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Arabidopsis capping protein senses cellular phosphatidic acid levels and transduces these into changes in actin cytoskeleton dynamics

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Abbreviations: ABPs, actin-binding proteins; CP, heterodimeric capping protein; DAG, diacylglycerol; G-actin, monomeric actin; F-actin, filamentous actin; NPC, non-specific PLC; PA, phosphatidic acid; DGK, diacylglycerol kinase; PIP_2, phosphatidylinositol (4,5)-bisphosphate; PLC, phospholipase C; PI-PLC, phosphoinositide-specific PLC; PLD, phospholipase D; VAEM, variable-angle epifluorescence microscopy

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Plants respond rapidly and precisely to a broad spectrum of developmental, biotic and abiotic cues. In many instances, signaling cascades involved in transducing this information result in changes to the cellular architecture and cytoskeletal rearrangements. Based originally on paradigms for animal cell signaling, phospholipids have received increased scrutiny as key intermediates for transmitting information to the actin cytoskeleton. Significantly, a wealth of biochemical data for plant actin-binding proteins (ABPs) demonstrates that many of these interact with phosphoinositide lipids in vitro. Moreover, phosphatidic acid (PA) has been identified not only as an abundant structural lipid in plants, but also as an intermediary in developmental and stress signaling pathways that lead to altered actin organization. Several years ago, the heterodimeric capping protein (CP) from Arabidopsis was demonstrated to bind PA and is negatively regulated by this lipid in vitro. Whether this form of regulation occurs in cells, however, remained a mystery. A new study, that combines live-cell imaging of cytoskeletal dynamics with reverse-genetic analyses in Arabidopsis, provides compelling new evidence that CP is inhibited from binding filament ends in the presence of PA in vivo. This allows rapid actin polymerization and increases in filament abundance following stimulation and could be one key factor in the physiological responses of plant cells to environmental stimuli.

Changes in Phosphatidic Acid (PA) Levels Correlate with Cytoskeletal Reorganization

Phosphatidic acid (PA) is one of the simplest structural phospholipids, comprising just a single phosphate group on a glycerol backbone attached to two fatty acyl chains (**Fig. 1**). In plant cells, PA can be quite abundant, representing 1–10% of plasma membrane lipid, and its levels change dramatically in response to developmental signals as well as a variety of biotic and abiotic stresses.[1-4] Two major, distinct signaling pathways contribute to the production of PA (**Fig. 1**). One is a direct cleavage of structural phospholipids, like phosphatidylcholine, through phospholipase D (PLD) activity. In the second two-step pathway, PA is produced by the phosphorylation of diacylglycerol (DAG) catalyzed by diacylglycerol kinase (DGK). Intriguingly, DAG can be generated by cleaving either phosphatidylinositol (4,5)-bisphosphate (PIP2) by phosphoinositide-specific PLC (PI-PLC) or the cleavage of structural phospholipids by non-specific PLC (NPC).[5] Over the previous decade, several key studies have shown that altering PA levels experimentally, either via exogenously-applied phospholipids or blocking its production through PLD with 1-butanol, results in marked changes in actin organization.[6-12] Elevated PA levels correlate with an overall increase in the density of actin filament arrays and perhaps actin filament polymerization, whereas decreased PA generally correlates with disassembly of filament

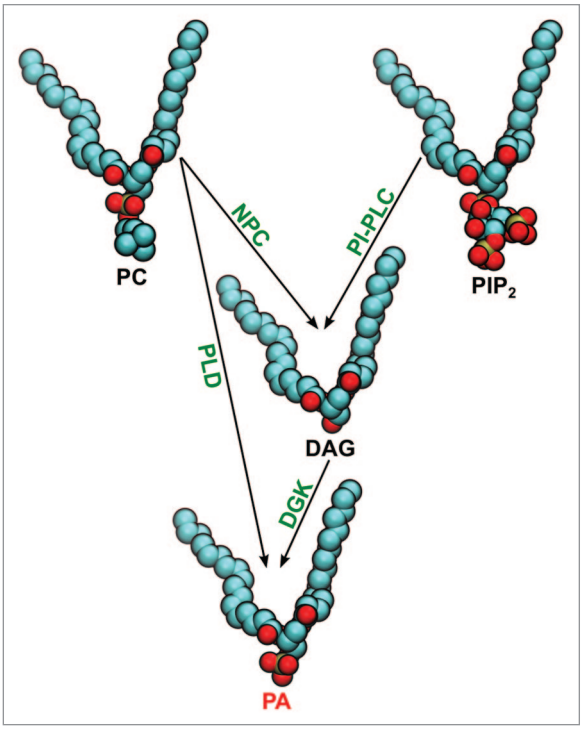

Figure 1. Distinct enzymatic pathways lead to PA production. Carbon atoms are shown as light blue balls, oxygen atoms are in red, phosphorus atoms are in brown and nitrogen atom of PC is represented as the dark blue ball. Hydrogen atoms are not shown for clarity. Abbreviations used: DAG, diacylglycerol, DGK, diacylglycerol kinase, NPC, non-specific phospholipase C, PA, phosphatidic acid, PC, phosphatidylcholine, PIP2, phosphatidylinositol (4,5)-bisphosphate, PI-PLC, phosphoinositide-specific phospholipase C, PLD, phospholipase D. This figure was prepared with VMD.[28]

