, all MAMPs and DAMPs failed to elicit an actin architecture change in rbohD as they did when applied to wild-type cells (Fig. 1), suggesting that RBOHD-mediated ROS generation is upstream of actin reorganization in response to MAMPs or DAMPs.

Treatment with H2O2 Mimics the Increase in Actin Filament Abundance Induced by MAMPs and DAMPs

To dissect the signaling cascades and response regulators that elicit this actin rearrangement in greater detail, we focused on flg22-induced innate immune signaling. In Arabidopsis, the molecular components involved in this pathway have been well characterized (Monaghan and Zipfel, 2012). Flg22 is perceived by a PRR complex comprising receptor kinase FLS2, coreceptor BAK1, and the cytoplasmic kinase BIK1. The perception of flg22 by FLS2 receptor complex further activates defense responses including ROS production (Monaghan and Zipfel, 2012). Several recent studies demonstrate that RBOHD is a component of the FLS2
receptor complex (Kadota et al., 2014; Li et al., 2014b). Upon flg22 perception, RBOHD can rapidly associate and be phosphorylated by BIK1. Moreover, RBOHD phosphorylation by BIK1 is critical for ROS production during MAMP signaling.

When flg22 was applied to wild-type plants for 5 to 15 min, we detected a dose-dependent increase in actin filament abundance (Supplemental Fig. S2A); however, no change in the extent of filament bundling was observed at any concentration tested (Supplemental Fig. S2B). It has been reported previously that flg22-induced actin remodeling requires the perception of flg22 by its cognate PRR (Henty-Ridilla et al., 2013b). In the homozygous PRR mutants fls2, bik1, as well as bak1-5, expressing GFP-fABD2, the cortical actin array was completely unresponsive to flg22 treatment, which is consistent with previous results (Fig. 2A; Henty-Ridilla et al., 2013b). Additionally, loss of RBOHD in Arabidopsis inhibited the actin remodeling in response to flg22 (Figs. 1, A and B, and 2A). In the PRR mutants and rbod mutant, flg22-induced ROS production was inhibited partially or completely (Supplemental Fig. S3). These data suggest that upon flg22 perception ROS production via RBOHD is necessary to induce actin remodeling. To test this hypothesis, we performed exogenous H2O2 treatment on wild-type plants, the PRR mutants, as well as the rbod mutant. In wild-type plants, treatment with H2O2 was sufficient to elicit a dose-dependent increase in actin filament abundance, mimicking the actin response that occurs after MAMP treatment (Supplemental Fig. S4). Moreover, exogenous H2O2 treatment recapitulated the flg22-induced actin remodeling in mutants of rbod and the PRR complex in the absence of MAMPs (Fig. 2C). Except for fls2, the extent of filament bundling showed no significant differences in any genotype tested after H2O2 treatment (Fig. 2D). These data confirmed that elevated ROS levels in cells are required for actin remodeling in response to flg22. In support of this, we used pharmaceutical agents, such as diphenyleneiodonium (DPI; Wilkins et al., 2011; Ben Rejeb et al., 2015) to inhibit NADPH oxidase activity, or the ROS scavenger TEMPOL (Scott and Logan, 2008; Wilkins et al., 2011) to decrease ROS level in wild-type cells. We found that both treatments blocked the increase in actin filament abundance induced by flg22 in a dose-dependent manner (Supplemental Figs. S5 and S6). Collectively, these results demonstrate that upon MAMP perception ROS production via RBOHD is required for actin remodeling during innate immune signaling.

The Actin Filament CP Is an Intermediary in ROS Signaling to the Actin Cytoskeleton during the Innate Immune Response

