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A DYNAMIC INTERPLAY BETWEEN MEMBRANES AND THE CYTOSKELETON CRITICAL FOR CELL DEVELOPMENT AND SIGNALING

Topic Editors
Clément Thomas and Christopher J. Staiger

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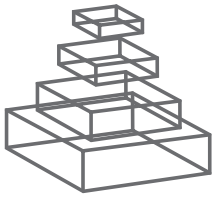

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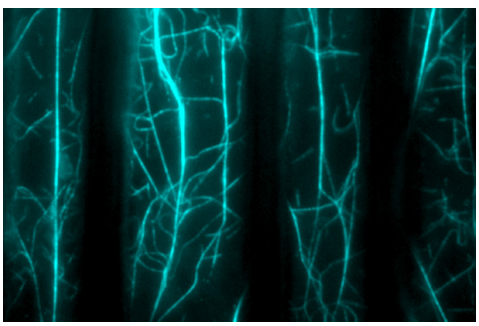
A DYNAMIC INTERPLAY BETWEEN MEMBRANES AND THE CYTOSKELETON CRITICAL FOR CELL DEVELOPMENT AND SIGNALING

Topic Editors:
Clément Thomas, Public Research Centre for Health, Luxembourg
Christopher J. Staiger, Purdue University, USA



The cover image (variable-angle epifluorescence microscopy, VAEM) shows the typical organization of actin filaments at the cortex of Arabidopsis hypocotyl epidermal cells. The fine, presumably single, actin filaments exhibit a random organization and undergo incessant changes in shape. The presence of many filament fragments, which mostly originate from the severing of longer filaments, illustrates the remarkably high rate of actin filament turnover near the cell membrane. The thickest/brightest, axially-oriented, elements correspond to less dynamic actin bundles made of several crosslinked filaments. Cortical actin filament nucleation, anchoring and behavior are controlled by membrane associated proteins and signaling molecules. In turn, membrane trafficking and organization largely rely on actin cytoskeleton remodeling.
Image kindly provided by Dr. Céline Hoffmann (CRP-Santé, Luxembourg).

Various cellular processes underlying plant development and response to environmental cues rely on a dynamic interplay between membranes and the cytoskeleton, e.g. vesicle and organelle trafficking, endocytosis, exocytosis, and signal transduction. In recent years, significant progress in the understanding of such interplay has been achieved and several critical links between membranes and the cytoskeleton have been characterized. As an example, recent work has clarified how auxin promotes the reorganization of cortical actin filaments by the activation of Rho GTPase pathways, and how such reorganization in turn locally modifies endocytosis and/or exocytosis and directs asymmetric distribution of PIN family of auxin transporters. Another recent achievement is the characterization of the Rho- and microtubule-driven mechanism by which the cell wall architecture is established. In particular, the elegant work by Oda and Fukuda (Science 337 p.1333, 2012) provides evidence that secondary wall patterning in xylem vessel primarily relies on two processes: a local activation of the plant Rho GTPase ROP11 and a mutual, MIDD1-mediated, inhibitory interaction between active ROP domains and cortical microtubules. Additional examples include

recent genetic evidence that microtubule and actin filament interacting/regulatory proteins, such as MAP65-1 and capping protein, function as transducers of membrane lipid signaling into changes in cytoskeleton dynamics and organization.

This Research Topic aims at collecting a comprehensive set of articles dealing with cellular processes involving membrane-cytoskeleton interactions. Its scope extends beyond the specific fields defined by the above examples and includes intracellular trafficking, host-pathogen interactions, response to biotic and abiotic stresses and hormonal regulation of growth. We hope that this Research Topic will also highlight critical questions that need to be addressed in the future. We welcomed Original Research Articles, Technical/Methodological Advances (e.g. analysis of cytoskeleton dynamics close to membranes), Reviews and Mini Reviews that can expand our understanding of how and why membranes and the cytoskeleton interact.

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EDITORIAL
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A dynamic interplay between membranes and the cytoskeleton critical for cell development and signaling

Clément Thomas[1]* and Christopher J. Staiger[2]*

[1] *Laboratory of Cellular and Molecular Oncology, Department of Oncology, Public Research Centre for Health, Luxembourg, Luxembourg*
[2] *Department of Biological Sciences, Purdue University, West Lafayette, IN, USA*
**Correspondence: clement.thomas@crp-sante.lu; staiger@purdue.edu*

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Markus Geisler, University of Fribourg, Switzerland

Keywords: actin, cytoskeleton, membrane trafficking, microtubules, signaling lipids

Diverse cellular processes that underlie plant development and response to environmental cues rely on a dynamic interplay between membranes and the cytoskeleton. The original research and review articles assembled in this Research Topic provide a valuable insight into our current understanding of how actin filaments and microtubules physically and functionally interact with membranes and cell wall and *vice versa*.

ACTIN-BINDING PROTEINS: OLD AND NEW

Actin-binding proteins come in hundreds of different varieties and are the governors for cellular cytoskeletal organization and turnover. The rate limiting step for generating actin filaments from a monomer pool *in vitro*, and presumably *in vivo*, is formation of a seed or nucleus of three actin subunits. Formins are conserved actin nucleation factors which, in plants, take on special relevance for the presence of secretion signals, transmembrane domains and lipid interaction motifs that permit their function on or near cellular membranes. In an up-to-date review, Cvrčková (2013) discusses the myriad ways that plant formins are thought to associate with membranes and their function(s) at various subcellular locations, especially the interface between cortical actin cytoskeleton–plasma membrane–cell wall.

The monomer-binding protein, profilin, was the first conventional plant actin-binding protein to be identified more than two decades ago. Sun et al. (2013) briefly review the long history of discovery research surrounding this abundant regulator of actin dynamcis. Biochemically, isoforms from maize and Arabidopsis have been well characterized and these bind three cellular ligands: monomer of G-actin, proteins with contiguous stretches of proline residues, and phosphoinositide lipids. More recently, it has been discovered that plant profilins are localized or function at cellular membranes and organelles via their interaction with nucleators like the formins; here, profilin-actin complexes likely provide a supply of subunits to polymerize new actin filaments. Whether this actin assembly powers vesicle or organelle movements, as in the case of mammalian endosomes or pathogenic intracellular bacteria, is an area for future investigations.

One striking difference between the repertoire of actin-binding proteins from plants and animals is that many types of actin-membrane adaptors present in animals are absent from plants. Plants in turn own a plant-specific superfamily of actin-binding proteins, called the Networked (NET) proteins; presumably these are able to connect actin filaments to various membrane compartments. Here, Hawkins et al. (2014) report a phylogenetic analysis including NET gene sequences across a large range of species. Their data support that the NET superfamily emerged early in the evolution of vascular plants and has continuously developed and diversified coincidentally with the complexity of plant species.

CORTICAL MICROTUBULES AND CELL WALL ASSEMBLY

A remarkable parallelism between membrane-associated microtubules in the cortical cytoplasm and cellulose microfibrils in the cell wall has captured the imagination of plant biologists for a half century. A review by Lei et al. (2014) synthesizes a broad range of research on this topic, from thoughts on how the primary wall regulates cell morphogenesis to the nature and dynamic visualization of the cellulose synthesis machine (CESA complex) that resides in the plasma membrane, and to how cortical microtubules guide or influence the trajectory of CESA movement in the plane of the membrane. Most importantly, these experts highlight the recent discovery and characterization of microtubule–CESA linkers, known as CSIs.

During secondary cell wall assembly, massive localized cellulose depositions in the cell wall are predicted and patterned by bands of cortical microtubules interspersed by regions devoid of microtubules. In elegant work by Oda and Fukuda (2013), a mutually inhibitory interaction beween cortical microtubule bundles and membrane domains is elucidated. They review the foundational work that reveals the switching on and off of ROP GTPases by GAP and GEF proteins that generate membrane domains. An effector of the activated ROP11, MIDD/ICR/RIP, regulates microtubule depolymerization via the kinesin-13A family member at these membrane domains.

TRAFFICKING ON THE CYTOSKELETON TOWARD THE PLASMA MEMBRANE AND THE NUCLEUS

Cytoskeleton-dependent trafficking of cell wall and membrane components is crucial for establishing and maintaining polar growth. Dissecting the underlying, highly coordinated, regulatory pathways remains a major challenge. In this context, mutations in genes encoding the unconventional plant kinesin ARK1 or the ARF-GTPase modulator AGD1 were reported to induce strikingly similar root hair developmental defects and cytoskeletal aberrations, suggesting that ARK1 and AGD1 specify root hair

tip growth via common molecular pathways. To further examine this possibility, Yoo and Blancaflor (2013) compared root hair defects in various double mutant combinations to *ark1* and *agd1*. Furthermore, they analyzed the distribution of the small GTPases ROP2 and RABA4b in both *ark1* and *agd1* root hairs. Their data confirm that ARK1 and AGD1 functionally overlap in maintaining the stability of small GTPases involved in root hair tip growth. They also point to divergences with ARK1 having a broad function during the entire root hair developmental program and AGD1 being more specifically involved in the early stages of root hair initiation and tip growth.

Batzenschlager et al. (2013) bring us into a functional study suggesting that the *Arabidopsis* γ-tubulin complex (γ-TuC) components GCP3-interacting protein1 (GIP1) and GIP2 are key determinants of nuclear shaping and nuclear envelope organization. The authors also report the identification of a novel and direct partner of AtGIP1, namely AtTSA1, which partially co-localizes with AtGIP1 at the nuclear envelope. Based on AtTSA1 domain signatures and previously identified partners, they suggest a model in which AtGIP1-AtTSA1 interaction is involved in the anchoring of γ-tubulin complexes at the outer nuclear envelope as well as in chromatin regulation within the nuclear compartment, positioning GIPs as a key regulator of a nucleo-cytoplasmic continuum.

LIPID REGULATION OF THE CYTOSKELETON AND THE EXOCYST COMPLEX

Protein–lipid interactions dominate signal transduction pathways, but also influence cortical cytoskeletal organization, and *vice versa*. In their article, Pleskot et al. (2014) summarize current knowledge about the regulation of actin dynamics by membrane phospholipid signaling molecules, with a particular focus on phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA). Following a brief description of the structural properties of PIP_2 and PA that are relevant to their functions at the plasma membrane, the authors discuss the interrelationships between PIP_2 and PA biosynthesis and the actin cytoskeleton. In addition, they review how PIP_2 and PA induce cytoskeletal changes via the regulation of specific actin-binding proteins, e.g., capping protein, or ROP small GTPases. Finally, the authors explain why they anticipate a synergistic regulation of cytoskeletal regulators by various phospholipids and an intense crosstalk between PIP_2 and PA signaling to the actin cytoskeleton.

The exocyst is a multiprotein complex, conserved across kingdoms, that tethers secretory vesicles to the plasma membrane during polarized growth. Synek et al. (2014) review linkages between this complex and the cortical actin and microtubule cytoskeletons, as well as small G-proteins. They further portray the exocyst as a key hub for coordinating exocytosis and signal transduction/sensing of cell wall stresses. Although most of this article summarizes influential studies and fundamental data from yeasts and mammalian cells, the path forward for plant biologists is clearly defined.

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Formins and membranes: anchoring cortical actin to the cell wall and beyond

Fatima Cvrčková*

Department of Experimental Plant Biology, Faculty of Science, Charles University, Prague, Czech Republic

Edited by:
Christopher J. Staiger, Purdue University, USA

Reviewed by:
Shanjin Huang, Institute of Botany, Chinese Academy of Sciences, China
Haiyun Ren, Beijing Normal University, China

****Correspondence:***
Fatima Cvrčková, Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, CZ 128 43, Prague, Czech Republic
e-mail: fatima@natur.cuni.cz

Formins are evolutionarily conserved eukaryotic proteins participating in actin and microtubule organization. Land plants have three formin clades, with only two – Class I and II – present in angiosperms. Class I formins are often transmembrane proteins, residing at the plasmalemma and anchoring the cortical cytoskeleton across the membrane to the cell wall, while Class II formins possess a PTEN-related membrane-binding domain. Lower plant Class III and non-plant formins usually contain domains predicted to bind RHO GTPases that are membrane-associated. Thus, some kind of membrane anchorage appears to be a common formin feature. Direct interactions between various non-plant formins and integral or peripheral membrane proteins have indeed been reported, with varying mechanisms and biological implications. Besides of summarizing new data on Class I and Class II formin-membrane relationships, this review surveys such "non-classical" formin-membrane interactions and examines which, if any, of them may be evolutionarily conserved and operating also in plants. FYVE, SH3 and BAR domain-containing proteins emerge as possible candidates for such conserved membrane-associated formin partners.

Keywords: formin, actin, plasmalemma, endomembranes, cell polarity, endocytosis, vesicle trafficking

INTRODUCTION

Formins (FH2 proteins) are a large family of evolutionarily conserved proteins sharing the well-defined FH2 domain (cd smart00498, pfam02181), originally identified in metazoans and fungi and later found to be ubiquitous among eukaryotes (Higgs, 2005; Higgs and Peterson, 2005; Chalkia et al., 2008; Grunt et al., 2008) and thus apparently dating back to the last eukaryotic common ancestor (see Vaškovičová et al., 2013). Land plants have three formin subfamilies, termed Class I, II and III (Deeks et al., 2002; Grunt et al., 2008), with only two of them (Class I and Class II) present in the angiosperms, and the third clade (Class III) found in mosses and lycophytes.

Formins were originally understood as multi-functional proteins involved in various aspects of cytoskeletal organization and intracellular signaling (see e.g., Frazier and Field, 1997; Heil-Chapdelaine et al., 1999). In the decade following the discovery that the FH2 domain can nucleate actin (Evangelista et al., 2002; Pruyne et al., 2002; Sagot et al., 2002) using an unique mechanism with the FH2 dimer acting as a leaky barbed end cap (Xu et al., 2004; Otomo et al., 2005), researcher's attention shifted mainly toward their actin-related roles. However, other functions of formins are coming back into focus, in particular their participation in microtubule organization and actin-microtubule co-ordination (reviewed in Bartolini and Gundersen, 2010; Chesarone et al., 2010; Wang et al., 2012).

Another (re)emerging frequent feature of formins is their association with cellular membranes. Here studies in plants have led the way, with typical Class I formins predicted and later experimentally proven to be directly inserted into membranes, especially the plasmalemma (Banno and Chua, 2000; Cvrčková, 2000; further experimental evidence reviewed below and in Cvrčková, 2012 and van Gisbergen and Bezanilla, 2013). Also Class II formins often possess a domain related to metazoan phosphoinositide phosphatase PTEN, which may mediate their peripheral association with membranes (Cvrčková et al., 2004). Indeed, in *Physcomitrella patens*, the PTEN domain of a Class II formin, For2A, was shown to bind plasmalemma phosphoinositides, especially PtdIns(3,5)P2 (van Gisbergen et al., 2012). The PTEN domain is also required for targeting the rice Class II formin FH5 to the chloroplast envelope (Zhang et al., 2011).

However, the structural and functional relationships between formins and membranes remain somewhat neglected in the literature. This review attempts to fill this gap by addressing the following questions:

(i) Which mechanisms, in addition to those described above for typical plant Class I and Class II formins, associate FH2 proteins to membranes in non-plant eukaryotic lineages?

(ii) What are the biological implications of formin-membrane association?

(iii) Which, if any, of the mechanisms and functions found in other lineages may operate also in plants?

A VARIETY OF MECHANISMS CAN ATTACH FORMINS TO MEMBRANES

The functionality (or value, in the neo-Darwinian terms) of a protein critically depends on its (intracellular) location, reminiscent of the well-known truth concerning real estate. Aside of regulating gene expression with far-reaching downstream effects, a protein can hardly exert a membrane-related function without physically associating with membranes. This may be accomplished by diverse mechanisms: by membrane insertion in integral membrane proteins, by direct binding (possibly following a post-translational

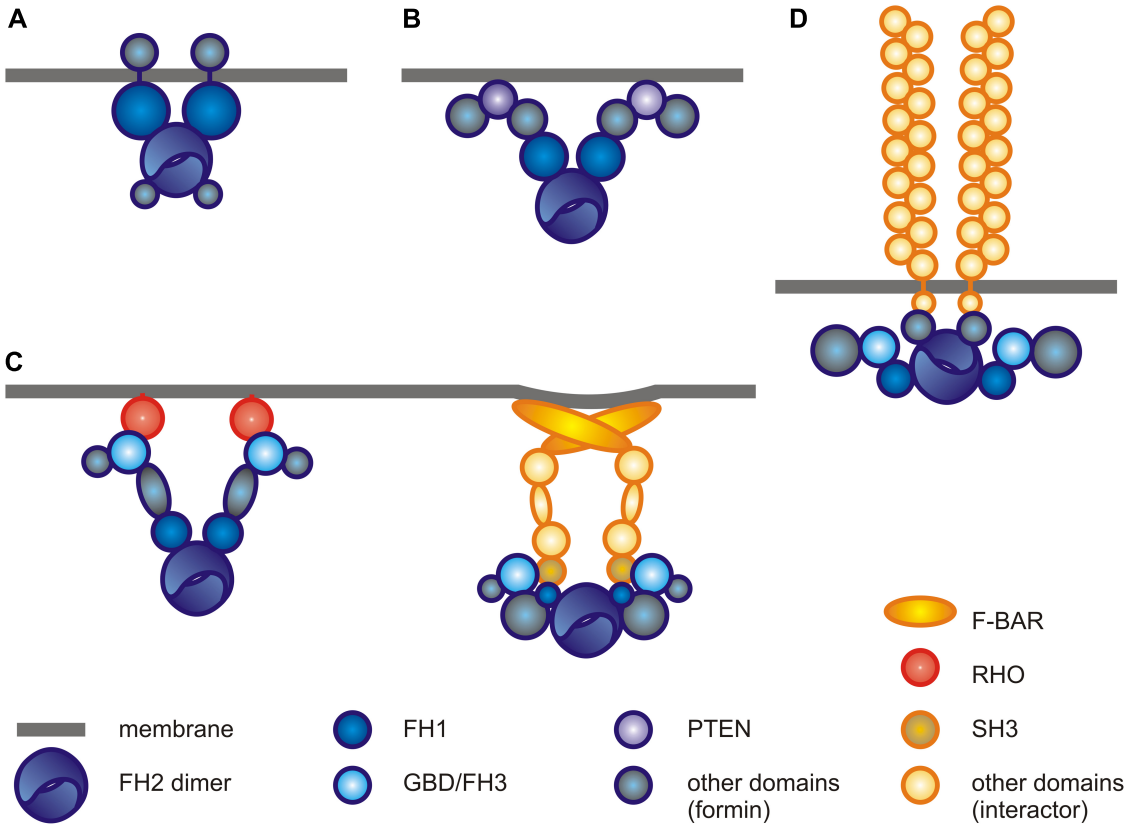

FIGURE 1 | Possible mechanisms of formin-membrane attachment. Protein domanins are drawn roughly to scale based on the sequences of proteins listed in parentheses (including *Arabidopsis* locus identifiers and/or GenBank or Uniprot accession numbers; interacting protein pairs were chosen based on cited literature). Formins are shown in shades of blue, their interactors in shades of orange, cytoplasmic side of the membrane faces down. Complex stoichiometry is speculative in the absence of data. **(A)** Direct insertion into the membrane, as in plant Class I formins (*Arabidopsis* AtFH1, At3g25500). **(B)** Peripheral membrane binding, as in plant Class II formins (*Arabidopsis* AtFH14, At1g31810). **(C)** Interaction with a peripheral membrane protein, such as a RHO GTPase or a FBAR protein (left: mouse mDia1, NP_031884.1 and Cdc42, NP_033991.1; right: human DAAM1, XP_005267487.1, and FBP17, Q96RU3.2). **(D)** Interaction with an integral membrane protein, as in mammalian formins binding to CD21 (human FHOS, NP_037373.2, and CD21, NP_001006659.1).

modification) in peripheral membrane proteins, and, last but not least, by binding to another integral or peripheral membrane protein (**Figure 1**).