arrays or extensive filament bundling. The latter changes could relate directly to PLD activity, or represent a feedback mechanism, since it has been shown that plant PLDβ isovariants bind and are regulated by both monomeric (G-) and filamentous (F-)actin.[12,13] G-actin inhibits PLDβ activity, whereas F-actin stimulates it.[12] Alternatively, PA could execute changes in cytoskeletal organization by interacting with specific effector proteins, altering their activity, function or localization, and these then could transduce cytoskeletal organization and dynamics changes. The list of PA-binding proteins in plants is growing,[3] but surprisingly few, if any, of these have established links to the actin cytoskeleton.

Heterodimeric Capping Protein is a PA-Binding Protein

The organization and turnover of the actin cytoskeleton is choreographed by hundreds of accessory proteins known collectively as actin-binding proteins (ABPs). Many ABPs have been characterized in plant systems and their biophysical and biochemical properties extensively studied in vitro. They can be roughly categorized according to their activities, which range from binding G-actin and modulating its polymerization; nucleation of new filaments; binding and stabilizing F-actin; severing actin filaments; and bundling or crosslinking into higher-order structures. Most ABPs are regulated, either positively or negatively, by post-translational modifications (e.g., phosphorylation) and/or by signaling second messengers, like Ca^{2+}, phosphoinositide lipids and pH.

The heterodimeric capping protein (CP; also known as CapZ in animal cells) is a conserved and ubiquitous regulator of actin filament dynamics.[14] In most plants, CP is encoded by single genes for α- and β-subunits, referred to as *CPA* and *CPB* respectively, and is an obligate heterodimer.[15] Recombinant CP binds to the barbed- or fast-growing end of actin filaments with high affinity and inhibits subunit addition or loss from that end.[10,16] It also dissociates slowly, with a $t_{1/2}$ for dissociation of 30–40 min in vitro,[10] and it competes with processive elongation factors, known as formins, for binding to barbed ends.[17] Thus, CP is an inhibitor of actin filament polymerization or depolymerization when bound to F-actin. Binding to filament ends also has consequences for another mechanism of filament turnover that is well-characterized in vitro, filament-filament annealing.[16] There are a few examples of this process in living cells,[18,19] but how prevalent is annealing and what contribution it makes to actin dynamics is poorly understood. Like other ABPs, CP is regulated by common signaling phospholipids. Arabidopsis CP binds with modest affinity to both PIP2 and PA, and this interaction inhibits its ability to bind filament barbed-ends and blocks filament-filament annealing reactions.[10,15,16] Moreover, signaling phospholipids can uncap filament ends perhaps though steric hindrance or through an allosteric mechanism, although the exact mechanism remains controversial.[20,21] It is worth noting that CP is the first and only eukaryotic ABP shown to interact directly with and be negatively regulated by PA.[10] Because of the cellular abundance of PA, even though its affinity for CP is rather modest, the negative regulation of CP is thought to be physiologically relevant.[10] However, evidence demonstrating that CP or any other ABP is linked via signaling lipids to cytoskeletal rearrangements in plants is completely lacking.

A Model Linking CP and PA to Altered Cytoskeletal Dynamics and Organization

Plant cells are somewhat unusual in that they contain a low amount of F-actin, just 5–10% of total actin, compared with the huge pool of monomer available for assembly.[22] It is predicted that this is due predominantly to the cooperative action of CP and the abundant monomer-binding protein, profilin.[22,23] Profilin prevents actin filament nucleation and inhibits addition of G-actin at filament pointed-ends. When barbed ends are blocked with CP, filament elongation is inhibited, as shown

in **Figure 2**. In contrast, when filament barbed-ends are uncapped, profilin-actin complex can add onto free ends contributing to rapid elongation. Thus, CP is a negative regulator of filament assembly and elongation. Loss of CP should result in new filament assembly and an increase in the density of actin filament arrays. If CP is, in turn, inhibited by PA and/or ends are uncapped by this lipid, then new filament assembly and filament-filament annealing will ensue (**Fig. 2**). Alternatively, if levels of PA are lowered, more ends will become capped and filament assembly will be blocked. This model predicts that altering PA levels in plant cells should change filament organization; specifically, increased PA should enhance polymerization and filament end dynamics, whereas lower PA should favor filament disassembly (**Fig. 2**). Further, if CP is the main sensor of PA levels in cell, then *cp* mutants are predicted to be insensitive to these changes. A new study by Li et al.[24] provides a critical test of this hypothesis by combining experimental alterations of PA levels, with advanced live-cell imaging of actin dynamics and use of several *cp* knockdown mutants in Arabidopsis.