We previously demonstrated that negative regulation of CP is required for actin remodeling during elf26 and chitin signaling in Arabidopsis hypocotyl epidermal cells (Li et al., 2015b). In addition, CP enhances plant resistance to bacterial pathogens; specifically, leaves from cp knockdown mutants supported greater growth of Pseudomonas syringae pv. tomato DC3000 compared to wild type, whereas a CP overexpression (OX) line was somewhat resistant to bacterial growth (Supplemental Fig. S7; Li et al., 2015b). Here, to test whether flg22-triggered actin reorganization also involves CP, we applied flg22 to knockdown and overexpression mutants of CP, and compared actin architecture with wild-type leaf epidermal pavement cells quantitatively. In the absence of flg22 treatment, actin arrays in cells from two cp knockdown mutant alleles were denser and less bundled compared with mock-treated wild-type cells (Fig. 3, A–C; Supplemental Fig. S8). Actin arrays in CP OX#1 cells showed the opposite phenotype to cp mutants (Fig. 3, A–C), which was consistent with our previous findings (Li et al., 2012, 2014a). After flg22 treatment, a significant increase in the density of actin filament arrays was observed in wild-type cells (Fig. 3, B and C). The organization of actin arrays in cpb-1 or cpb-3 mutants treated with flg22 did not show any significant differences when compared to mock treatment (Fig. 3, B and C; Supplemental Fig. S8), even when high concentrations of flg22 were used (Supplemental Fig. S9). In CP OX#1 cells, the actin filament density after MAMP treatment increased significantly, but to a lesser extent when compared with wild type (Fig. 3B). Collectively, these data suggest that, in addition to elf26 and chitin (Li et al., 2015b), CP is also necessary for actin remodeling in response to flg22 signaling.

To investigate whether CP acts downstream of ROS production, exogenous H2O2 was applied to wild type and CP mutants. As shown in Fig. 3, A and D, an increase in actin filament density occurred in wild-type cells treated with H2O2. However, H2O2 treatment failed to elicit actin remodeling in the cpb-1 mutant, but did restore the increase in actin filament density in CP OX#1 cells to a level comparable to wild type (Fig. 3D). The extent of bundling, on the other hand, decreased after H2O2 treatment in wild type and CP OX#1, not in cpb-1 mutant (Fig. 3E). Even when 10 μM H2O2 was applied to cpb-1 mutant cells, no significant change in actin filament density or extent of bundling was observed (Supplemental Fig. S9). In addition, the inhibitory effect of DPI on flg22-induced actin remodeling was completely abrogated in cpb-1 mutant when compared with wild type (Fig. 4). Taken together, these findings suggest that CP regulates flg22-induced actin remodeling by responding to ROS signaling in Arabidopsis.

Perturbation of Actin Dynamics Enhances flg22-triggered ROS Production

During plant innate immunity, actin remodeling is required for a variety of cellular defense responses. These include transcriptional reprogramming of defense-responsive gene networks and fortification of the cell
wall by callose deposition (Henty-Ridilla et al., 2014; Li et al., 2015b). Here, we examined whether ROS production, as one hallmark feature of innate immunity, requires CP and actin remodeling.

To test this, we compared the flg22-induced ROS production between wild-type plants and CP mutants. In wild-type leaves treated with flg22, a robust ROS production was triggered. A significantly enhanced ROS production after flg22 treatment occurred in both cp knockdown and CP overexpression mutants compared to wild type (Fig. 5A; Supplemental Fig. S10). Moreover, enhanced ROS production in the cpb-1 mutant depends completely on RBOHD, because the cpb-1 rbohD double mutant failed to exhibit an ROS response after elicitation with flg22 (Supplemental Fig. S11). These data suggest that CP-dependent actin remodeling negatively regulates ROS production in response to flg22. Furthermore, disrupting actin arrays with the actin polymerization inhibitor, LatB, also resulted in a significant increase in flg22-induced ROS production (Fig. 5B). Treatment with LatB alone, however, did not trigger ROS production in the absence of MAMP. In the rbohD mutant, no ROS production was detected among any of the treatments tested (Fig. 5B). These data further confirm that actin remodeling plays a role in ROS production during flg22 signaling, and the regulation of flg22-induced ROS production by actin dynamics is completely RBOHD-dependent. Interestingly, we found that LatB treatment of CP mutants did not enhance flg22-induced ROS production even further; on the contrary, it diminished the increased ROS production in these mutants after flg22 treatment.