The only formins experimentally proven to be integral membrane proteins are the members of the plant Class I clade. Outside plants, secretory and transmembrane peptides were predicted only in several uncharacterized invertebrate and protist formins, without experimental proof that these proteins are membrane-located, albeit in one Caenorhabditis case there is at least cDNA evidence that the gene is expressed (Grunt et al., 2008). Some metazoan formins can also bind to membranes peripherally, similar to plant Class II formins. Drosophila Diaphanous, a prototype member of the large metazoan Diaphanous related formin (DRF) clade (Goode and Eck, 2007), directly binds PtdIns(4,5)P2 through an N-terminal basic domain. However, its membrane association requires simultaneous binding to a RHO GTPase (see below), i.e., binding a membrane phosphoinositide alone does not yet make the formin a peripheral membrane protein (Rousso et al., 2013).

Association of fungal and metazoan formins with membranes is thus usually indirect, mediated by binding to peripheral or integral membrane proteins. Numerous formin interactors have been identified, most of them cytoplasmic (Aspenström, 2010). The best characterized membrane-associated ones are notorious formin regulators – the small GTPases of the RHO family, which can attach to membranes thanks to their hydrophobic post-translational modifications. Many formins, including fungal ones and metazoan DRFs, contain a conserved N-terminal GTPase binding domain (GBD/FH3) whose binding to an active (GTP-loaded) RHO alleviates autoinhibition mediated by a C-terminal autoinhibitory domain (Watanabe et al., 1997). The GBD/FH3 domain is probably evolutionarily ancient, although it appears to be absent in plants (Rivero et al., 2005).

Formins can bind some other peripheral membrane proteins. The N-terminal portion of mammalian FMNL1, a classical GBD/FH3 containing formin, interacts with AHNAK (desmoyokin), a huge phosphoprotein binding the plasmalemma as a part of a larger multiprotein complex (Haase, 2007; Dempsey et al., 2012). Rather than attaching itself to the membrane via AHNAK, the formin, bound to a RHO GTPase, participates in recruiting AHNAK from the cytoplasm to the plasmalemma

(Han et al., 2013). Association of related (FMN family) mammalian formins with compartments of the endomembrane system is, among other interactions, mediated by their binding to FYVE domain-containing proteins, including the Spir proteins that themselves can nucleate actin *in vitro* (Quinlan et al., 2005; Kerkhoff, 2011; Dietrich et al., 2013).

Proteins containing the conserved F-BAR domain, an extended version of the membrane-binding BAR domain (Heath and Insall, 2008; Roberts-Galbraith and Gould, 2010) may also contribute to interaction-mediated membrane localization of formins (albeit also here the localization may work in both directions, as F-BAR proteins are involved in large multiprotein complexes including RHO GTPases as well). Yeast and mammalian formin interactors such as FBP1/FBP17/Rapostlin (Wakita et al., 2011), FNBP1L/Toca (Huett et al., 2009) or CIP4 (Aspenström et al., 2006) all share a common architecture with an N-terminal F-BAR domain and C-terminal SH3 domain, with a coiled coil motif in between (Roberts-Galbraith and Gould, 2010). A mammalian homolog of CIP4, a prototype protein of this family originally identified as a Cdc42 (RHO GTPase) effector, interacts with the DAAM1 formin via its SH3 domain, raising thus the possibility that other SH3-containing proteins may bind formins as well (Aspenström et al., 2006). This is not surprising, as SH3 domains associate with proline-rich proteins (Alexandropoulos et al., 1995), and the majority of formins contain an extremely Pro-rich domain, termed FH1, in front of the hallmark FH2 domain. Indeed, the same study identified a Src family non-receptor tyrosine kinase as a DAAM1 binding partner, confirming thereby previous observations that other metazoan formins can bind Src (Uetz et al., 1996).

SH3 domain-containing proteins often interact with integral membrane proteins, and some are themselves inserted into membranes, such as, e.g., the budding yeast protein Fus1p (not to be confused with the fission yeast formin Fus1) which can bind to the Bni1p and Bnr1p formins via its SH3 domain (Tong et al., 2002). Another SH3-containing transmembrane protein, the osmosensor Sho1p, participates in a larger protein complex with Bni1p and Fus1p (Nelson et al., 2004).

Additional integral membrane proteins directly bind formins. The zebrafish plasmalemma protein Antxr2 (anthrax toxin receptor 2) participates in a ternary complex involving also a RHO GTPase and a DRF type formin (Castanon et al., 2013). The glutamate receptor delta2 (Grid2) from mammalian neurons binds to delphillin, an unusual formin that contains a PDZ domain that appears to be required for this apparently delphillin-specific interaction (Miyagi et al., 2002). In *Aspergillus*, MesA, a protein possibly post-translationally inserted into membranes, may be contributing to the localization of the SepA formin in the plane of the membrane (Pearson et al., 2004). Even a membrane transporter – the PKD2 cation channel – was reported to bind a DRF type formin (Rundle et al., 2004). Remarkably, the cytoplasmic domain of human complement receptor 2 (CD21) binds to the FHOS/FHOD1 formin and facilitates its localization to the plasmalemma upon viral infection (Gill et al., 2004), demonstrating that interactions with membrane proteins can indeed recruit formins to membranes.

Formins in non-plant lineages thus appear to have explored in evolution a variety of membrane association mechanisms which have not been documented, or even suspected, to exist in plants.

WHAT ARE THEY DOING THERE: NON-PLANT FORMINS IN MEMBRANE TRAFFICKING

Detailed discussion of the RHO-controlled, actin nucleation or actin-microtubule co-ordination-based cortical processes in non-plant lineages, including formation of invasive structures such as e.g., metazoan filopodia, would be out of scope of this review, and can be found elsewhere (e.g., Chesarone et al., 2010; Yang and Svitkina, 2011; Vaškovičová et al., 2013). What follows is a summary of biological implications of the formin-membrane interactions discussed in the previous section.

Some of these mechanisms may localize formins within the plane of the plasmalemma, participating thus in the control of cell polarity, or delimiting cell surface domains with increased membrane expansion or turnover (including polar or tip growth; for the concept of "activated cortical domains" in plant cells compare Žárský et al., 2009). Phosphoinositide interaction of Drosophila Diaphanous is required for targeting the formin to the epithelial apical membrane (Rousso et al., 2013), and interaction with the F-BAR protein CIP4 may inhibit Diaphanous in lateral and basal membrane domains (Yan et al., 2013). However, other metazoan F-BAR proteins may stimulate formin activity while connecting the plasmalemma and the cortical cytoskeleton during actin-driven membrane tubulation and ruffling (Toguchi et al., 2010) or during formation of dendritic spines in neurons (Wakita et al., 2011). *Aspergillus* formin interactor MesA promotes formin localization to growing tips of hyphae (Pearson et al., 2004), reminiscent of the function of some plant formins in tip growth (see below). Similarly, formin-containing complexes of budding yeast Fus1p localize at the tip of mating protrusions, or "shmoos" (Nelson et al., 2004). In zebrafish, complexes involving RHO, a DRF type formin and Antxr2a exhibit polar localization at the plasmalemma and contribute to division plane positioning (Castanon et al., 2013).

Formins also associate with the endomembrane system and participate in vesicle trafficking. The above-described metazoan Spir/formin complexes engage in actin-dependent vesicle transport, possibly via actin nucleation on vesicle membranes (see Kerkhoff, 2011; Dietrich et al., 2013). Formins, bound to RHO GTPases, also participate in spatially restricted endocytosis and in endosome dynamics in both yeasts (Gachet and Hyams, 2005; Prosser et al., 2011) and metazoans, where interaction with Src appears to be contributing as well (Gasman et al., 2003). It has to be noted, though, that all the endosome- and endocytosis-associated formins described so far contain the GBD/FH3 domain which can engage in endocytosis regulation also outside the formin context, as in the Entamoeba EhNCABP166, which lacks the FH2 domain (Campos-Parra et al., 2010). The F-BAR family formin interactors are also predominantly involved in endocytosis (Feng et al., 2010), as well as in autophagy, also an endosome-dependent process (Huett et al., 2009). The F-BAR domain's ability to increase or stabilize membrane curvature may play an important role in generating endocytotic membrane vesicles, a process facilitated by dynamin (Roberts-Galbraith and Gould, 2010).

While most reports on formin-endomembrane associations point to endocytotic pathways or compartments, genetic data from fission yeast suggest that the For3 formin participates in exocytosis, as a synthetic thermosensitivity phenotype was observed upon combining mutations affecting For3 and Mug33, a transmembrane protein involved in polarized secretion and co-localizing with the exocyst complex (Snaith et al., 2011). Also the formin binding partner AHNAK has been implicated in the delivery of Ca2+ channels to the plasmalemma repair of cell membrane lesions, i.e., in processes that, on the first glance, appear to be exocytosis-driven, albeit they have a non-separable endocytotic component as well (Idone et al., 2008).

To summarize, numerous lines of evidence point to formins being involved in various aspects of endosome trafficking or endomembrane system organization. Recent reports even indicate that the ER associated formin INF2 (Chhabra et al., 2009) participates in the division of mitochondria, which involves a dynamin-related protein (Korobova et al., 2013), and other formins contribute to actin rearrangements involved in Toxoplasma apicoplast division (Jacot et al., 2013). However, as most of the reported interactions involve proteins so far found only in opisthokonts, it remains to be seen if similar mechanisms operate also in plants.

MEMBRANE-ASSOCIATED FORMINS IN PLANTS: THE KNOWN AND THE POSSIBLE

Insertion of typical plant Class I formins into membranes, as well as membrane association of PTEN domain-containing formins, is experimentally well documented. As far as biological function is concerned, plant formins, often plasmalemma-associated, were shown to participate in the control of the cell cortex architecture during cell growth, including both tip growth (e.g., Cheung and Wu, 2004; Deeks et al., 2005; Yi et al., 2005; Vidali et al., 2009; Ye et al., 2009; Cheung et al., 2010) and isodiametric or polar expansion (Favery et al., 2004; Rosero et al., 2013), as well as in cytokinesis (Ingouff et al., 2005; Li et al., 2010). The *Arabidopsis* AtFH1 formin mediates trans-membrane anchorage of the cortical actin to the cell wall, exhibits restricted lateral mobility due to its cell wall attachment, and localizes to microtubule-free cortical areas (Martiniere et al., 2011, 2012), providing thus a possible mechanism for attenuating cortical microtubule dynamics. Consistent with this hypothesis, mutants

Table 1 | Candidate plant membrane-associated formin interactors.

Protein or domain(s)	Non-plant query	Land plant candidates	Notes
AHNAK	NP_001611.1 (human AHNAK isoform 1)	N.A.	Best plant BLAST hit with E-value 5e-06 only matches a low compexity region of AHNAK
Spir (FYVE)	NP_001246101.1 (Drosophila spire isoform F)	N.A.	
other FYVE	cd00065 (FYVE domain)	At4g33240, FAB1A At3g14270, FAB1B	Many plant FYVE domain protein exist; for candidate selection see text.
F-BAR-SH3	NP_004231.1 (human CIP4) NP_055848.1 (human FBP1) NP_060207.2 (human FNBP1)	N.A.	No *bona fide* plant F-BAR domains but several proteins have an analogous BAR-SH4 domain layout with a plant-specific shorter BAR domain (cd07607) instead of FBAR (see BAR-SH3).
Fus1 (SH3)	NP_009903 (*Saccharomyces cerevisiae* Fus1p)	N.A.	
other BAR-SH3	cd07607 (BAR domain of the plant SH3 domain-containing proteins)	At1g31440, AtSH3P1 At4g34660, AtSH3P2 At4g18060, AtSH3P3	No additional *Arabidopsis* paralogs identified by Blast with AtSH3P3 query.
Antxr2	XP_005165376.1 (zebrafish Antxr2a isoform X1)	N.A.	
MesA	Q5BGR2.2 (*Aspergillus nidulans* MesA)	N.A.	
Grid2	NP_001501.2 (human Grid2)	Numerous glutamate receptors exist in plants but formin association unlikely.	PDZ domain in the formin partner required for binding, not founds in plant formins.
CD21	NP_001006659.1 (human CD21 isoform 1)	N.A.	
PKD2	NP_032887.3 (mouse polycystin-2)	N.A.	PKD2 homologs found in Micromonas and volvocal algae.

GenBank/Uniprot accession numbers are provided for protein sequences used as queries, and NCBI conserved domain database accessions for domains. N.A., not available (not found in standard Blast searches of the Viridiplantae section of the NCBI protein database using the listed non-plant sequences as queries). For proteins and domains where land plant candidates were found, only Arabidopsis proteins are shown (referred to using standard A. thaliana locus nomenclature), albeit non-Arabidopsis homologs without experimental data exist as well.

lacking AtFH1 have more dynamic microtubules (Rosero et al., 2013).

Similar to other eukaryotic lineages, also in plants formins may be closely involved in membrane turnover or associated with endomembranes. *Physcomitrella patens* Class II formin For2A specifically localizes to PtdIns(3,5)P2-rich sites of active plasmalemma turnover (van Gisbergen et al., 2012). Overexpressed microtubule-associated Class I *Arabidopsis* formin AtFH4 can decorate the endoplasmic reticulum and co-align it to the microtubule cytoskeleton (Deeks et al., 2010), and its relative AtFH8 is targeted to the nuclear envelope (Xue et al., 2011). Loss of tip polarity in formin-overexpressing pollen tubes (Cheung and Wu, 2004; Cheung et al., 2010) or root hairs (Yi et al., 2005), as well as irregular cell wall thickening observed in rice mutants lacking the Class II formin FH5 (Yang et al., 2011) might be understood as disturbance of the exocytosis/endocytosis co-ordination. Thus, the biological implications of formin-membrane association may be conserved, and it is worth examining the molecular mechanisms underlying membrane localization of formins.

Non-classic angiosperm formins lacking the transmembrane (in Class I) or PTEN-like (in Class II) domains might heterodimerize with their membrane-bound paralogs. Surprisingly, FH2-mediated formin heterodimerization has been neither documented nor excluded yet in any organism, albeit dimerization via other domains was reported (see Cvrčková, 2012).

The Rop GTPases represent a plant branch of RHO proteins (see Mucha et al., 2011), often understood as general formin regulators. However, plant formins lack the RHO-binding GBD/FH3 domain, and the only putative RHO interaction motif found in land plant FH2 proteins is a RHO GTPase activating protein (RhoGAP)-related domain in non-angiosperm Class III formins (Grunt et al., 2008). Thus, Rops are unlikely to provide the means for direct formin-membrane binding in angiosperms, albeit they may participate in larger multi-subunit complexes.

Few, if any, clear homologs of other non-plant membrane associated formin interactors can be identified in database searches (**Table 1**). Two protein families may, nevertheless, deserve a closer look.

While there is no direct plant homolog of Spir, numerous plant proteins harbor FYVE domains. The 15 FYVE-containing proteins of *A. thaliana* can be divided into five groups according to their domain architecture (Wyvial and Singh, 2010). Most of these proteins are experimentally uncharacterized, and none exhibit a significant match to any of the previously described formin interactors in BLAST searches. However, the only two experimentally characterized *Arabidopsis* FYVE-containing proteins encoded by the FAB1A and FAB1B genes are members of type III phosphatidylinositol 3-phosphate 5-kinase, or PIKfyve, family which has been implicated in endocytosis and actin dynamics in metazoan cells, albeit with no evidence for direct formin participation (Shisheva, 2008). Intriguingly, in *Arabidopsis*, mutations in FAB1A/B cause extensive vacuolization and collapse of pollen grains (Whitley et al., 2009), disrupt endocytosis and vacuole pH regulation, and perturb auxin transporter recycling (Hirano and Sato, 2011; Hirano et al., 2011; Bak et al., 2013). While these effect may be due to various regulatory effects of PtdIns(3.5)P2 produced by PIKfyve, a possible involvement of formins (including Class II members binding to PtdIns(3.5)P2-containing membranes) may deserve attention.

Likewise, no direct homolog of yeast Fus1p (a transmembrane SH3-containing protein) has been found. However, members of the coiled-coil-SH3-containing family of AtSH3Ps associate with the plasmalemma and endomembranes and participate in clathrin-mediated endocytosis (Lam et al., 2001), albeit there is yet no evidence of their interaction with formins. AtSH3P2 appears to be upregulated in pollen tubes, whose growth is formin-dependent (Wang et al., 2008). Intriguingly, these proteins contain a N-terminal BAR domain, a plant-specific variant of a shorter version of the F-BAR domain (which is absent in plants); and perhaps they might represent a plant counterpart of the yeast and metazoan F-BAR formin interactors.