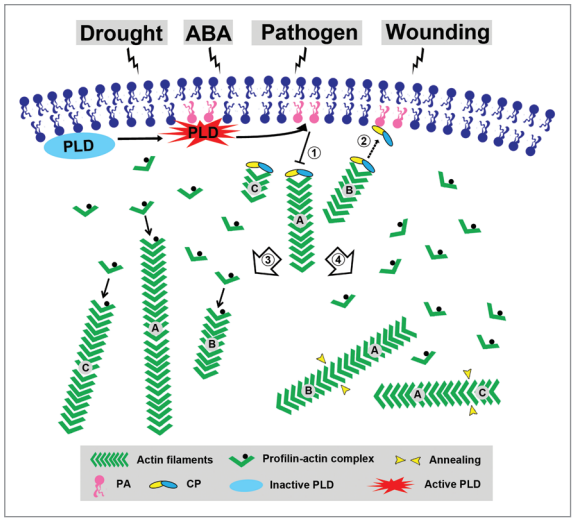

Figure 2. A model for the modulation of CP activity and dynamic behavior of actin filament ends in response to PA. CP binds to the barbed ends of actin filaments and prevents profilin-actin complex addition onto this end; i.e., actin polymerization is inhibited in the presence of CP. (A, B and C represent individual actin filaments). Reduced CP levels results in more available dynamic ends, which leads to new actin filament assembly (3) and enhanced filament-filament annealing (4). The end-capping activity of CP is negatively regulated by PA (1). When cellular PA levels increase, direct binding to PA dissociates CP from barbed ends (2), causes uncapping of the actin filaments, and leads to enhanced dynamic behavior of filament ends (followed by processes (3) and (4)).

Genetic and Pharmacological Support for CP as a Key Sensor of Cellular PA Levels

The recent work of Li and coworkers[24] makes use of several *cp* knockdown mutants and capitalizes on the ability to measure single actin filament turnover in living hypocotyl epidermal cells with variable-angle epifluorescence microscopy (VAEM;[25,26]). Moreover, a set of tools developed by Higaki and coworkers[27] was applied to quantify the architecture of actin filament arrays, including filament abundance or *density*, and filament bundling or *skewness*. Finally, a new set of metrics that focus on generation of new filaments and the behavior of filament barbed-ends were developed for this study. In wild-type plant cells, actin filament turnover in the cortical array has been described as 'stochastic dynamics', with individual filaments that assemble rapidly at available barbed ends (~1.7 μm/s) and slow depolymerization from pointed ends (~0.3 μm/s), balanced by prolific stochastic severing and disassembly events.[19,24-26] Recently, it was demonstrated that ADF4 contributes, at least partially, to filament disassembly through its severing activity in vivo.[26] In the new work, it was found that filament-filament annealing occurs at modest levels in wild-type cells (2–3% of free barbed ends), but this could still represent a physiologically-relevant mechanism for filament assembly given that thousands of subunits can be added simultaneously to an elongating filament.

Three independent *cp* knockdown alleles (*cpa-1*, *cpb-1* and *cpb-3*) were found to have similar actin-based phenotypes.[24] Reductions in CP levels in Arabidopsis epidermal cells resulted in a 5–15% increase in the density of actin filament arrays, consistent with new actin polymerization, and increased the dynamic behavior of filament barbed-ends. Specifically, the growth of new filaments from filament ends nearly doubled and filament-filament annealing was enhanced by as much as 6-fold in *cp* mutants. If CP is negatively regulated by PA, then increasing cellular PA levels in wild-type epidermal cells should phenocopy the *cp* mutants. This was indeed the case; PA applied exogenously to wild-type epidermal cells led to a rapid increase in the density of filament arrays and increased the availability of filament barbed ends for growth and annealing. Moreover, lowering of PA levels with 1-butanol had the opposite effect on wild-type cells, it significantly reduced actin filament density by as much as 20%. If PA transmits changes to the cytoskeleton predominantly through the regulation of capped filament ends, then it is predicted that *cp* mutants would be insensitive to elevated or reduced PA levels. That too appears to be the case; reduction in CP levels by genetic mutations completely abrogates the response of epidermal cells to exogenous PA or 1-butanol treatments. Collectively, these data demonstrate that the signaling lipid, PA, inhibits CP activity in plant cells and thereby is capable of transducing this message into changes in actin array organization and filament dynamics in vivo. And, this provides the first genetic and cytological evidence for regulation of the filament end-capping activity of CP through signaling phospholipids in any eukaryotic cell. These findings further predict that *cp* mutants will be altered in their response to diverse signaling events and developmental processes that depend upon PLD activity and changes in PA levels.

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