Figure 2. Actin rearrangement upon flg22 perception requires ROS signaling through the cognate PRR. Actin architecture measurements were performed on epidermal pavement cells from wild-type, rbohD, fls2, bik1, and bak1-5 mutants treated with mock and 1 μM flg22 (A and B) or 1 μM H2O2 (C and D) for 5 min to 15 min. A and B, Treatment with 1 μM flg22 significantly increased the actin filament abundance in wild-type cells, whereas actin arrays in rbohD and PRR mutants failed to remodel in response to the stimuli (A). No significant differences in the extent of filament bundling were observed in all genotypes tested except for bik1 (B). C and D, A significant increase in actin filament abundance occurred in wild-type, rbohD, and PRR mutants treated with H2O2 (C). No significant differences in the extent of filament bundling were observed in all genotypes tested except for fls2 (D). Values given are means ± se (n = 50 cells for each treatment and genotype; a, P ≤ 0.05 between mock and treatment of the same phenotype; b, no significant difference between treatment within the same genotype; c, no significant difference compared to wild type treated with mock; d, P ≤ 0.05 when compared with mock-treated wild type. Student’s t test; results from two additional biological replicates are shown in Supplemental Fig. S13).
The distinct effect of LatB on wild type and CP mutants suggest that precise control of actin architecture and dynamics is essential for cells to accurately execute defense responses during innate immunity.

DISCUSSION

In this study, we show that the cortical actin array in plant cells responds to a diverse array of danger signals. Within minutes after treatment with diverse MAMPs and DAMPs, a significant increase in actin filament abundance occurs in Arabidopsis epidermal pavement cells. Actin remodeling requires a common innate immune signal, ROS, generated by the defense-associated NADPH oxidase, RBOHD. Moreover, ROS-induced actin remodeling in response to flg22 requires activation of RBOHD through the cognate FLS2 receptor complex. By combining advanced live-cell imaging with genetic dissection, we find that actin in mutants of CP fails to respond to flg22 and H$_2$O$_2$, suggesting that CP acts downstream of ROS during PTI. In addition, chemical and genetic perturbation of actin dynamics enhances flg22-induced ROS production, revealing negative feedback regulation between these two processes (Fig. 5D).

The actin cytoskeleton in Arabidopsis epidermal cells has been shown to respond to diverse microbes and external elicitors (Henty-Ridilla et al., 2013a, 2013b). Here, we observed the occurrence of actin remodeling not only in response to various MAMPs, but also to endogenous DAMP signaling. Our data further support a role for actin cytoskeleton as basal defense machinery during innate immunity. Consistent with Henty-Ridilla et al. (2013b), we failed to detect actin remodeling after treatment with a peptide mimic of the bacterial elongation factor EF-Tu, elf26. This may result from: (1) the cortical actin array may only respond to particular MAMPs/DAMPs; (2) the receptor for elf26, EFR, may be not expressed in epidermal pavement cells of rosette leaves but be present in other cell types in this tissue, which leads to a lack of detectable actin response in epidermal cells, whereas other PTI responses can still take place in the whole tissue; (3) the actin response to elf26 does not occur as rapidly as to other MAMPs/DAMPs, and is undetectable by SDM over the time-scale (5 min to 15 min) we monitored. Both MAMPs and DAMPs elicit an increase in actin filament abundance, which likely represents a conserved PTI response in
plants. Actin remodeling associates with a variety of cellular defense responses during innate immunity. Actin rearrangements are required for receptor-mediated endocytosis of ligand including the flagellin receptor FLS2, as vesicle dynamics are significantly reduced after treatment with the flagellin polymerization inhibitor LatB (Robatzek et al., 2006; Beck et al., 2012). In addition, genetic disruption of MAMP-induced actin remodeling leads to impaired callose deposition, transcriptional reprogramming of defense genes (Henty-Ridilla et al., 2014; Li et al., 2015b), as well as generation of ROS (this study). These data suggest that the actin cytoskeleton integrates innate immune signaling to coordinate broad defense responses. The actin cytoskeleton responds not only to diverse MAMPs and DAMPs, but it can also be remodeled by bacterial effectors. During infection of Arabidopsis by *P. syringae* DC3000, increased actin filament bundling occurs at late time points, and is dependent on both T3SS and bacterial T3SS effectors (Henty-Ridilla et al., 2013b). The enhanced actin filament bundling is suggested to associate with effector-triggered susceptibility and cell death (Henty-Ridilla et al., 2013b). Shimono et al. (2016) have recently demonstrated that a *P. syringae* T3SS effector, HopG1, is involved in the increase in actin filament bundling via its interaction with an Arabidopsis mitochondria-localized kinesin. In addition, HopW1 from *P. syringae* pv. *maculicola* directly targets and disrupts actin filaments in vitro and in vivo (Kang et al., 2014). Collectively, these data further support the argument that the actin cytoskeleton is an essential component of host defense machinery involved in multilayered responses during dynamic plant-microbial interaction.