Last but not least, plant formins may be attached to membranes by lineage-specific mechanisms. A gene encoding a protein with unique combination of FH2 and Sec10 domains, physically linking a formin and a subunit of the membrane-associated Exocyst complex, exists in Physcomitrella (Grunt et al., 2008; Cvrčková et al., 2012), and the first identified plant formin interactor, FIP2 (At5g55000; Banno and Chua, 2000) contains a domain corresponding to the oligomerization interface of voltage-gated potassium channels, and might perhaps interact with them.

In summary, there may be more to the association of plant formins with membranes than just the transmembrane and PTEN-like domains characterizing the two angiosperm formin clades, and a comparison with non-plant systems does provide some candidates that may be worth closer investigation.

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Profilin as a regulator of the membrane-actin cytoskeleton interface in plant cells

Tiantian Sun, Shanwei Li and Haiyun Ren*

Key Laboratory of Cell Proliferation and Regulation Biology of Ministry of Education, College of Life Science, Beijing Normal University, Beijing, China

Edited by:
Christopher J. Staiger, Purdue University, USA

Reviewed by:
Rashid Ali, University of Connecticut, USA
Frantisek Baluska, University of Bonn, Germany

****Correspondence:***
Haiyun Ren, Key Laboratory of Cell Proliferation and Regulation Biology of Ministry of Education, College of Life Science, Beijing Normal University, No. 19, Xin Jie Kou Wai Street, Beijing 100875, China
e-mail: hren@bnu.edu.cn

Membrane structures and cytoskeleton dynamics are intimately inter-connected in the eukaryotic cell. Recently, the molecular mechanisms operating at this interface have been progressively addressed. Many experiments have revealed that the actin cytoskeleton can interact with membranes through various discrete membrane domains. The actin-binding protein, profilin has been proven to inhibit actin polymerization and to promote F-actin elongation. This is dependent on many factors, such as the profilin/G-actin ratio and the ionic environment of the cell. Additionally, profilin has specific domains that interact with phosphoinositides and poly-L-proline rich proteins; theoretically, this gives profilin the opportunity to interact with membranes, and a large number of experiments have confirmed this possibility. In this article, we summarize recent findings in plant cells, and discuss the evidence of the connections among actin cytoskeleton, profilin and biomembranes through direct or indirect relationships.

Keywords: actin cytoskeleton, profilin, plasma membrane, organelle, vesicle, plants

INTRODUCTION

The membrane is a lipid bilayer that functions to divide and separate the cells and organelles. It undergoes many dynamic morphological changes during cellular processes such as endocytosis, exocytosis, vesicular transport, and morphogenesis. Growing evidence has demonstrated that actin cytoskeleton dynamics are involved in these processes. However, the interactions between microfilaments and membranes vary in different cell types and locations. Some cytoskeletal elements may interact with membranes directly. Transmembrane proteins can regulate membrane-cytoskeleton interactions directly or indirectly through adaptor proteins or adaptor complexes. Furthermore, some proteins have domains that can associate with the membrane, and domains that can interact with cytoskeletal components. These are the main types of membrane-cytoskeleton interactions (Doherty and McMahon, 2008). The extracellular matrix (ECM) of animals mainly consists of proteinaceous materials. However, the plant cell wall, which deviates plant cell from spherical shapes, mainly consists of carbohydrates. This implies that there are differences in the intermolecular interactions that occur in membrane-cytoskeleton of animal and plant. In mammals, cytoskeletal proteins that can function as adaptors, such as talin (Heise et al., 1991), vinculin (Geiger et al., 1980), and filamin (Stossel et al., 2001) bind the actin cytoskeleton to membranes; homologs of these proteins are absent from plants (Hussey et al., 2002). There are many plant-specific linker molecules. For example, myosin VIII binds directly or indirectly to plasma membrane-localized callose synthase complexes (Verma and Hong, 2001; Ostergaard et al., 2002) and it also binds to actin filaments in the cytoplasm, which implies that myosin VIII associates plasma membrane with actin filaments in plants. Moreover, a plant-specific Networked (NET) superfamily of actin-binding proteins is found in *Arabidopsis*. Members of the NET superfamily localize to the actin cytoskeleton and specify different membrane compartments. NET1A is located at the plasma membrane and binds directly to actin filaments through a novel actin-binding domain. The NET superfamily is grouped into four phylogenetic clades, and other members have functions at the tonoplast, nuclear membrane, and pollen tube plasma membrane, which suggest that this superfamily is involved in regulating actin-membrane interactions (Deeks et al., 2012).

A large amount of literature has fostered our current understanding of the membrane, the actin cytoskeleton, and of actin-binding proteins that mediate membrane and actin cytoskeleton components. Profilins are actin-binding proteins, and have the capacity to interact with three classes of ligands. In addition to G-actin, they also associate with poly-L-proline (PLP) which can interact with the binding cleft formed from the N-terminal and C-terminal helices of profilin (Metzler et al., 1994; Mahoney et al., 1999) and phosphoinositides (Gibbon and Staiger, 2000; Jockusch et al., 2007) which offers the possibility that profilin interacts with the membrane. In recent years, much evidence has been verified that profilins can interact with membranes directly or indirectly. In this review, we will summarize recent findings and focus predominantly on the functions of profilins in the direct or indirect relationships among actin cytoskeleton, profilin and membranes in plant cells.

MULTIFUNCTIONAL PROFILINS

Genomic DNA sequences of putative profilins contain three exons; these may be separated by introns of different sizes (Huang et al., 1996), and are dispersed throughout the genome. Comparing the amino acid sequences of different profilins reveal that profilins have less than 25% identity across different kingdoms (Pollard and Quirk, 1994), but are highly conserved, with at

least 70% identity, across various plant species (Mittermann et al., 1995; Vidali et al., 1995). This is consistent with the analysis of the phylogenetic tree shown in **Figure 1**. Although the secondary and tertiary structures of all profilins are well conserved (Fedorov et al., 1997; Thorn et al., 1997; Jockusch et al., 2007), the fact that many varieties of profilins isoforms exist in different species, and even in the same organism, may indicate that members of the profilin family have diverse functions. Plant profilins are from multigene families and can be divided into two major groups: the vegetative group, in which profilins exist extensively and are constitutively expressed in all plant tissues; and the reproductive group, where profilins are expressed in reproductive tissues (Kandasamy et al., 2002). The *Arabidopsis* profilin family includes five highly different isoforms: AtPRF1–AtPRF5; AtPRF1–AtPRF3 belong to the vegetative class, and AtPRF4 and AtPRF5 to the reproductive class (Christensen et al., 1996; Kandasamy et al., 2002). AtPRF1 has much higher affinities for both PLP and G-actin than AtPRF2 (Wang et al., 2009). The tobacco profilin gene, pronp1, is prominently expressed in mature pollen, elongating pollen tubes, and the root hairs of developing seedlings. Pronp1 represents a unique profilin as it has activities in two kinds of tip-growing cells, the pollen tubes and root hairs, which rapidly regulate the organization of the actin cytoskeleton (Swoboda et al., 2001). In tomato, LePRO1 was found to be expressed only in pollen grains, and not in other parts of the anther or in other organs using a non-radioactive labeling method (Yu et al., 1998). RcPRO1, a *Ricinus communis* phloem profilin, is expressed in epidermal, cortex, pith, and xylem tissue. In the sieve-tube exudates, RcPRO1 has 15-fold molar excess to actin, which suggests that actin filament formation is blocked in the assimilate stream (Schobert et al., 2000). In maize, five profilins have been identified (ZmPRO1–ZmPRO5); ZmPRO1–ZmPRO3 are major profilin isoforms of a pollen-abundant class, whereas ZmPRO4 and ZmPRO5 appear to be members of a predominantly endosperm profilin class. Furthermore, ZmPRO1 inhibits hydrolysis of membrane phosphatidylinositol-4, 5-bisphosphate (PIP_2) by phospholipase C more effectively than ZmPRO5. Conversely, ZmPRO5 has higher affinity for PLP and sequesters more monomeric actin to inhibit actin polymerization better than ZmPRO1 (Staiger et al., 1993; Kovar et al., 2000). Currently, there are over 400 profilins from 100 plant species, which are effective at NCBI GenBank database (Pruitt et al., 2007; Jimenez-Lopez et al., 2012). All of the above evidences support that profilins are multifunctional proteins according to their expressions and locations.

PROFILIN IS INVOLVED IN PLASMA MEMBRANE-ACTIN CYTOSKELETON INTERACTIONS

Binding interactions between the plasma membrane and the actin cytoskeleton define cell functions such as cytoplasmic streaming, cytokinesis, and endocytosis. Profilin is one of the crucial linkers of the membrane-cytoskeleton interaction. The inherent interaction of the actin cytoskeleton with the plasma membrane is through the relationship between actin-binding proteins and PIP_2, which itself localizes to the inner side of the plasma membrane (Nebl et al., 2000; Caroni, 2001). PIP_2 can bind to transmembrane adhesion protein, and also interacts with several actin-binding proteins including profilin (Goldschmidt-Clermont et al., 1990; Heiska et al., 1998; Couchman et al., 2002). Immunofluorescence analysis revealed that at the plasma membrane of maize root cells, PIP_2 is targeted to discrete domains that resemble profilin-enriched domains. PIP_2 redistributes and the actin cytoskeleton remodels following treatment with phospholipase C activator mastoparan (Baluska et al., 2001). Therefore, profilin may be a linker between the plasma membrane and actin cytoskeleton through PIP_2. Furthermore, profilins can interact with the proteins that contain PLP stretches of at least eight to ten prolines in continuous or discontinuous sequences (Schluter et al., 1997). In eukaryotes, formins are a group of actin-binding proteins that contain the FH1 domain with different numbers of PLP stretches; they are considered to act as morphological regulation proteins that direct the assembly of unbranched actin filaments (Paul and Pollard, 2009). Profilins or actin/profilin complexes can interact with the PLP stretches of different formins to promote actin filament polymerization (Chang et al., 1997; Pruyne et al., 2002; Kovar et al., 2006; Paul and Pollard, 2009). Additionally, type I formins contain an N-terminal transmembrane domains; this is the region of formin association with the plasma membrane in plants (Cvrckova et al., 2004). For example, in *Arabidopsis*, formin homology 6 (AtFH6) interacting with profilin locates at the plasma membrane and is uniformly distributed (Favery et al., 2004). Furthermore, AtFH1 and AtFH5 are reported to associate with the cell membrane (Banno and Chua, 2000; Cheung and Wu, 2004; Ingouff et al., 2005). This verifies that plant type I formins are likely to be membrane-bound, with AtFH8 being the exception, as it is targeted to the nuclear envelope (Xue et al., 2011). The site of the profilin binding FH1 PLP tracks is on the opposite face of the actin binding site of profilin (Schutt et al., 1993), and this explains why profilin can bind PLP and actin simultaneously without mutual influence (Tanaka and Shibata, 1985; Perelroizen et al., 1994). Profilin has an indirect connection and possibly acts as a regulator in the linkage of the plasma membrane and the actin cytoskeleton.

The plant cell is able to defend itself from infection by exogenous fungi. During this process, the cytoskeleton reorganizes and the papilla localizes at penetration sites, this leads to a thick cell wall being formed to prevent pathogen ingress (Schmelzer, 2002). Material is site-directed to arrive at positions around the fungal infection structure beneath the cell wall, and the actin filament and microtubule re-orientate their structures toward the penetration site (Schmelzer, 2002; Takemoto et al., 2003). In cultured parsley cells, undergoing attack from infection with the oomycetous plant pathogen *Phytophthora infestans*, profilin is expressed and accumulates at the site of infection on the plasma membrane, and the actin cables focus at the penetration site where Rop GTPases also accumulate (Schutz et al., 2006). In addition, in developing microspores and mature pollen of *Zea mays*, profilin is associated with the plasma membrane (von Witsch et al., 1998). Profilin accumulates in the tip zone near the plasma membrane in root hairs of *Arabidopsis* (Braun et al., 1999; Baluska et al., 2000). These results suggest that profilins play a role in both signal transduction and linkage between the plasma membrane and actin cytoskeleton

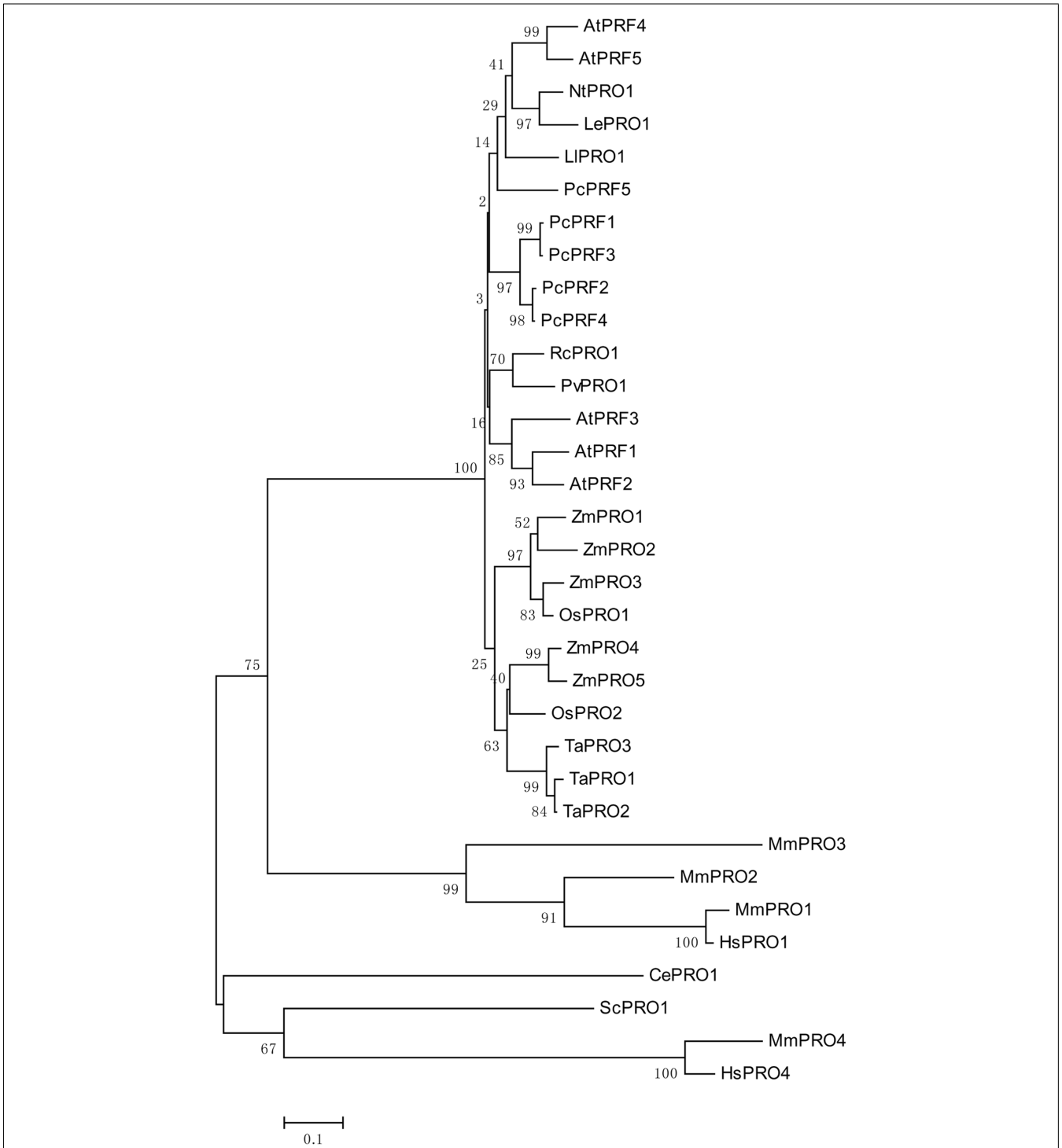

FIGURE 1 | An unrooted phylogenetic tree of profilins. The plant genes are *Arabidopsis thaliana* AtPRF1–AtPRF5 (AT2G19760, AT4G29350, AT5G56600, AT4G29340, AT2G19770), *Petroselinum crispum* PcPRF1–PcPRF5 (AY900012-AY900016), *Zea mays* ZmPRO1–ZmPRO5 (X73279, X73280, X73281, AF032370, AF201459), *Oryza sativa* OsPRO1–OsPRO2 (LOC_Os10g17680, LOC_Os06g05880), *Triticum aestivum* TaPRO1–TaPRO3 (X89825-X89827), *Nicotiana tabacum* NtPRO1 (pronp1 AJ130969), tomato LePRO1 (U50195), *Ricinus communis* RcPRO1 (AF092547), *Phaseolus vulgaris* PvPRO1 (CAA57508), *Lilium longiflorum* LlPRO1 (AF200184). Selected fungal and metazoan sequences are included: *Mus musculus* MmPRO1–MmPRO4 (NP_035202, NP_062283, NP_083579, AK013595), *Homo sapiens* HsPRO1 and HsPRO4 (BC057828, BC029523), *Caenorhabditis elegans* CePRO1 (PFN-1, NP_493258) *Saccharomyces cerevisiae* ScPRO1 (PFY1, NP_014765). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

PROFILIN IS INVOLVED IN ORGANELLE LOCATION WITH THE ACTIN CYTOSKELETON

There is much evidence, that in various eukaryotic cells the cytoskeleton is involved in organelle movements. In plant cells, the role of the actin cytoskeleton in organelle movements has been reported for movements of chloroplasts (Kandasamy and Meagher, 1999), the endoplasmic reticulum (ER; Boevink et al., 1998), and the Golgi apparatus (Boevink et al., 1998; Nebenfuhr et al., 1999).

In *Arabidopsis*, CHUP1 (*Chloroplast unusual positioning 1*) which is a 112 kDa protein that is closely related with chloroplast movement (Kasahara et al., 2002; Oikawa et al., 2003) is directly targeted to the outer envelope of the chloroplast; this is dependent on its N-terminus domain (Oikawa et al., 2003). In addition to the N-terminus domain, the CHUP1 protein has four other domains, including two leucine-zippers, an actinin-type actin binding domain (Gimona et al., 2002), and a proline-rich motif (PRM) that is similar to PRM1 identified as a profilin binding motif (Holt and Koffer, 2001). A fusion protein which includes GST and the actin binding domain of CHUP1 can bind F-actin *in vitro* (Oikawa et al., 2003). The *in vitro* biochemical analyzes revealed that CHUP1 interacts with profilin as a modulator of actin polymerization through the PRM of C-terminal part of CHUP1 (CHUP1-CT). The experiment of CHUP1-CT titrated to a mixture of profilin and actin confirmed that the trimeric complex of actin, profilin, and CHUP1-CT is more stable than the individual binary complex. Though CHUP1 can bind F-actin directly, profilin has been reported to enhance the connection between chloroplasts and actin filaments (Schmidt von Braun and Schleiff, 2008).