The conserved actin-binding protein CP participates in multiple MAMP signaling pathways in different tissues and cell types. It was reported previously that in hypocotyl epidermal cells, CP regulates actin dynamics in response to elf26 and chitin (Li et al., 2015b). Whether CP is required for flg22 signaling remained unclear, because flg22 fails to activate actin response in this tissue due to lack of its receptor FLS2 (Ma et al., 2005; Henty-Ridilla et al., 2014). In this study, we provide evidence that CP is involved in flg22-induced actin remodeling in epidermal pavement cells from leaves. Our results further demonstrate that CP is a convergence point that transduces multiple MAMP signaling events to actin dynamics.

Based on genetic evidence herein, CP acts downstream of ROS in response to flg22; however, it is unclear how ROS signaling impacts on CP activity. Phosphatidic acid (PA) is an abundant membrane phospholipid increasingly recognized as a signaling intermediate during innate immunity (Zhao, 2015). In rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*) suspension-cultured cells, PA accumulates within a few minutes upon treatment with various MAMPs (van der Luit et al., 2000; Yamaguchi et al., 2003, 2005; Bargmann et al., 2006; Laxalt et al., 2007; Lanteri et al., 2011; Raho et al., 2011). Nod factors have also been reported to induce PA production in vivo (den Hartog et al., 2001). Recognition of pathogen effectors leads to increased PA level in host cells (de Jong et al., 2004; Andersson et al., 2006; Kirik and Mudgett, 2009). Pharmacological studies suggest that actin remodeling upon perception of elf26 and chitin requires PLD-dependent PA signaling (Li et al., 2015b). Moreover, the negative regulation of CP by PA is essential for MAMP-induced actin

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**Figure 4.** The NADPH oxidase inhibitor DPI blocks actin remodeling in wild type after flg22 treatment. A, Percentage of occupancy or density was measured on epidermal pavement cells of wild type and cpb-1 mutant treated with 50 μM DPI for 10 min, and followed by treatment with 1 μM flg22 for 5 min to 10 min. The actin abundance in wild-type cells treated with DPI failed to increase after flg22 treatments. By contrast, actin filament abundance in the cpb-1 mutant was not altered by either treatment. B, No significant differences in the extent of filament bundling were observed in wild-type and cpb-1 mutant cells after flg22, DPI, or both treatments. Values given are means ± se (n = 50 cells for each treatment and genotype; a, P < 0.01 between mock and treatment of the same phenotype; b, P < 0.01 when compared with wild type treated with mock; c, no significant difference compared to mock control of the same genotype. Student's *t* test; results from two additional biological replicates are shown in Supplemental Fig. S15).
reorganization (Li et al., 2015b). The activation of PLD/PA signaling by ROS has been documented in plant cells. Treatment with H$_2$O$_2$ induces rapid PA accumulation in rice suspension cells, which is required for elicitor-induced biosynthesis of phytoalexin (Yamaguchi et al., 2004). Arabidopsis PLD$_d$ is activated in response to H$_2$O$_2$, resulting in increased cellular PA levels (Zhang et al., 2003). Thus, ROS may regulate CP activity through PLD/PA signaling. Another possible mechanism is the redox modification of CP by ROS. ROS is likely involved in redox signaling during plant defense, mainly through regulation of protein activities via glutathionylation of protein Cys thiol residues (Lehmann et al., 2015). Both subunits of human CP are glutathionylated during oxidative stress (Lind et al., 2002). Whether CP undergoes redox modification by ROS and its physiological relevance in plant innate immunity needs to be investigated in the future.

Actin remodeling participates in flg22-induced ROS production, but the underlying mechanism remains unknown. In mammalian cells, the activity of NADPH oxidase is directly regulated by actin dynamics. Neutrophil NADPH oxidase binds to actin in vitro (Tamura et al., 2000a, 2000b, 2006). Actin polymerization activates NADPH oxidase, whereas depolymerizing actin deactivates it. These data suggest a simple correlation between NADPH oxidase activity and the state of actin dynamics (Morimatsu et al., 1997; Tamura et al., 2000a, 2000b). In vivo, however, actin polymerization and depolymerization both activate NADPH oxidase. Close
examination of actin dynamics, such as F/G-actin ratio as well as actin turnover rates further disprove the in vitro correlation (Rasmussen et al., 2010). In our study, disruption of actin dynamics by up- and down-regulation of CP, or by LatB treatment, all lead to an enhancement of flg22-induced ROS production, suggesting that the repertoire of mechanisms that controls NADPH oxidase activation by actin remodeling is more complex than expected.