Although profilin can bind to formin, the type II formins do not contain the transmembrane domains present in type I formins (Cvrckova et al., 2004). In rice, like other plant type II formins, formin homology 5 (FH5) has a characteristic N-terminal phosphatase tensin (PTEN)-related domain that may interact with membranes (Cvrckova et al., 2004). The experiments of transiently expressing the PTEN-RFP fusion protein in tobacco (*Nicotiana tabacum*) cells and immunostaining analysis using rice leaf cells revealed that the PTEN-like domain of FH5 is sufficient to confer localization of the protein to the chloroplast surface. This suggests that the PTEN domain of FH5 may be a bridge between chloroplasts and the actin cytoskeleton (Zhang et al., 2011). Furthermore, FH5 was capable of nucleating actin assembly from the actin/profilin complex *in vitro* biochemical analyzes (Yang et al., 2011; Zhang et al., 2011). Therefore, profilin is indirectly involved in the localization of chloroplast to the actin filaments. In *Arabidopsis*, observations of living cells in stable transgenic plants revealed that 35S:: GFP-AtPRF1 forms a filamentous network likely associated with actin filaments; this was verified by treatment with latrunculin A, and through a recovery experiment involving the removal of latrunculin A. Whereas, 35S:: GFP-AtPRF2 forms polygonal meshes resembling ER in the same latrunculin A treatment conditions (Wang et al., 2009). Furthermore, in plants, profilins possibly participate in the linkage of the nuclear envelope and the actin cytoskeleton during the interphase of *Arabidopsis*; this is because AtFH8 locates primarily to the nuclear envelope at this stage (Xue et al., 2011).

PROFILIN IS INVOLVED IN VESICLE TRAFFICKING

Profilins are known to play an important role in endocytosis and membrane trafficking in lower eukaryotes (Wolven et al., 2000; Pearson et al., 2003). In mammalian cells, profilins may also be involved in membrane trafficking. It has been reported that profilin 1 exists in budding Golgi vesicles, and that dynamin 2 recruitment to the Golgi is dependent on profilin 1 (Dong et al., 2000). Moreover, in mammalian cells, there are multiple phosphoinositide 3-kinases (PI3Ks), and these can be grouped into three main classes. Class I and II PI3Ks can induce receptor-dependent trafficking processes, such as phagocytosis. Class III PI3Ks, which represent the most ancient form of PI3Ks, and are the only ones conserved in lower eukaryotes, mammals, and plants. Class III PI3Ks mainly regulate receptor-independent trafficking events, such as endocytic membrane traffic (Lindmo and Stenmark, 2006). In animal cells, PI3Ks have been reported to play many different roles in vesicle trafficking, and inhibition of PI3Ks induces the inhibition of clathrin-dependent endocytosis (Martys et al., 1996; Spiro et al., 1996). In plant cells, Class III PI3K protein complexes may have a regulatory function during vesicle trafficking (Matsuoka et al., 1995; Kim et al., 2001; Jung et al., 2002). In *Phaseolus vulgaris*, in addition to the N- and C-terminal PLP-binding domain, profilin has a domain around Tyr72; this can recognize and bind PLP and PI3K (Aparicio-Fabre et al., 2006). Profilin can bind directly to Class III PI3Ks in a manner reliant upon the tyrosine phosphorylation status of the PLP domain in profilin. This interaction between profilin and Class III PI3Ks suggests that profilin may participate in membrane trafficking, and may act as a linker between the endocytic pathway and the actin reorganization dynamics (Aparicio-Fabre et al., 2006).

With advances in biotechnology, diverse pharmaceutical drugs have been used to study the interaction between vesicular trafficking and cytoskeleton. Brefeldin A (BFA) is a drug that inhibits the recycling of vesicular trafficking, and disrupts secretion in yeast, mammalian, and plant cells (Vogel et al., 1993; Samaj et al., 2004; Citterio et al., 2008; Robinson et al., 2008). In *Arabidopsis* roots, BFA-compartments can be formed due to the accumulation of trans-Golgi network (TGN) secretory and recycling vesicles, which gather together following BFA treatment (Geldner et al., 2003). During this process, profilin 2 is up-regulated and accumulates in the BFA-compartments, which then interacts with the actin to remodel the actin cytoskeleton. This study suggested that profilin 2 may bridge vesicular trafficking to the actin cytoskeleton in a BFA-dependent manner (Takac et al., 2011). **Table 1** lists the profilins cited in the present article and emphasizes some of their cellular functions. Therefore, the recently investigated interactions between membranes and the actin cytoskeleton have revealed profilins to be of particular interest, this is because they may act as linkers and regulate communication and cooperation between the two cellular members in plants. Currently available studies suggest that diverse interaction mechanisms are required to satisfy the different structural and dynamic requirements of particular systems. Future

Table 1 | Profilin and its cellular functions in plant cells.

Profilin involving in the cellular pathway	Profilins	Cells or ligands	Reference
Plasma membrane-actin cytoskeleton interaction	ZmPRO3	Root cells of maize PIP2	Baluska et al. (2001)
	AtPRF1 etc	*Arabidopsis* seed endosperm, root cells etc AtFH1, AtFH5, and AtFH6	Banno and Chua (2000); Cheung and Wu (2004), Ingouff et al. (2005), Favery et al. (2004)
	PcPRF1	Cultured parsley cells	Schutz et al. (2006)
Organelles location with the actin cytoskeleton	Profilins from *Arabidopsis*	*Arabidopsis* mesophyll protoplasts CHUP1	Oikawa et al. (2003); Schmidt von Braun and Schleiff (2008)
	NA	Rice leaf cells OsFH5	Zhang et al. (2011); Yang et al. (2011)
	AtPRF2	*Arabidopsis* epidermal cells, trichomes, stem epidermal cells ER	Wang et al. (2009)
	NA	*Arabidopsis* root cells AtFH8	Xue et al. (2011)
Vesicle trafficking with the actin cytoskeleton	PvPRO1	*Phaseolus vulgaris* root nodules Class III PI3Ks	Aparicio-Fabre et al. (2006)
	AtPRF2	*Arabidopsis* roots TGN	Takac et al. (2011)

NA, not available.

research is required to unravel how membrane-actin cytoskeleton interactions are regulated through profilins and their different ligands.

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The evolution of the actin binding NET superfamily

Timothy J. Hawkins[1], Michael J. Deeks[2], Pengwei Wang[1] and Patrick J. Hussey[1]*

[1] School of Biological and Biomedical Sciences, Durham University, Durham, UK
[2] College of Life and Environmental Sciences, University of Exeter, Exeter, UK

Edited by:
Clément Thomas, Public Research Centre for Health, Luxembourg

Reviewed by:
Andreas Nebenführ, University of Tennessee, USA
Andre Steinmetz, Public Research Centre for Health, Luxembourg

***Correspondence:**
Patrick J. Hussey, School of Biological and Biomedical Sciences, Durham University, South Road, Durham, DH1 3LE, UK
e-mail: p.j.hussey@durham.ac.uk

The *Arabidopsis* Networked (NET) superfamily are plant-specific actin binding proteins which specifically label different membrane compartments and identify specialized sites of interaction between actin and membranes unique to plants. There are 13 members of the superfamily in *Arabidopsis*, which group into four distinct clades or families. NET homologs are absent from the genomes of metazoa and fungi; furthermore, in plantae, NET sequences are also absent from the genome of mosses and more ancient extant plant clades. A single family of the NET proteins is found encoded in the club moss genome, an extant species of the earliest vascular plants. Gymnosperms have examples from families 4 and 3, with a hybrid form of NET1 and 2 which shows characteristics of both NET1 and NET2. In addition to NET3 and 4 families, the NET1 and pollen-expressed NET2 families are found only as independent sequences in Angiosperms. This is consistent with the divergence of reproductive actin. The four families are conserved across Monocots and Eudicots, with the numbers of members of each clade expanding at this point, due, in part, to regions of genome duplication. Since the emergence of the NET superfamily at the dawn of vascular plants, they have continued to develop and diversify in a manner which has mirrored the divergence and increasing complexity of land-plant species.

Keywords: networked superfamily, actin cytoskeleton, actin binding proteins, membrane adaptors, evolution

INTRODUCTION

The Networked (NET) proteins are a superfamily of plant-specific actin-binding proteins which localize simultaneously to the actin cytoskeleton and specific membrane compartments and are suggested to couple these membranes to the actin cytoskeleton in plant cells (Deeks et al., 2012). Metazoans utilize a variety of adaptor proteins, including α-actinin, spectrin, filamin, and FERM-domain proteins to produce specialized sites of interaction between membrane and actin. Notably, however, all of these protein families are absent from plants, despite actin-membrane interactions remaining critical for the plant cell with actin filaments dominating microtubules during organelle and endomembrane trafficking (Boevink et al., 1998; Kandasamy and Meagher, 1999; Van Gestel et al., 2002; Langowski et al., 2010). Evidence is accumulating that the plant cell employs analogous factors of its own, including those of the NET superfamily, to fulfill this role. In light of this plant specialization, it is rewarding to consider the evolutionary significance of the NET proteins and chart the development of the superfamily through plant evolution.

The founding member of the superfamily, NET1A, was originally identified as a 288 amino-acid fragment that labels a filamentous network during screening of an *Arabidopsis thaliana* cDNA (GFP)-fusion expression library. Residues 1–94 of this 288 aa region are sufficient to associate directly with actin filaments. This minimal actin binding region, referred to as the NET actin-binding (NAB) domain, represents a new actin binding motif unique to plants with no apparent primary sequence homology to previously identified actin binding domains. The NAB domain defines the NET superfamily, of which there are 13 members in the *Arabidopsis thaliana* proteome, ranging in size from 25 to 199 kDa. These proteins divide into four families based on the NAB domain sequence and the structural organization and length of the C termini. The C terminal region, beyond the NAB domain, is variable between families but within each family the members share several areas of conservation throughout this portion. Despite primary sequence differences, the C terminal domains of all NET proteins are predicted to take on a coiled-coil secondary structure which may provide an interface for protein-protein interactions with itself, other NETs or additional binding partners (Deeks et al., 2012).

RESULTS

THE NET SUPERFAMILY AND EXPRESSION PROGRAMMES

The NET Superfamily separates into four phylogenetic families: 1–4 (**Figure 1A** and Deeks et al., 2012). There is high sequence conservation within the NAB domain across all of the four families, often with amino acid differences still representing residues of the same nature. In *Arabidopsis*, the NAB domain always starts with three conserved tryptophan residues, WWW, a motif whose worldwide web connection gives added significance to the NET family name (**Figure 1B**). The C terminal half of the domain is very highly conserved, more so than the N terminal. There are several residues which are identical in all NET NAB domains ($W_{15}W_{16}W_{17}$,H_{20},S_{25},W_{27},L_{32},D_{51},A_{57},P_{65},R_{79}, L_{81},A_{82}) suggesting that, in addition to conserved motifs, these residues are likely to be essential for the structure of the domain and potentially its actin affinity. In general, NET3A and B have the most divergent NAB domain. Downstream of the core domain, there is further conservation between NET1 and 2 isoforms, indicating that these families may share a recent ancestor and/or

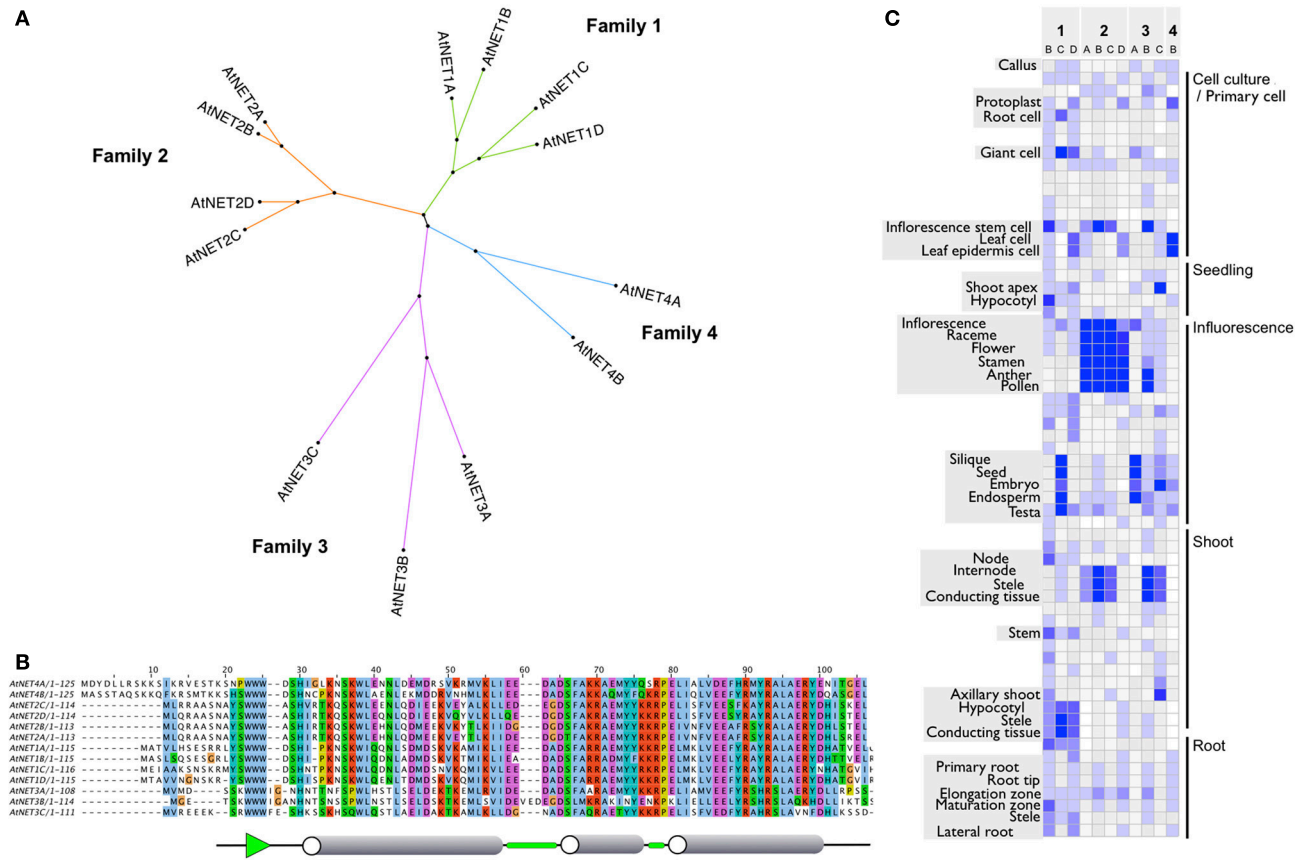

FIGURE 1 | (A) Radial cladogram of the *Arabidopsis* NET protein superfamily. Protein sequences fall into four distinct clades or families. **(B)** NAB domain protein sequence alignment. ClustalX default residue coloring scheme based on physiochemical properties. Consensus secondary structure is shown below. Cylinders represent α helices, green sections show β turns, and green arrow represents β sheet. **(C)** Heatmap of NET family transcript level profiles. Adapted from publicly available DNA microarray data visualized with gene investigator.

common function. The predicted secondary structure of the domain includes three major α helices connected by β turns with the WWW motif predicted to form a β sheet. NET3B is unique in having an insertion in the sequence at turn 2.

Components of the NET superfamily show distinct expression profiles. Often, members of each family have primary zones in common but with subtle differences, where they are unique or predominant. **Figure 1C** gives an overview of the general expression profiles of the NET family and is adapted from publicly available DNA microarray data, visualized with gene investigator (Zimmermann et al., 2004) see Supplementary Figure 1 for complete tissue annotation. The NET1 family show peak expression levels in stele and conducting tissues of the root and hypocotyl with some lower expression in the elongation zone and tip. NET1C is the only NET1 family member which also has high expression in the tissues within the silique. Members of the NET2 family show peak expression within pollen and are almost exclusively found here. NET2B, however, does show a second peak within conductive tissue of the shoot. The NET3 family show more diversity. NET3A shows peak expression in the seed/endosperm/embryo and is almost exclusively found within these tissues of the silique. NET3B shows peak expression within pollen and the conductive tissue of the shoot and NET3C has peak expression at the shoot apex, embryo and hypocotyl. NET4B shows peak expression within the leaf with lower levels found in the root and silique.

NET1A is absent from this analysis as the probe used in the construction of the At22K chip not only covers the NET1A sequence but also the gene which resides next to it in the genome. We have previously shown, however, that a GUS reporter line for the NET1A promoter shows high levels of expression within the root. NET4A is not included as it is not represented on the chip. Again, we have demonstrated that the NET4AGFP fusion protein expressed under the control of the native NET4A promoter is expressed in the epidermis of the root elongation zone (Deeks et al., 2012).

EVOLUTION OF THE SUPERFAMILY

To chart the emergence and development of the NET proteins, the presence of the NET genes across a diverse range of species was assessed, ranging from the bryophyte *Physcomitrella patens* through many Tracheophyta to the crop Angiosperm *Zea Mays*. Tracheophyta genomes and ESTs surveyed were: Lycophytes (Spikemoss, *Selaginella moellendorffi*); Ferns (*Pteridium*

*aquilinum, Adiantum capillus-v*eneris); Gymnosperms (*Picea abies, Pinus banksiana, Picea sitchensis*) and Angiosperms, (basal Angiosperm—*Amborella trichopod*a, Magnolids—*Aristolochia fimbriata*, *Persea americana*, Eudicots—*Arabidopsis thaliana, Populus trichocarpa*, and Monocots *Brachypodium distachyon*, *Zea mays*). No examples of NET proteins exist in non-plant genomes. (**Figure 2A**, see Supplementary Table 1 for a full list of NET orthologs).

Interestingly, members of the NET superfamily are completely absent from the genome of Bryophytes (mosses) and more ancient extant plant clades, which lack vasculature and can only be identified within the genomes of Tracheophytes (vascular plants). NET sequences are present in the genomes of all Tracheophyte species analyzed with the number of families represented increasing coincidentally with distinct stages of plant evolutionary complexity. Importantly, Bryophytes and

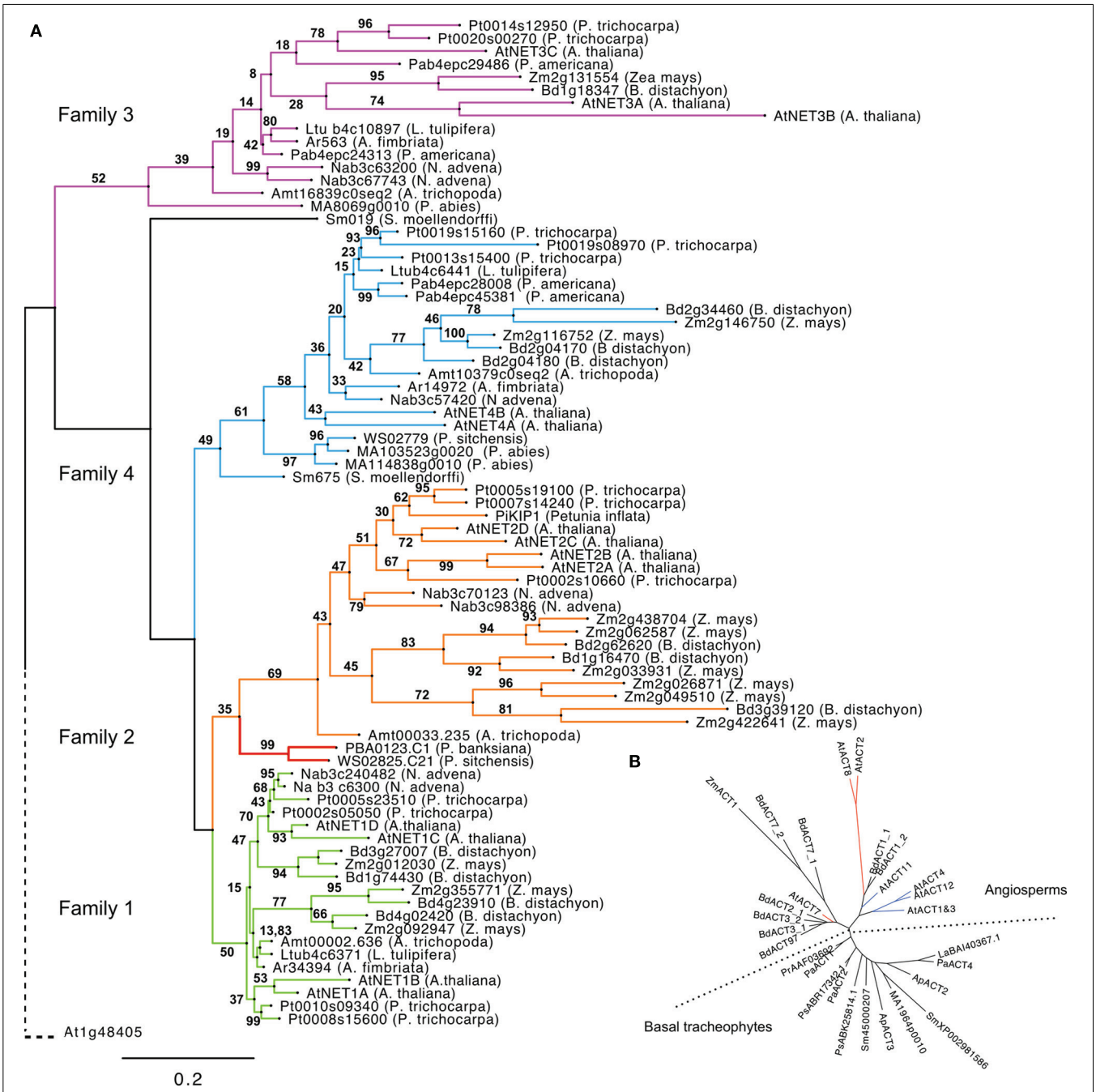

FIGURE 2 | (A) Phylogeny of NET proteins calculated from the alignment of NAB domains. Branches are colored according to NET family. Gymnosperm 1/2 hybrid forms are separate in red. The phylogeny is rooted using the NAB-like N terminus of *Arabidopsis thaliana* protein At1g48405. **(B)**. Radial cladogram drawn from alignment of actin protein sequences from tracheophytes. The reproductive actins as defined by Kandasamy and Meagher (1999) are colored blue. Vegetative actins are red.

Tracheophytes differ in the molecular mechanisms that couple the actin cytoskeleton to cell growth, for example genetic analysis of *P. patens* has shown that Bryophyte cell expansion requires the ARP2/3 complex, whereas Angiosperms appear considerably more resilient to equivalent genetic lesions (Harries et al., 2005).

NET proteins first emerge within the completed genome of the spikemoss, *Selaginella moellendorffii*. There are two examples present; one possesses a NAB domain that groups in phylogenetic analysis with the NET4 family and a second that does not, yet reciprocal BLAST searches indicate that the NAB domain of this protein most closely resembles that of NET4 proteins. This classification is further supported by analysis of the C-terminal portions of these proteins which exhibit the regions of homology found to be conserved between NET4 proteins in *Arabidopsis*. No examples of NET proteins of the remaining families are present. It does remain however that this more divergent NET4 like protein may be an orphan descendant of a distinct NET protein class.

The next major branch on the plant evolutionary tree of life is that of the ferns; here, information is limited. So far, whole-genome sequencing of a fern has not been economically feasible. However, despite this lack of a completed fern genome, some limited mining of NET sequences is still possible following the recent sequencing of the gametophyte transcriptome of bracken fern, *Pteridium aquilinum* (Der et al., 2011) and the fern EST database AcEST. Interrogation of the AcEST database revealed no NET sequences, however the much larger gametophye transcriptome contained a single example. This bracken NET sequence contains a NAB domain which shows most similarity to one of the two present in *Selaginella*, a NET4; although the sequence is truncated and as such not included in the ML tree. Ferns may, like *Selaginella*, only have NET proteins of family 4. However, without a complete genome and a transcriptome restricted to that of the gametophyte, it is reasonable that other NET examples may still exist in ferns.

Gymnosperms are a group of land plants, comprising Cycads, Ginkgo, Gnetophytes, and conifers, which first appeared more than 300 million years ago (Nystedt et al., 2013). There are several Gymnosperm EST databases and fortunately, very recently, the complete genome sequence of Norway Spruce has been completed (Nystedt et al., 2013). Considering sequences from both sources, it appears that Gymnosperms contain examples of the NET4 and NET3 families along with a NET protein which falls within the NET2 clade but possess a "hybrid" NAB domain with regions of homology characteristic of both family 1 and 2 NAB domains (**Figure 3**).

The next branch of the evolutionary tree to be surveyed is that of the Angiosperms, which differ from Gymnosperms primarily in their reproductive development and water-conducting xylem cells (Nystedt et al., 2013). The Angiosperm Eudicot, *Arabidopsis thaliana*, possesses examples of all four NET families, but how early in Angiosperm evolution did NET independent isoforms of the NET1 and 2 families emerge? To answer this question we need to consider a genome at the root of Angiosperm divergence. Until recently, the majority of sequenced Angiosperm genomes resided on just two limbs within the Angiosperm branch of the Tree of Life (Jansen et al., 2007; Moore et al., 2007; Soltis et al., 2008), yet many key Angiosperm innovations first appeared among the basal Angiosperm lineages (Soltis et al., 2002, 2005; Williams and Friedman, 2002; Friedman, 2006). Recently, the full genome sequence of *Amborella trichopoda* has been completed, a species identified as the single "sister species" to all other living flowering plants and is situated "between" Gymnosperms and all other Angiosperms (Soltis et al., 2008). Therefore, this genome was interrogated for the presence of NET proteins and revealed that *A. tricopoda* possesses NET proteins which fall into all four NET subclades, indeed a single example of each. This suggests that the emergence of the NET2 clade occurred early in the divergence of the Angiosperm lineage. The observation that NET1 and NET2 isoforms are found separately here, analogous to the two distinct families found in *Arabidopsis thaliana,* is of particular significance as NET2 proteins are pollen-specific NET isoforms and their emergence as individual proteins occurs at a point in plant evolution corresponding to the divergence of reproductive actin (**Figure 2B**) (Kandasamy et al., 2002). The timing of this specialization can also be seen in the diversification of ADF and Profilin families into forms adapted to interact with these two distinct subclades of actin isoforms (Kandasamy et al., 2007) which may suggest a co-evolution of the NET proteins with actin and known actin binding proteins. To fully substantiate such an hypothesis, a further rigorous comparison of evolutionary patterns in additional actin binding protein families would be required.

Modern Angiosperms in general can be classified into one of three sister clades: Monocots, Eudicots, and Magnolids. Inspection of the genomes of Monocot *Zea mays and Brachypodium distachyon* reveal that examples of all four NET families are also present here and that NET1s and NET2s

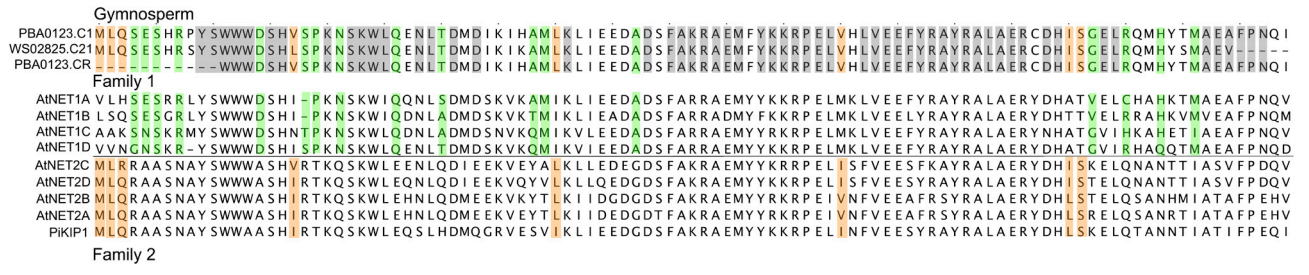

FIGURE 3 | Protein sequence alignment of Gymnosperm NET1/2 hybrid NAB domains with *Arabidopsis* NET1 and NET2 NAB domains. Gray areas highlight those residues found in both NET1 and NET2 NAB domains. Green areas highlight those residues unique to NET1 NAB domains with orange areas highlighting residues unique to NET2 NAB domains.

are found separately, analogous to the two distinct families found in *Arabidopsis thaliana* and other Eudicots. In particular, maize has more NET2 isoforms than any other genomes analyzed.

Pertinently, even though three Magnolid EST databases were searched, no examples of NET2 isoforms can be identified, despite all three collections containing cDNA isolated from mature pollen. As described above, the basal Angiosperm genome of *Amborella trichopoda* does possess a recognizable NET2 sequence; yet in contrast to *Arabidopsis*, there is only a single example. NET2 sequences may be under-represented in these Magnolid transcriptomes and as no full Magnolid genome is currently available one cannot be certain, but it does appear to be a possibility that Magnolids have lost the progenitor NET2 found in basal Angiosperms, whereas Monocots and Eudicots have not only retained this clade but expanded it.

Further sequence analysis reveals that domain architecture is consistent across members of each clade from all species analyzed, although Gymnosperm NET4 examples are much larger than their counterparts in other species.

NAB SEQUENCE CONSERVATION ACROSS SPECIES

When multiple sequence alignments of these NAB domains across species and complexity are considered, several features are conserved (Supplemental Figure 2). At the N terminus of NET3 isoforms from Monocot and Magnolid examples there is WWFD, which is also found in 3C (WWWFD as opposed to WWW). The region upstream of WWW appears to be less conserved when compared to other NET families. Magnolid and basal Angiosperm examples have an additional Serine residue before WWW, whereas Mono and Eudicots do not. The latter half of the domain is more highly conserved than the first part with the majority of sequences most similar to NET3C.

Interestingly, when one considers the NAB domain sequence of NET3 isoforms across species, the only example found to have the 3 amino acid insertion (VED) is *Arabidopsis* NET3B. This suggests that this occurred recently and uniquely in the genome of *Arabidopsis.* This sequence has been confirmed experimentally. The predicted secondary structure for this region of the domain is that of a loop, a structure where perhaps such an extension can be tolerated without abolishing the actin affinity of the NAB domain. Indeed, in GFP fusion experiments, AtNET3B does associate with actin filaments (data unpublished).

Cross species forms of the NET1 NAB domain are highly conserved throughout the domain. Upstream of WWW, these NAB domains have conserved RXYS. This YS preceding the WWW is a feature common with NET2 NAB domains, again pointing toward the shared ancestry of the NET1 and NET2 clades.

NET2 NAB domains are very conserved at the amino terminus, preceding the WWW motif. This sequence MLQRA is conserved in all Angiosperms examples and in many cases extends slightly beyond this (ASNAYSWWWASHIR), with the progenitor NET2 found in *Amborella trichopoda* only exhibiting small differences (E>G & A>SS). In fact, this sequence could be considered the defining feature of a NET2 type protein and may suggest that this sequence is important for the punctate localization at the pollen plasma membrane seen with NET2A.

Comparison of the NAB domain sequence of Gymnosperm NET1 like isoforms with *Arabidopsis* NET1 and NET2 proteins, reveals that it possesses features found to be common to both but also features which are unique to either form. This suggests that these Gymnosperm NAB domains represent a hybrid form predating a split which gave rise to the two independent forms found in Angiosperms and consistent with the functional divergence of reproductive actin (**Figure 3**). Specifically, the hybrid Gymnosperm NAB domain has part of the NET2 family defining MLQ sequence preceding the WWW motif, although the sequence following this region is predominately more characteristic of NET1. However, here there are still several residues which are found only in NET2 forms. The *Arabidopsis* NET1 to which these Gymnosperms seem most closely related is NET1D. If a complete fern genome sequence becomes available, it will be vitally important to identify if there are NET1 sequences within it to help pinpoint the emergence of NET1 isoforms.

EXPANSION AND MULTIPLICITY WITHIN SUBCLASSES

Following the emergence of new families, the number of members of each family rapidly multiplies in higher Angiosperms, specifically Monocots and Eudicots. In this analysis, we have primarily considered those complete genomes which can provide a full representation of NET isoforms present; however, it is intriguing that multiple Magnolid EST databases suggest that here such expansion is less prominent. The *Arabidopsis* genome contains numerous collinear clusters of genes which reside in large duplicated chromosomal segments, encompassing 60% of the genome. The sequence conservation between duplicated genes varies, as does the proportion of homologous genes in each duplicated segment, ranging from 20 to 47% (The *Arabidopsis* Genome Initiative, 2000).

In order to ascertain whether such duplications could account for the family expansions observed in *Arabidopsis*, we performed an analysis of collinear clusters across all 5 chromosomes to identify those which contained NET protein isoforms (**Figure 4**). Here indeed, there are three significant genome duplications of regions in which NET genes reside. When the chromosomal locations of the *Arabidopsis* NET genes are plotted onto a chromosome schematic, it is striking that both chromosome 1 and 4 contain a NET1 isoform and a NET3 isoform in close proximity in the same orientation. Following collinear analysis, these genes are found to be within a duplicated region (multiplicon 23641) NET1C—NET1D and NET3A—NET3B. Secondly, there is a duplication and inversion between the mid regions of chromosome 3 and 4 (multiplicon 20748). Within this region are NET1A and NET1B. Thirdly, there has been an internal duplication within chromosome 1 (multiplicon 19672) leading to the duplication of NET2A and NET2B. Therefore, large scale comparatively recent genome duplications can, in part, account for expansion within NET families in *Arabidopsis*. Further analysis of chromosomal localities of NET isoforms in other Angiosperms, including Monocots, suggests that similar duplications have also occurred here. Additionally, the tandem duplication of NET coding sequences in the genome of maize may account for the large NET2 family found in this species.

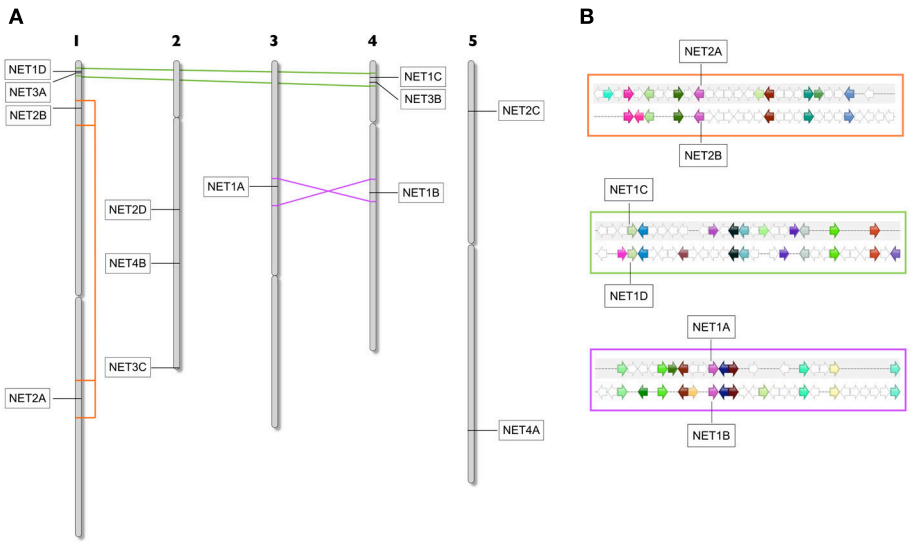

FIGURE 4 | (A) *Arabidopsis* chromosome schematic showing areas of duplication which contain *Arabidopsis* NET genes. Duplication between Chr1 and 4 contain NET1D/NET3A and NET1C/NET3B. Duplication and inversion between Chr3 and 4 contains NET1A and NET1B. An internal Duplication in Chr1 contains NET2A and NET2B. **(B)** Zoomed area of multiplicon showing the position, conservation, and orientation of genes within duplications.

In some cases, there are duplicated segments where the NET gene is not present on both copies of the duplication, for example both NET2C and NET4A reside in regions which have been duplicated within chromosome 5 but in both cases the gene is absent from the other copy. One explanation is that one copy has been lost, which suggests that most of the remaining *Arabidopsis* NET sequences have been maintained by natural selection.