Successful immunity often requires dynamic membrane trafficking and cytoplasmic rearrangements to precisely localize immune receptors and defense compounds, these processes are dependent on the actin cytoskeleton (Schmidt and Panstruga, 2007; Yang et al., 2014). A hallmark of PTI in Arabidopsis is ligand-induced endocytosis of the flagellin receptor FLS2 (Robatzek et al., 2006). FLS2 endocytosis has been implicated in flg22-signal attenuation, because impaired FLS2 internalization often correlates with altered downstream defense signaling (Robatzek et al., 2006; Salomon and Robatzek, 2006; Chinchilla et al., 2007). A recent study showed that loss of Dynamin-Related Protein 2B inhibits FLS2 endocytosis, leading to enhanced ROS production (Smith et al., 2014). Pharmacological studies imply that actin dynamics is required for FLS2 receptor internalization and/or vesicle trafficking, as vesicle dynamics are reduced after treatment with either LatB or the actin stabilizer endosid1 (Robatzek et al., 2006; Beck et al., 2012). Thus, it is possible that the impaired flg22-induced ROS production may result from the involvement of actin remodeling in FLS2 internalization.

During plant interactions with fungi and oomycetes, the recruitment of plasma membrane-localized NADPH oxidase toward the penetration site and its subsequent activation involve an intact actin cytoskeleton (Hardham et al., 2007; Schmidt and Panstruga, 2007). A study from Hao et al. (2014) indicated that the RBOHD enzymatic activity is closely related to its dynamics at the plasma membrane. Treatment with flg22 significantly increases the mobility and aggregation of surface-localized RBOHD (Hao et al., 2014). However, whether changes in RBOHD localization are associated with flg22-induced ROS production, and the involvement of actin remodeling in this process, remain to be examined.

CONCLUSION

During innate immunity, production of ROS is an early hallmark of cellular signaling. Similarly, remodeling of the network of filaments or cytoskeleton is an important facet of recognition and response to signals from microbial invaders. However, whether ROS signaling impinges on cytoskeletal rearrangements and its underlying molecular mechanisms remains poorly understood. Using high performance, live-cell imaging approaches, we find that epidermal pavement cells from Arabidopsis plants respond to a diverse array of MAMPs and DAMPs, by significantly elevating the density of actin filament arrays. Additionally, these actin rearrangements require NADPH oxidase-dependent ROS production and can be recapitulated with exogenous addition of H$_2$O$_2$. We further demonstrate that the conserved heterodimeric, actin filament CP is a key downstream intermediary of ROS signaling during innate immunity. Finally, disrupting actin dynamics by LatB or regulation of in vivo CP levels leads to enhanced ROS production, suggesting a negative feedback loop between ROS signaling and actin remodeling during innate immunity. Collectively, our data provide compelling genetic evidence that CP is a key transducer of ROS signaling into changes of actin dynamics during plant immunity.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The homozygous fls2 (SALK_062054), fkh1 (SALK_005291), rbohD (Torres et al., 2002), as well as cpb-1 (SALK_014783) and cpb-3 (SALK_101017) single mutants were crossed to wild-type Arabidopsis (Arabidopsis thaliana) Col-0 expressing the GFP-fABD2 reporter (Sheahan et al., 2004; Staiger et al., 2009) and homozygotes were recovered from F2 populations. The homozygous fkh1-5 mutant (Schwesinger et al., 2011) and CP OX81 mutant (Li et al., 2014a) was transformed with a binary vector for the actin reporter GFP-fABD2 by the floral dip method (Zhang et al., 2006) and selected on plates containing antibiotics. T3 plants were used for analysis of actin organization. The expression levels of CP in cp mutants and CP OX81 line were examined previously by quantitative real-time PCR and semi-quantitative immunoblotting (Li et al., 2012, 2014a). The transcripts and protein levels for both CP subunits were decreased approximately 2-fold in cp mutants, and increased up to 5-fold in CP OX81 line compared with wild-type plants (Li et al., 2012, 2014a). Seeds were sown onto soil and stratified at 4°C for 3 d. Flats were transferred to a growth chamber and plants grown under long-day conditions (16 h light, 8 h dark) at 21°C for 3 to 4 weeks.

Plant Treatments with MAMPs and DAMPs

Before use, flg22, elf26, Pep1 (Neobiosc), chitin (Sigma-Aldrich), or OG (Dr. Bruce Kohorn, Bowdoin College) were diluted in 1× PBS at various concentrations. Immediately before imaging, the abaxial surface of rosette leaves from 3- to 4-week-old plants was infiltrated with MAMPs and DAMPs using a 1-mL needleless syringe until the intercellular space was filled with solution (~200 to 300 µL per leaf). As a negative control, plants were treated with solvent used to dilute the elicitors.

Image Acquisition and Quantitative Analyses of Cortical Actin Array Architecture

The extent of actin filament bundling (skewness) and the percentage of occupancy (density) were measured as described in Henty-Ridilla et al. (2013b). The cortical actin array in epidermal pavement cells was imaged using SDCM by collecting 15 to 20 steps of 0.5 µm each starting at the plasma membrane. SDCM was performed using a Yokogawa CSU-1X1 mounted on an Olympus IX83 motorized microscope equipped with a 100×/1.4 NA objective. Illumination was from a solid-state 50-mW, 488-nm laser with AOTF control over excitation wavelength. The emission was captured with an Andor iXON ultra 888 EMCCD camera. The SDCM was operated using the software MetaMorph (V. 7.1; Molecular Devices). A fixed exposure time and gain settings were selected for all the genotypes or treatments and their respective controls. Images were analyzed with Image J (National Institutes of Health) using the methods described in Henty-Ridilla et al. (2013b). Each experiment was repeated three times independently. The data presented are from one replicate. Data of two additional replicates are shown in Supplemental Figures S12 to S15. All image collection and data analyses were performed as double-blind experiments.

Measurement of ROS Production

Leaf disks (4-mm diameter) from 3- to 4-week-old plants were floated on water overnight in a 96-well plate (one disk per well). Apoplastic ROS released by the leaf tissue were measured by a luminol-dependent assay (Hese et al., 2007).
Before detection, the water was replaced with 200 μL of a solution containing 34 μg/mL luminol (Santa Cruz Biotechnology), 20 μg/mL horseradish peroxidase (Sigma-Aldrich) and elicitors. Luminescence over time was recorded with a Synergy 2 plate reader system (Biotek). For LatB treatment, leaf disks were pretreated by floating in water containing 1 μM LatB for 30 min before subsequent flg22 elicitation. Each experiment was repeated three times independently. The data presented are from one replicate. Data of two additional replicates are shown in Supplemental Fig. S16.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL databases under accession numbers AT5G47910 (RBOHD), AT5G46330 (FLS2), AT3G33430 (BAK1), At3g05520 (AtCPA), and At1g7190 (AtCPB).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. RBOHD is required for ROS production in response to various stimuli.

Supplemental Figure S2. The increase in actin filament abundance triggered by flg22 treatment is dose-dependent.

Supplemental Figure S3. ROS production in response to flg22 is inhibited by mutants in the cognate PRR complex.

Supplemental Figure S4. Exogenously applied H2O2 increases actin filament abundance in a dose-dependent manner.

Supplemental Figure S5. Diphenyleneiodonium treatment blocks flg22-triggered actin remodeling.

Supplemental Figure S6. Actin remodeling induced by flg22 treatment is inhibited by the ROS scavenger TEMPOL.

Supplemental Figure S7. CP contributes to plant resistance against bacterial pathogens.

Supplemental Figure S8. Actin architecture in cpb-3 mutant cells fails to respond to flg22 treatments.

Supplemental Figure S9. Actin remodeling in cpb-1 mutant cannot be elicited with high concentrations of flg22 or H2O2.

Supplemental Figure S10. The flg22-dependent ROS production is significantly enhanced in cpb-3 mutant.

Supplemental Figure S11. The increase in flg22-induced apoplastic ROS in the cpb-1 mutant depends on RBOHD.

Supplemental Figure S12. Results from two additional replicates for the experiment shown in Figure 1.

Supplemental Figure S13. Results from two additional replicates for the experiment shown in Figure 2.

Supplemental Figure S14. Results from two additional replicates for the experiment shown in Figure 3.

Supplemental Figure S15. Results from two additional replicates for the experiment shown in Figure 4.

Supplemental Figure S16. Results from two additional replicates for the experiment shown in Figure 5.

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LITERATURE CITED


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