This expansion in families may represent the evolution of additional unrecognized subclasses or diversification within the subclasses to generate isoforms that function in particular developmental, environmental, or physiological contexts as has been suggested for RAB GTPases (Rutherford and Moore, 2002). Indeed, inspection of microarray data does suggest that this is likely to be the case for the NET superfamily, although in some cases functional redundancy may also be evident.

DISCUSSION

Our analysis reveals the emergence and development of the NET superfamily through plant evolution. Significantly NET proteins are plant specific, with no examples found within the genomes of metazoa or yeast and are also absent from nonvascular plants including Bryopytes, first emerging in Sellaginella at the beginning of the vascular plant linage. From here the superfamily have continued to develop and diversify in a manner which has mirrored the divergence and complexity of plant species through evolution (**Figure 5**). Importantly a significant proportion of the NAB domain is conserved not only within all families but also across evolutionary diverse species. Strikingly several residues are identical in every NET protein identified. This suggests that these residues must be essential in providing surfaces, moieties or conferring the conformation required for actin binding. The differences may represent different affinities for F-actin, a preference for binding different actin isoforms or provide family specific control regions.

When considering the emergence of NET proteins in Sellaginella, it is notable that the Lycophytes represent a critical development in the evolutionary complexity of land plants, signifying the onset of vasculature development, with its origins dating as far back as the late Silurian/early Devonian period (Banks, 2009). A particular difference between vascular and nonvascular plants is the morphological complexity of the sporophyte generation; its function being to produce haploid spores, which in non-seed plants represent the principal method of reproductive dispersal. The very first land plant sporophyte was extremely basic, constituting a short, rootless cylinder complete with terminal sporangium (Kenrick and Crane, 1997). Therefore, in order to increase the efficiency of reproductive dispersal, one method would be to increase the height of the sporophyte, which correspondingly requires the evolution of specialized tissues facilitating the transport of water, nutrients and hormones, resistant to the effects of increased wind speed and gravitational forces (Raven, 1993; Banks, 2009).The co-occurrence of NET proteins with the development of vasculature suggests that NET proteins may have supported the changes observed in this step in plant complexity including transport tissues and resistance to increased mechanical, gravitational and osmotic pressures. It is tempting to speculate that perhaps a rigid structure surrounding the vacuole is beneficial in the tonoplast membrane to adapt to increases in turgor pressure required for land plant structural integrity, in particular in those cells at the surface of the tissue, the epidermis, where NET4A is found to be expressed.

Furthermore, there may be added significance to the fact that it is the NET4 class which emerge first and specifically at this point. Plant cells predominantly utilize actin microfilaments for the spatial regulation of their major membrane components, including the ER (Sheahan et al., 2004; Runions et al., 2005), Golgi (Boevink et al., 1998; Nebenfuhr et al., 1999), and importantly, the Vacuole (Ovecka et al., 2005; Higaki et al., 2006). Actin

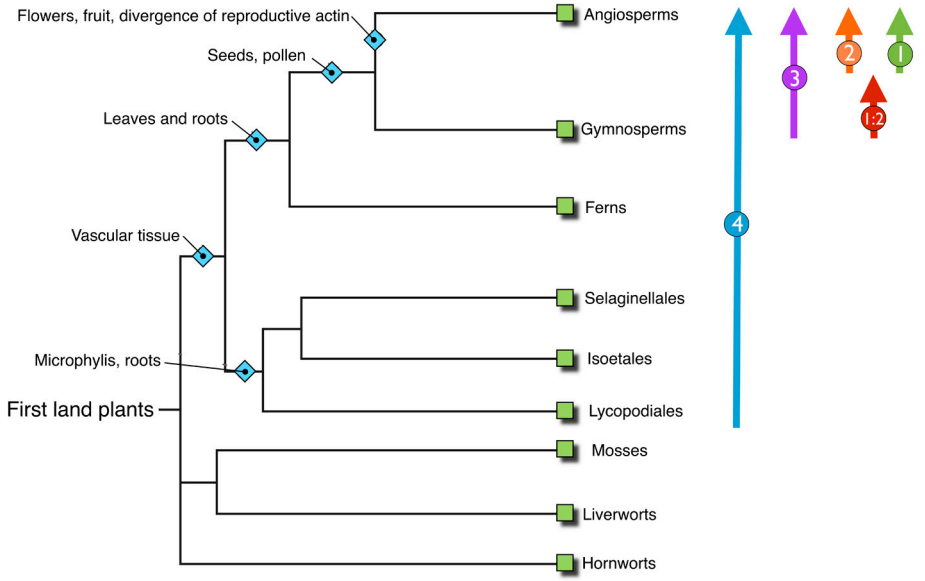

FIGURE 5 | Overview phylogenetic tree of land plants, inferring the progress of plant evolution. NET families identified within those genomes are shown on the right illustrating the emergence and development of the NET family concurrent with steps in plant evolution and complexity.

filaments colocalize with the vacuole membrane and following the breakdown of actin microfilaments by anti-actin agents vacuoles are seen to deform, fragment and lose their dynamics (Kutsuna et al., 2003; Ovecka et al., 2005). However, in direct contrast to plant cells, recent studies in *Physcomitrella patens* have demonstrated that here microtubules are required in maintaining the structure and distribution of the vacuole rather than actin (Oda et al., 2009). In Physcomitrella, microtubules and vacuolar membranes co-localized with elongating microtubules appearing to "tug" vacuolar membranes. Furthermore, actin depolymerization agents had little effect on vacuolar morphology whereas the microtubule depolymerization herbicide, oryzalin, clearly affected the vacuolar structures (Oda et al., 2009). These findings suggest the possibility of a divergence in the regulatory system of vacuolar structures by the cytoskeleton during land plant evolution. AtNET4A decorates actin filaments which lie upon the surface of the vacuole at the tonoplast membrane where they may be an adaptor responsible for linking the two, possibly aiding in the encaging of the vacuole (Deeks et al., 2012). It is notable that NET proteins, and in particular the tonoplast associated NET4 isoform, emerge at a point in plant evolution where there has been a shift to actin as the predominant regulator of vacuole structure and hence the requirement for novel factors to co-ordinate or link actin and the vacuole. It is tempting to speculate that here the NET superfamily emerge to fill this role.

Further on in plant evolution, the genome of the more complex Gymnosperms contain three NET isoforms, NET4, NET3 and a unique form of NET2 whose NAB domain is a hybrid NET1/2 form, sharing several features unique to 1 and 2 isoforms, including the upstream NET2 sequence MLQ. We have previously shown that an *Arabidopsis* member of the NET1 family, NET1A, is localized to the plasma membrane and particularly enriched at the plasmodesmata (PD) in root cells (Deeks et al., 2012). PD occur in all higher plants (Cook and Graham, 1999); indeed, the most closely related examples of Charophycean green algae from which Embryophytes evolved also possess PD, although the majority of extant Charophyceae do not. Studies have suggested a minimum of two and possibly more independent origins of PD in the Viridiplantae at the algal level and Heterokontophyta (Raven, 1997) Interestingly though, algal PDs differ in structure from that of higher plants with the absence of the desmotubule (Cook et al., 1997; Raven, 1997; Cook and Graham, 1999). It is considered that PD in Bryophytes and all vascular plants are comparable (Cook and Graham, 1999; Raven, 2005), although differences in the frequencies and development of PDs have been seen between ferns and Angiosperms within the shoot apical meristem (Imaichi and Hiratsuka, 2007; Jones, 1976; Cooke et al., 1996; Ehlers and Kollmann, 2001). Therefore, evolutionary development of PD significantly predates the emergences of NET1 isoforms; however, these forms may appear at a point where the structural complexity of the PD has advanced and here, new components are required to maintain this structure and assist in its functionality. The NET1 and 2 families are first seen as independent families at the divergence of Angiosperms, with the emergence of the pollen specific NET2 family at a point which corresponds to the divergence of pollen and vegetative actin isoforms.

The growth of the NET family can be compared to the sequence in which major terrestrial plant groups have appeared in the fossil record. Each new group has successfully competed against ancestral clades to dominate the land. These successes are attributed to increasing complexity and sophistication in cells and tissues that enhance reproduction, transpiration, nutrient transport, and plant structural integrity. From the pattern of NET diversification, it is tempting to speculate that the NETs have somehow contributed to driving these adaptations; however, this suggestion raises several broader questions including:

Do other proteins that shape cell architecture show similar complexity-associated patterns of diversification across plants and other major groups? and does such an interpretation rely too heavily on considering plant evolution as a linear sequence of events?

This expansion of sub-types for cytoskeletal proteins is not true for every super-family. Bryophytes and Lycophytes contain three very distinct classes of formins, yet Angiosperms have retained only two classes (Grunt et al., 2008). Diversity has been lost along the journey as well as gained. The NETs are not, however, a single isolated example of diversification as the recently identified DUF593 super-family of myosin cargo receptors follow a similar trajectory of increasing subfamily variety (Peremyslov et al., 2013).

Considering plant evolution as a linear sequence misses the ever-branching tree of speciation and adaptation where most spurs are dead-ends and only occasional outgrowths give rise to the living examples from which we can collect molecular data. This pruning through fitness and bad luck (such as mass extinctions) occurs at the smaller scale of gene families and has perhaps led to the loss of NET2 within the extant mangolids.

Inevitably, our sample of genomic data is biased toward the plant species we find most valuable and the species with compact genomes that can be sequenced most efficiently. In this study, we have been limited by the absence of a completed fern genome, likely because of the costs incurred by their complex karyotype (Barker and Wolf, 2010). More high-quality data from many extant species are needed, including complete genome sequences, molecular cellular phenotyping and expression data that shows precisely where in time and space new genetic innovations are exploited. It will then be possible to realize one of the key aims of evolutionary-developmental studies: to make a major contribution to the functional analysis of novel proteins.

MATERIALS AND METHODS

PHYLOGENETIC AND DOMAIN ANALYSIS

Arabidopsis thaliana NET sequences were identified by screening the TAIR and Genbank sequence databases using the BLASTP and TBLASTN algorithms (Altschul et al., 1997). To identify NET homologs in other species, BLASTP and TBLASTN algorithms were used to screen: non-redundant and EST databases of Genbank; Norway spruce genome (http://congenie.org/); *Selaginella* genome (http://genome.jgi-psf.org/) Amborella genome http://www.amborella.org/; Magnolid EST databases, ancestral Angiosperm genome project (http://ancangio.uga.edu/); Fern EST databases AcEST (http://togodb.dbcls.jp/acest); and braken gametophyte unigenes at NCBI. Further BLAST analysis was conducted using the resources available at phytozome (http://www.phytozome.net/). Reciprocal TBLASTN screening of the TAIR database was used to validate sequences.

Multiple alignments were assembled in ClustalX (Larkin et al., 2007) Manual adjustment and cropping of multiple alignments were made using CINEMA (Parry-Smith et al., 1997) and exported as graphics using Jalview (Waterhouse et al., 2009). Secondary structure prediction of *Arabidopsis* NAB domains was performed by Jpred3 (Cole et al., 2008). The Arabidopsis NET family tree was generated from multiple alignments by applying the neighbor-joining method to a bootstrapped dataset with 1000 replicates (Saitou and Nei, 1987).

The Maximum likelihood method was chosen for the NET evolution phylogenetic tree as this method has been identified as one of the most robust optimality criterion (Felsenstein, 1981, 2004; Swofford et al., 2001) Maximum Likelihood trees were calculated in the MetaPIGA software package (Helaers and Milinkovitch, 2010), using stochastic heuristics for large phylogeny inference with the Metapopulation Genetic Algorithm (metaGA) (Lemmon and Milinkovitch, 2002). MetaGA is an evolutionary computation heuristic in which several populations of trees exchange topological information which is used to guide the Genetic Algorithm (GA) operators for much faster convergence. The MetaGA algorithm was chosen as it resolves the problems inherent to classical GAs including the need to choose between strong selection (speed) and weak selection (accuracy) by maintaining high inter-population variation even under strong intra-population selection. Furthermore, MetaGA generates branch support values that approximate posterior probabilities. Dataset quality control included testing for identical sequences and excessively ambiguous or excessively divergent sequences and automated trimming of poorly aligned regions using the trimAl algorithm. Within MetaPIGA statistical methods, including Likelihood Ratio Test, Akaike Information Criterion, and Bayesian Information Criterion were used to select the amino-acid substitution model that best fitted the data. MetaPIGA calculations were stopped when the mean relative error of 10 consecutive consensus trees stayed below 5% using trees sampled every 5 generations or the Likelihood stopped increasing after 200 iterations. Trees were drawn and exported as graphical files from FigTree (Andrew Rambout, University of Edinburgh).

GENOME ANALYSIS

The comparative genomics tools available at PLAZA, (Bel et al., 2012) (http://bioinformatics.psb.ugent.be/plaza/) including WGDotplot, were used to identify *Arabidopsis* genome duplications and the presence of NET proteins within these regions.

AUTHOR CONTRIBUTIONS

Timothy J. Hawkins conducted the analysis; Michael J. Deeks contributed additional bioinformatics data; Pengwei Wang contributed additional experimental data; Timothy J. Hawkins, Michael J. Deeks, and Patrick J. Hussey wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2014.00254/abstract

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Dissecting the molecular mechanism underlying the intimate relationship between cellulose microfibrils and cortical microtubules

Lei Lei, Shundai Li, Logan Bashline and Ying Gu*

Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA, USA

Edited by:
Christopher J. Staiger, Purdue University, USA

Reviewed by:
Ram Dixit, Washington University in St. Louis, USA
Yoshihisa Oda, The University of Tokyo, Japan

****Correspondence:***
Ying Gu, Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA 16802, USA
e-mail: yug13@psu.edu

A central question in plant cell development is how the cell wall determines directional cell expansion and therefore the final shape of the cell. As the major load-bearing component of the cell wall, cellulose microfibrils are laid down transversely to the axis of elongation, thus forming a spring-like structure that reinforces the cell laterally and while favoring longitudinal expansion in most growing cells. Mounting evidence suggests that cortical microtubules organize the deposition of cellulose microfibrils, but the precise molecular mechanisms linking microtubules to cellulose organization have remained unclear until the recent discovery of cellulose synthase interactive protein 1 , a linker protein between the cortical microtubules and the cellulose biosynthesizing machinery. In this review, we will focus on the intimate relationship between cellulose microfibrils and cortical microtubules, in particular, we will discuss microtubule arrangement and cell wall architecture, the linkage between cellulose synthase complexes and microtubules, and the feedback mechanisms between cell wall and microtubules.

Keywords: cellulose synthase complex, cell wall, microtubules, CESA interactive proteins, cell expansion

INTRODUCTION

Microtubules were first observed in plant cells and have been characterized as essential components of the cell division apparatus (Ledbetter and Porter, 1963). Microtubules are present in all eukaryotic cells and are important for cell division, cell expansion, and cell morphogenesis. In contrast to yeast and animal cells, plant cells do not have well-defined microtubule organizing centers such as the centrosomes of animal cells and the spindle pole bodies of yeast cells (Vaughn and Harper, 1998). In post-mitotic plant cells, nucleation of new microtubules occurs at dispersed sites at the cell cortex, the area that is in very close proximity to the plasma membrane within the cell (Nakamura et al., 2010). The microtubules of the plant cortex are arranged into a cortical array, a feature that is unique to plants. Cortical microtubules migrate across the cortex by means of a hybrid treadmilling mechanism, which consists of intermittent depolymerization at the lagging end and polymerization-biased dynamic instability at the leading end (Shaw et al., 2003). The unique behavior of cortical microtubules determines the overall organization of the cortical microtubule array and thereby determines the asymmetric cell growth of plant cells.

In addition, plant cell shape is largely dictated by the opposing forces of turgor pressure and cell wall tension (Skotheim and Mahadevan, 2005; Dumais and Forterre, 2012). Cellulose microfibrils of the cell wall are the major load-bearing component that supports the cell wall tension and that enforces asymmetric cell expansion (Green, 1962). Cellulose microfibrils, composed of β-1, 4-linked glucan chains, are laid down outside of the plasma membrane of plant cells (Somerville, 2006; Carpita, 2011; Endler and Persson, 2011). In cylindrical fast growing cells, cellulose microfibrils are mostly arranged in a transverse orientation that is perpendicular to the axis of elongation. As a consequence of transversely oriented cellulose microfibrils, radial cell expansion is restricted while longitudinal cell expansion is promoted (Roelofsen and Houwink, 1951, 1953). Green's hypothesis of plant cellular morphogenesis states that the shape of plant cells is determined by the orientation of cortical microtubules because the orientation of newly synthesized cellulose microfibrils is dictated by the cortical microtubule array (Green, 1962). In support of Green's hypothesis, disruption of either microtubules or cellulose microfibril organization by pharmacological or genetic means leads to cell expansion defects (Hepler and Palevitz, 1974; Itoh, 1976; Hardham and Gunning, 1979; Arioli et al., 1998; Fagard et al., 2000; Lane et al., 2001; Cano-Delgado et al., 2003). Despite the observation that cellulose microfibrils co-align with cortical microtubules, mechanistic details regarding how microtubules and cellulose microfibrils work together to effect cell expansion are lacking. Together with genetic and biochemical methods, recent developments in the live imaging of fluorescent protein-tagged cellulose synthase (CESA) proteins and tubulin isoforms has provided unprecedented opportunities to dissect the molecular mechanisms underlying the intimate relationship between cellulose microfibrils and cortical microtubules.

THE MACHINERY FOR CELLULOSE BIOSYNTHESIS

In higher plants, cellulose microfibrils are synthesized at the plasma membrane by transmembrane protein complexes, known as cellulose synthase complexes (CSCs; Somerville, 2006). CSCs were initially visualized by freeze fracture electron microscopy in vascular plants as hexagonal rosettes (Mueller and Brown, 1980; Haigler and Brown, 1986). Immunogold labeling studies have shown that these rosettes contain CESA proteins, the only verified

component of CSCs in higher plants (Herth, 1983; Kimura et al., 1999). Although the exact composition and stoichiometry of CSCs remains to be discerned, the most popular model predicts that each of the six rosette subunits contains six individual CESA proteins. Assuming that each CESA protein is enzymatically active and synthesizes a single glucan chain, this model would suggest that each CSC could synthesize an elementary cellulose microfibril comprised of 36 glucan chains. However, using advanced techniques in spectroscopy and microscopy, recent measurements of elementary cellulose microfibrils in both primary and secondary cell walls indicate that an 18 or 24-glucan chain model best fits the size of an elementary fiber (Fernandes et al., 2011; Thomas et al., 2013; Zhang et al., 2013). These measurements suggest that either there are less than 36 CESA proteins in each CSC or that not all CESAs of a single CSC are enzymatically active.

In *Arabidopsis*, there are 10 *CESA* genes (*CESA1–10*; Holland et al., 2000; Richmond, 2000). Analyses of mutants with xylem cell defects have revealed that CESA4, CESA7, and CESA8 are each required for cellulose biosynthesis of secondary cell walls (Taylor et al., 2000, 2003). A similar requirement for three different CESA proteins exists in cellulose biosynthesis in primary cell walls (Arioli et al., 1998; Scheible et al., 2001; Beeckman et al., 2002; Burn et al., 2002; Desprez et al., 2002). For cellulose synthesis of primary cell walls, CSCs are composed of CESA1, CESA3, and CESA6 or CESA6-like proteins (CESA2, CESA5, and CESA9; Desprez et al., 2007; Persson et al., 2007). The distinction between primary and secondary CESAs might not be as strict as initially defined (Lei et al., 2012b). For example, CESA7 can partially rescue the growth defect of *cesa3*^{je5} when under the expression of the CESA3 promoter (Carroll et al., 2012). Similarly, CESA1can partially rescue the phenotype of the *cesa8*^{irx1} null mutant when driven by the CESA7 promoter (Carroll et al., 2012; Li et al., 2013). These results suggest that primary CESAs may have structural properties that allow its incorporation into secondary CSCs and vice versa.

VISUALIZATION OF CELLULOSE SYNTHASE COMPLEXES

Green fluorescent protein (GFP) fused CESA7, the first fluorescent protein tagged CESA, was shown to complement *irx3-1*, a mutant of *CESA7* (Gardiner et al., 2003). CESA7-GFP formed thick bands that marked the sites of cell wall deposition in the developing xylem of *Arabidopsis* (Gardiner et al., 2003). However, several characteristics of developing xylem cells prevented the accurate measurement of CESA dynamics. First, developing xylem cells are embedded deep within seedlings at a focal plane that is near the maximum working distance of confocal lenses and therefore difficult to image clearly. As another obstacle to imaging, the thick banding pattern of CESAs in developing xylem cells prevented the accurate measurement of individual CSC particles. To circumvent these difficulties, a similar strategy was developed to visualize and measure the dynamics of fluorescent protein tagged primary CESAs in epidermal cells that synthesize primary cell walls. In etiolated *Arabidopsis* hypocotyls, functional yellow fluorescent protein (YFP) tagged CESA6 (YFP-CESA6) was visualized as distinct particles at the plasma membrane (Paredez et al., 2006). Fluorescent protein fusions of several additional primary CESAs (CESA1, 3, and 5) have since been developed and visualized using similar approaches (Paredez et al., 2006; Desprez et al., 2007; Bischoff et al., 2011; Miart et al., 2014). FP-tagged CESAs, that presumably represent rosette CSCs, exhibited linear motility in the plane of the plasma membrane, traveling an average speed of 300–350 nm/min. The trajectories of plasma membrane localized FP-CESA particles are predicted to represent the position of newly deposited cellulose microfibrils (Li et al., 2014).

Cellulose synthase complexe rosettes are believed to be assembled in Golgi apparatus due to evidence from electron micrographs that showed rosette structures at the trans face of the Golgi apparatus (Haigler and Brown, 1986) and in vesicles close to the plasma membrane (Giddings et al., 1980). Consistent with these early observations, live cell imaging has shown that both primary and secondary CSCs accumulate in Golgi bodies and in vesicles that are close to the plasma membrane (Gardiner et al., 2003; Paredez et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009). Pausing events of CSC-containing Golgi bodies at cortical microtubules were reported in both etiolated hypocotyls (Crowell et al., 2009) and developing xylems (Gardiner et al., 2003) and were proposed to be associated with the secretion of CSCs to the plasma membrane. However, CSC delivery events that occur independently of Golgi pausing events have also been observed in hypocotyls (Sampathkumar et al., 2013). Recent evidence from the spatiotemporal analysis of primary CESAs during cell plate formation revealed that multiple routes of CSC delivery to the cell plate exist from phragmoplast-associated compartments, from Golgi-derived vesicles, and from direct transfer from the plasma membrane (Miart et al., 2014).

In addition to Golgi and plasma membrane localization, CESA is often associated with intracellular small CESA-containing compartments (SmaCCs) upon induction by osmotic stress or cellulose synthesis inhibitor treatment (Gutierrez et al., 2009). A similar population of CESA-labeled compartments was simultaneously described by another research team and referred to as microtubule-associated cellulose synthase compartments (MASCs), and has since been considered to be a subset of the SmaCC population (Crowell et al., 2009, 2010). SmaCCs/MASCs exhibit extended periods of pausing at cortical microtubules with intermittent instances of rapid motility that is driven by microtubule depolymerization (Crowell et al., 2009; Gutierrez et al., 2009). It has been hypothesized that microtubule-tethered SmaCCs/MASCs may function in the delivery or the internalization of CSCs. After relief from osmotic stress, some CSC delivery events coincided with microtubule-tethered SmaCCs that showed microtubule tip-tracking behavior before the CSC delivery, suggesting that microtubules might control CSC trafficking and delivery to the plasma membrane, although the delivery rate of CSCs to the plasma membrane was unaffected by pharmacological microtubule depolymerization (Gutierrez et al., 2009). Actin also plays a role in controlling the distribution of CSCs during the synthesis of both primary and secondary cell walls. In the epidermal cells of etiolated hypocotyls, treatment with actin depolymerizing agents, Cytochalasin D or Latrunculin B, caused CESA-containing Golgi bodies to aggregate and led to reduction of CSCs at the plasma membrane in areas that were devoid of aggregated Golgi bodies (Crowell et al., 2009; Gutierrez et al., 2009). The distribution of CSCs during secondary cell wall synthesis is also dependent on the

actin cytoskeleton. Latrunculin B treatment resulted in a loss of actin filaments that are typically positioned close to CSC bands in xylem cells and consequently resulted in a loss of CSC bands (Wightman and Turner, 2008). These results suggest that the plant cytoskeleton is involved in CSC distribution and trafficking during both primary and secondary cell wall synthesis.

The characterization of primary CSC behavior has been more successful than secondary CSC characterization because of the ease of imaging primary cell wall synthesizing tissues, such as epidermal cells, which are exposed at the surface of the plant, as opposed to secondary cell wall producing tissues, which are typically buried deep within the plant. Cellulose microfibrils in the secondary wall are presumably longer and more bundled than those in the primary cell wall. The production of more bundled microfibrils may be due to an increased clustering of CSCs at distinct sites of the plasma membrane underneath the secondary cell wall. As proof of concept, in algae, it has been proposed that CSC clustering is responsible for the formation of cellulose microfibrils with a diameter of 50 nm, which is indicative of a high degree of microfibril bundling (Hogetsu, 1983; Giddings and Staehelin, 1988). If imaging of secondary cell wall producing cells could be improved, some parameters of secondary CSCs may provide helpful insight into secondary CSC velocity and clustering as well as how these parameters affect the properties of the cellulose microfibril.

CELLULOSE MICROFIBRIL ORIENTATION AND MICROTUBULE ARRANGEMENT

The presence of cortical microtubules that are adjacent to the plasma membrane is a unique feature of plant interphase cells (Ledbetter and Porter, 1963; Baskin, 2001; Shaw et al., 2003). The formation of organized cortical microtubule arrays is believed to be generated by a self-organizing process that is mainly driven by two characteristics: the treadmilling behavior of microtubules and interactions between microtubules (Dixit and Cyr, 2004a,b). In rapidly elongating cells, such as epidermal cells of the root elongation zone, cortical microtubules uniformly organize into arrays that are perpendicular to the elongation axis of the cell (Sugimoto et al., 2000; Granger and Cyr, 2001). Newly deposited cellulose microfibrils of the cell wall are organized in a similar transverse pattern that mirrors the cortical microtubule array on the inner face of the plasma membrane. The co-alignment between cellulose microfibrils and cortical microtubules suggests that these two molecular components are intimately associated with one another. Interestingly, long before the observation of cellulose microfibril and microtubule co-alignment, the observation that colchicine, a microtubule-depolymerizing reagent, disrupted the organization of newly deposited cellulose microfibrils led to Green's hypothesis that a cytoplasmic structure (microtubules had not yet discovered) determines the orientation of cellulose microfibrils (Green, 1962; Ledbetter and Porter, 1963; Heath, 1974; Herth, 1980). Since then, cellulose microfibril and microtubule co-alignment has been observed in many types of plant cells, but exceptions have also been documented (Baskin, 2001). The simultaneous live imaging of YFP-CESA-labeled CSCs and CFP-tubulin-labeled microtubules has revealed an intimate association between CSCs and microtubules in which motility of active CSCs follows trajectories that co-align with underlying cortical microtubules in both primary and secondary cell wall synthesizing plant cells (Gardiner et al., 2003; Paredez et al., 2006). In support of the alignment hypothesis, changes in microtubule orientation resulted in a correlated shift in CSC trajectories. Complete removal of microtubules by the microtubule-depolymerizing agent, oryzalin, resulted in a uniform distribution of CSCs (Gardiner et al., 2003; Crowell et al., 2009; Li et al., 2011). Most of the early observations of the co-alignment were made on fixed tissues so that the dynamic features of cortical microtubules were unattainable. With newly developed live cell imaging tools, we can now examine the molecular details and dynamics of the relationship between cellulose and microtubules.

During cell growth, cortical microtubule arrays constantly undergo reorganization due to the dynamic instability of microtubules. A striking example of microtubule reorganization during cell expansion is the rotary movement of the cortical microtubule arrays at the outer surface of epidermal cells of the hypocotyl (Chan et al., 2007). CSC trajectories rotate simultaneously with cortical microtubules. This rotational readjustment of CSC trajectories causes successive layers of cellulose microfibrils to be deposited at progressively varying angles. Pharmacological disruption of the rotary movement of microtubules inhibited the rotation of CESA trajectories, suggesting that microtubules predominantly guide the rotation of CSC trajectories, thereby affecting the multi-angle cellulose deposition during cell wall assembly (Chan et al., 2007, 2010; Chan, 2012). Recently, multiple angles of cellulose microfibrils were observed at the inner, youngest layers of hydrated onion epidermal cell walls using atomic force microscopy (AFM; Zhang et al., 2013). The multi-angle pattern of cellulose microfibrils in successive cell wall layers may be a common feature during anisotropic cell expansion in many cell types. However, in epidermal cells of the root elongation zone in *Arabidopsis*, neither microtubule arrays nor CSC trajectories undergo rotary movement. Instead, in this cell type, the establishment of a multi-angled pattern of cellulose microfibrils in cell wall layers has been proposed to result from passive reorientation of cellulose microfibrils as cell expansion occurs (Lloyd, 2011). In support of this idea, cellulose microfibrils of the root elongation zone that were labeled with Pontamine fast scarlet 4B (S4B) dye were shown to exhibit varying angles, gradually changing the orientation from perpendicular at inner layers to parallel to the elongation axis at outer layers (Anderson et al., 2010). While the biological significance of varying cellulose microfibril orientation in successive cell wall layers is currently unknown, one possible function might be to provide strength and rigidity to the cell wall.

THE LINKAGE BETWEEN CELLULOSE SYNTHASE COMPLEXES AND MICROTUBULES

Two models have been put forward to explain the alignment between cellulose microfibrils and cortical microtubules: the direct guidance model and the bumper model. The direct guidance model postulates that some type of direct linkage exists between CSCs that are actively synthesizing cellulose and cortical microtubules (Heath, 1974; Somerville, 2006) while the bumper model suggests that cortical microtubules define channels within which

active CSCs move at the plasma membrane without any physical link between the CSCs and the cortical microtubules (Giddings and Staehelin, 1991). One important quality of a linker protein between CSCs and microtubules is the ability to interact with microtubules. In *Arabidopsis*, many microtubule-associated proteins and microtubule motor proteins have been identified. One such protein, the fragile fiber 1 (FRA1) kinesin motor protein, was proposed to be a possible linker protein between CSCs and microtubules due to the abrogation of cellulose microfibril organization in secondary cell walls of fiber cells in the *fra1* mutant (Zhong et al., 2002). However, further characterization of FRA1 suggests that FRA1 does not act as a CSC-microtubule linker protein. In an *in vitro* analysis, the motor domain of FRA1 was observed to travel along microtubules at a velocity that is about 100 times faster than the average velocity of CSC movement (Zhu and Dixit, 2011). Moreover, FRA1 exhibited unidirectional movement toward the plus end of microtubules while CSCs move bidirectionally along microtubules.

Aside from being able to interact with microtubules, a CSC-microtubule linker protein must also have the ability to interact (directly or indirectly) with the CSC. In an attempt to identify candidates that interact with CESAs, a yeast two-hybrid screen was performed using the central cytosolic domain of primary CESAs (Gu and Somerville, 2010; Gu et al., 2010). Cellulose synthase interactive protein 1 (CSI1) was identified among several dozen putative CESA-interacting proteins. Consistent with CSI1 playing a role in cellulose biosynthesis, *csi1* null mutants displayed a reduction in crystalline cellulose content and reduced anisotropic cell expansion in *Arabidopsis* hypocotyls and roots (Gu et al., 2010). Several lines of evidence suggest that CSI1 is a linker between active CSCs and cortical microtubules. CSI1 interacted with CESA3 and CESA6 in a split-ubiquitin yeast two-hybrid assay and CSI1 interacted with microtubules in an *in vitro* microtubule-binding assay (Li et al., 2011; Lei et al., 2012a). *In planta*, fluorescent protein-tagged CSI1 co-localized with CESA3 and CESA6 and traveled together with CESA3 and CESA6 along trajectories that co-aligned with cortical microtubules and at velocities that are typical of active CSCs (Gu et al., 2010; Li et al., 2011; Lei et al., 2012a). Furthermore, the association between CSCs and microtubules was disrupted in *csi1* mutants, suggesting that CSI1 is essential for the alignment between CSC trajectories and cortical microtubules.

In addition to its essential role in associating CSCs with microtubules, CSI1 is also critical in maintaining the normal dynamics of CSCs. CSCs move along cortical microtubules at an average velocity of 300–350 nm/min in the epidermal cells of etiolated *Arabidopsis* hypocotyls (Paredez et al., 2006; Gu et al., 2010; Li et al., 2011). In *csi1* null mutants, the average CSC velocity was reduced to about 70% of that of wild type (Gu et al., 2010; Li et al., 2011). Although a 200 nM dose of oryzalin, a microtubule-depolymerizing drug, had no affect on CSC velocity in hypocotyls (Chan et al., 2010), a prolonged 20 μM dose of oryzalin reduced the velocity of CSCs to a similar extent as that in *csi1* mutants. However, the removal of microtubules also affects the localization of CSI1 so it is not clear whether the CSC velocity reduction is influenced solely by the loss of microtubules or due to compromised CSI1 function (Li et al., 2011). Recent studies suggest that CSC velocity is correlated with cellulose crystallinity. For example, a point mutation in the catalytic region of CESA1 (*cesa1*^{D604N}) reduces CSC velocity and crystallinity (Fujita et al., 2013). In *mor1* mutants where the total microtubule mass is reduced, cellulose crystallinity and CSC velocity remain high (Fujita et al., 2011). While the mechanism of the influence of CSI1 on CSC velocity remains unknown, evidence suggests that microtubules are capable of regulating CSC velocity. In etiolated *cesa6*$^{prc1-1}$ hypocotyls, the removal of cortical microtubules led to a significant increase in GFP-CESA5 velocity (Bischoff et al., 2011). In another case, the asymmetric distribution of CSC velocity directionality caused by the expression of a CESA1 variant was shown to be dependent on the presence of cortical microtubules (Chen et al., 2010). Presumably, the removal of microtubules also disrupts the function of microtubule-associated proteins. Therefore, it is likely that microtubules together with microtubule-associated components contribute to regulating the velocity of CSCs. The molecular mechanism by which CSI1 remains associated to both CSCs and microtubules is also of special interest because both of these components are highly dynamic. There are two CSI1-like proteins in *Arabidopsis*, referred to as CSI2 and CSI3. CSI1 shares about 60% sequence similarity with CSI2 and CSI3. Promoter::GUS transcriptional analyses revealed that CSI3 was expressed in many tissues while CSI2 expression was undetectable (Lei et al., 2013). Similar to CSI1, CSI3 interacted with multiple primary CESAs in a split-ubiquitin yeast two-hybrid assay and CSI3 co-localized with CSCs and traveled along cortical microtubule tracks at comparable velocities. However, *csi3* null mutants did not display any defect in cell expansion nor did *csi3* affect the CSC velocity or the co-alignment of CSCs and microtubules. The functional difference between CSI1 and CSI3 was further supported by the inability of *ProCSI1:GFP-CSI3* to complement the phenotype of *csi1-3*. Although *csi3* mutants lack an apparent phenotype, *csi1csi3* double mutants displayed enhanced cellulose biosynthesis-related phenotypes, suggesting that CSI3 plays a role in cellulose biosynthesis (Lei et al., 2013).

While CSI1 was shown to mediate the interaction between active CSCs and cortical microtubules at the plasma membrane, CSI1 was also shown to label cortically localized SmaCCs/MASCs, indicating that CSI1 is potentially involved in CESA trafficking and/or delivery to the plasma membrane (Bringmann et al., 2012; Lei et al., 2012a). Interestingly, CSI1 puncta only localize to the plasma membrane and cortical region so CSI1 does not localize to CESA-containing Golgi bodies. Therefore, it is likely that CSI1 only associates with CSCs after they are fully assembled and within proximity to the plasma membrane. It remains to be determined how CSI1 is recruited to the plasma membrane and how CSI1 mediates the association between CSCs and cortical microtubules. The recruitment of the CSI1 protein to the plasma membrane may be the function of the C-terminal C2 domain of CSI1. The first identification of a C2 domain occurred using a membrane-associated protein kinase C, and many C2 domains have been shown to target proteins to cell membranes by binding to phospholipids in a calcium-dependent or independent manner (Davletov and Sudhof, 1993; Ochoa et al., 2001; Rickman and Davletov, 2003). Consistent with the role of the CSI1 C2 domain in targeting the CSI1 protein to the plasma membrane, a C2 domain

deletion variant of CSI1, YFP-CSI1ΔC2, did not complement the *csi1* mutant phenotype, nor did it localize to CESA complexes at the plasma membrane (Bringmann et al., 2012).

The putative lipid-binding activity of the C-terminal C2 domain of CSI1 may also allow CSI1 to influence the organization lipid micro-domains that contain CSCs at the plasma membrane. Studies in mammalian cells, have shown that lipids and proteins are not uniformly distributed at the plasma membrane, but instead specialized lipid environments can be organized into discrete islands or micro-domains and certain proteins prefer to be partitioned into these specialized lipid environments (Simons and van Meer, 1988; van Meer and Simons, 1988). CSCs are large transmembrane complexes and have been speculated to form membrane micro-domains together with specific lipids and other associated proteins (Guerriero et al., 2010; **Figure 1A**). If CSCs are partitioned into islands of special lipid content, some properties of CSC-containing islands, such as membrane fluidity, may differ from the properties of the surrounding plasma membrane. Cortical microtubules have been proposed to direct the formation of plasma membrane micro-domains that could influence the activities of CSCs (Fujita et al., 2012; Schrick et al., 2012). It is possible that a relationship exists between CSI1, CSCs, cortical microtubules, and specialized lipid micro-domains to provide a mechanism for microtubule-dependent organization of CSC-containing islands in which the proper function of CSCs is contingent on the integrity of each of these components

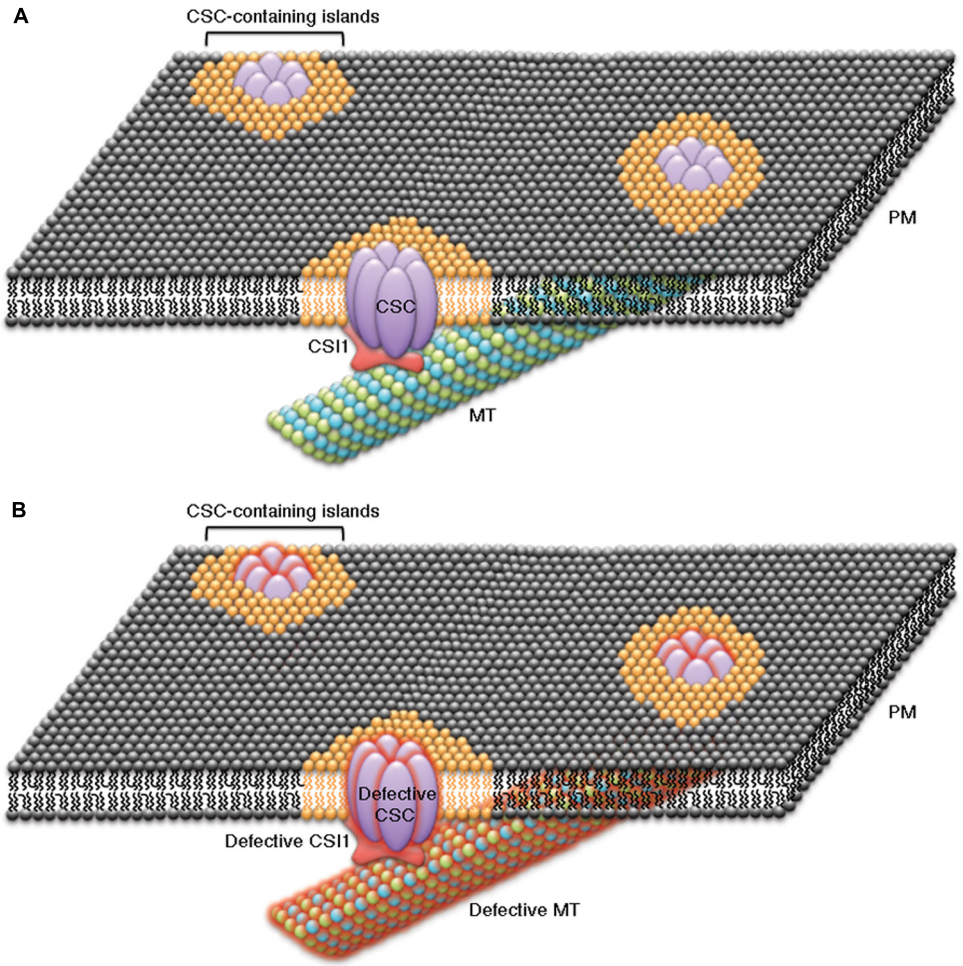

FIGURE 1 | Schematic representation of the cellulose biosynthesis machinery displays a continuum between cellulose synthase complexes (CSCs), CSC-associated proteins (e.g., CSI1), cortical microtubules (MT), and the plasma membrane. The transmembrane CSCs might be embedded in lipid islands or lipid micro-domains of specialized lipid content (orange lipids) that are surrounded by a sea of plasma membrane of normal composition (black lipids). CSI1 might act as a scaffold between CSCs, microtubules, and specialized lipids of CSC-containing islands by interacting with all three of these components to form a CSC, CSI1, microtubule, and lipid continuum. **(A)** The components in the continuum, including CSCs, CSC-interacting proteins, specialized lipids, and cortical microtubules, are closely associated. Under normal conditions CSCs that are within CSC-containing islands move along cortical microtubules during cellulose biosynthesis in a CSI1-dependent manner. **(B)** A defect in any single component of the CSC continuum causes a disruption of each of the other components of the continuum. Examples of experimental evidence that supports the CSC continuum model include: disruption of cortical microtubules influences the distribution and dynamics of CSCs and CSI1, genetic disruption of CSI1causes changes in both CSCs and cortical microtubules, and genetic disruption of CSCs or CSC-associated proteins (e.g., KOR1) affect the organization of cortical microtubules.

(**Figure 1B**). Several lines of evidence are consistent with this model. First, disruption of the cortical microtubules affected the distribution and dynamics of both CSCs and CSI1 puncta (Li et al., 2011). Second, lack of CSI1 in *csi1* null mutants led to defects in both CSCs and cortical microtubules (Li et al., 2011; Bringmann et al., 2012; Mei et al., 2012; Landrein et al., 2013). Third, mutants with defective CSCs or CSC associated proteins affected the organization of cortical microtubules (Paredez et al., 2008). Further evidence must be obtained to validate the existence of specialized CSC-containing lipid islands and the dependency of these structures on the integrity of CSCs, CSI1, and cortical microtubules.

FEEDBACK MECHANISM BETWEEN CELL WALL AND CYTOSKELETON

The concept of a "dynamic reciprocity" between the intracellular cytoskeleton and the extracellular matrix (ECM) was first postulated in reference to mammalian cells (Edelman, 1983). Despite the different composition of the mammalian ECM and the plant cell wall, it has been postulated that plant cells might regulate the perception and transduction of positional information using similar sensing mechanisms that involve a feedback interaction between the cell wall and the cytoskeleton (Wyatt and Carpita, 1993). Although plants lack the counterparts of most of the mammalian components involved in the relationship between the cytoskeleton and the ECM, several lines of evidence suggest that feedback exists between the cell wall and the cytoskeleton. For example, physically separating the cell wall from the plasma membrane by plasmolysis induced microtubule disintegration, suggesting that a physical connection between the plasma membrane and the cell wall is important for microtubule organization (Komis et al., 2002). Both pharmacological and genetic studies have shown that feedback from the cell wall regulates microtubule organization. Isoxaben, a cellulose biosynthesis inhibitor, caused reorientation of microtubules in plant cells (Fisher and Cyr, 1998; Himmelspach et al., 2003; Paredez et al., 2008). The reorganization of cortical microtubules upon isoxaben treatment can be attributed to a reduction in CSC activity since isoxaben treatment depleted CSCs from the plasma membrane (Gutierrez et al., 2009). Two cellulose biosynthesis deficient mutants, a null allele of *CESA6* and a new allele of *KORRIGAN (KOR)*, were identified in a screen for *Arabidopsis* mutants that are hypersensitive to oryzalin, a microtubule-depolymerizing drug. Both *kor1-3* and $cesa6^{prc1-20}$ exhibited altered orientation and stability of cortical microtubules in root cells and reduced CSC velocity (Paredez et al., 2008). Together, the observations that CSC velocity is reduced in cases where either CESA6, KOR, or microtubules are missing and that *kor1–3* and $cesa6^{prc1-20}$ mutants affect microtubule organization, supports the idea that a two-way feedback regulation mechanism exists between the cytoskeleton and the cell wall. Since attempts to purify integrin-like and spectrin-like proteins in plants using heterologous probes and searches for genes with sequence homology have been unsuccessful, the components involved in feedback between the cell wall and the cytoskeleton in plants may be unconventional (Nick, 2013). The function of cortical microtubules in plant cells is certainly not limited to regulating cellulose synthesis, so the feedback between microtubules and the cell wall may potentially be integrated with other microtubule-related functions. The unique dynamic features of microtubules add another layer of complexity to the investigation of the feedback regulation between the cytoskeleton and the cell wall in plants.

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The dynamic interplay of plasma membrane domains and cortical microtubules in secondary cell wall patterning

Yoshihisa Oda[1,2] * and Hiroo Fukuda[1] *

[1] *Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Tokyo, Japan*
[2] *Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency, Saitama, Japan*

Edited by:
Christopher J. Staiger, Purdue University, USA

Reviewed by:
Edouard Pesquet, Umea Plant Science Centre, Sweden
Ying Fu, China Agricultural University, China

****Correspondence:***
Yoshihisa Oda and Hiroo Fukuda, Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
e-mail: oda@biol.s.u-tokyo.ac.jp; fukuda@biol.s.u-tokyo.ac.jp

Patterning of the cellulosic cell wall underlies the shape and function of plant cells. The cortical microtubule array plays a central role in the regulation of cell wall patterns. However, the regulatory mechanisms by which secondary cell wall patterns are established through cortical microtubules remain to be fully determined. Our recent study in xylem vessel cells revealed that a mutual inhibitory interaction between cortical microtubules and distinct plasma membrane domains leads to distinctive patterning in secondary cell walls. Our research revealed that the recycling of active and inactive ROP proteins by a specific GAP and GEF pair establishes distinct *de novo* plasma membrane domains. Active ROP recruits a plant-specific microtubule-associated protein, MIDD1, which mediates the mutual interaction between cortical microtubules and plasma membrane domains. In this mini review, we summarize recent research regarding secondary wall patterning, with a focus on the emerging interplay between plasma membrane domains and cortical microtubules through MIDD1 and ROP.

Keywords: secondary cell wall, ROP GTPase, MIDD1, cortical microtubule, xylem

INTRODUCTION

The cellulosic cell wall plays a central role in shaping cells and determining cell function in plants. For example, physically rigid cellulose microfibrils restrict the direction of cell expansion and lead to the distinct shape of plant cells. Cortical microtubules play a central role in regulating the development of cell wall structures by controlling how the cellulose synthase complex is targeted to the plasma membrane (Paredez et al., 2006; Crowell et al., 2009; Gutierrez et al., 2009). Although cortical microtubules are closely and tightly anchored to the plasma membrane, they exhibit dynamic behaviors, such as growth, shrinkage, and branching (Shaw et al., 2003; Murata et al., 2005; Chan et al., 2009; Nakamura et al., 2010) and exhibit bundling (Dixit and Cyr, 2004), which leads to the self-organization of parallel microtubule arrays (Wasteneys and Ambrose, 2009). Several microtubule-associated proteins (MAPs) have been discovered that regulate the global dynamics of cortical microtubules in the cell. However, the molecular mechanisms underlying local regulation of cortical microtubule dynamics, for formation of structures such as patterned secondary cell walls in xylem vessels, are not well understood.

Xylem vessels are water-conductive tubes composed of dead cells called tracheary elements. Differentiating xylem cells deposit rigid water-impermeable secondary cell walls to avoid collapse of the vessel due to the negative pressure exerted as a consequence of water transport. In xylem vessels, secondary cell walls are deposited in various patterns, such as annular, spiral, reticulate, and pitted. Usually, protoxylems develop annular and spiral secondary walls while metaxylems exhibit reticulate and pitted formations. Perforations in the end walls of xylem cells and secondary wall pits in the side walls contribute to effective longitudinal and lateral water transport, respectively. Thus, the patterning of secondary cell walls is tightly coupled to function in xylem vessels (Esau, 1977).

The involvement of cortical microtubules in the regulation of secondary cell wall deposition during xylem vessel differentiation has been revealed through a number of studies. Dense cortical microtubule bundles are found beneath secondary wall thickenings and, if these cortical microtubules are pharmacologically depolymerized, the secondary cell wall patterns become severely disorganized (Hepler, 1981; Gunning and Hardham, 1982). Direct visualization of the cellulose synthase complex confirmed that patterned cortical microtubules regulate the localization of the cellulose synthase complex in xylem vessels (Wightman and Turner, 2008). Studies using isolated zinnia mesophyll cells revealed that a dramatic rearrangement of cortical microtubules leads to distinct patterns of secondary cell walls during tracheary element differentiation (Falconer and Seagull, 1985, 1986, 1988; Kobayashi et al., 1987, 1988; Fukuda and Kobayashi, 1989). Recent live cell imaging of differentiating xylem cells using *Arabidopsis* cell cultures further revealed that bundling and disassembly of cortical microtubules gives rise to distinct patterns of secondary cell walls (Oda et al., 2005, 2010; Oda and Hasezawa, 2006; Pesquet et al., 2010). These studies also revealed that several MAPs regulate the dynamic behavior of cortical microtubules during xylem differentiation (Oda et al., 2010; Pesquet et al., 2010; Oda and Fukuda, 2012b, 2013b). In addition, a recent study revealed that ROP GTPases generate plasma membrane domains that play critical roles in the spatial regulation of cortical microtubule dynamics, and that mutual inhibitory interactions between the plasma membrane domains and cortical microtubules establishes distinct patterns of secondary cell

walls (Oda and Fukuda, 2012a). In this review, we focus on the emerging role of the dynamic interplay between plasma membrane domains and cortical microtubules in secondary cell wall patterning.

MICROTUBULE-DEPOLYMERIZING PLASMA MEMBRANE DOMAIN LEADS SECONDARY WALL PIT

We previously established an *Arabidopsis* cultured cell line for *in vitro* xylem differentiation (Oda et al., 2010). In this system, VND6, a master transcription factor that prompts metaxylem vessel differentiation (Kubo et al., 2005), was introduced under the control of an estradiol inducible promoter (Zuo et al., 2000), allowing synchronous induction of metaxylem vessel differentiation at high frequency. Using this system, we found that there are several cellular regions in which cortical microtubules are unstable. Cortical microtubules are eventually depleted in these regions, resulting in the formation of secondary wall pits. This suggests that local depolymerization of cortical microtubules is the key event for formation of secondary wall pits (Oda et al., 2010). Cortical microtubules are closely anchored to the plasma membrane (Hardham and Gunning, 1978; Seagull and Heath, 1980; Lancelle et al., 1986; Giddings and Staehelin, 1988), and specific regulators of microtubule dynamics are thus expected to be locally present at the plasma membrane, mediating the formation of microtubule-depolymerizing plasma membrane domains.

MIDD1 IS LOCALIZED TO MICROTUBULE-DEPLETING PLASMA MEMBRANE DOMAINS

Microtubule-associated protein MIDD1 (microtubule depletion domain 1) is preferentially associated with depolymerizing cortical microtubules in the future pit region of secondary walls in xylem cells. Knockdown of MIDD1 inhibits the local disassembly of cortical microtubules, resulting in the loss of secondary wall pits. Conversely, overexpression of MIDD1 in non-xylem cells reduces cortical microtubule density. MIDD1 is composed of two coiled-coil domains: the N-terminal domain, which binds directly to microtubules, and the C-terminal domain, which is associated with specific plasma membrane domains. MIDD1 is thus thought to promote depolymerization of cortical microtubules in the plasma membrane domains, leading to the formation of secondary wall pits (Oda et al., 2010).

MIDD1 RECRUITS AtKinesin-13A TO LOCALLY DEPOLYMERIZE CORTICAL MICROTUBULES

Microtubule depletion domain 1 does not exhibit *in vitro* microtubule depolymerization activity. MIDD1 is therefore likely to interact with other proteins to induce microtubule disassembly. Recently, AtKinsein-13A was found to interact with MIDD1 in yeast (Mucha et al., 2010). AtKinesin-13A belongs to the kinesin-13 family (Lu et al., 2005), whose animal members have microtubule depolymerization activity (Desai et al., 1999). As expected, AtKinesin-13A exhibits microtubule depolymerization activity *in vitro* (Oda and Fukuda, 2013a). Knockdown of *AtKinesin-13A* results in smaller secondary wall pits, while overexpression of *AtKinesin-13A* enlarges secondary wall pits. AtKinesin-13A co-localizes and interacts with MIDD1 in secondary wall pits

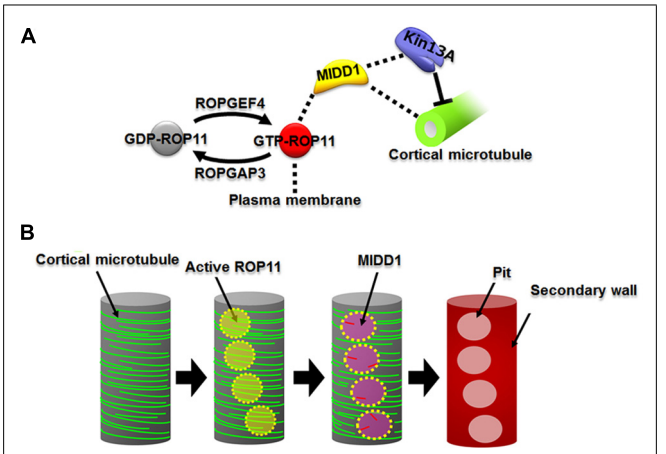

FIGURE 1 | The ROP11-MIDD1 pathway produces the microtubule-depolymerizing domain. (A) MIDD1 links plasma membrane, cortical microtubules, and AtKinesin-13A. ROP11 is locally activated by ROPGEF4 and ROPGAP3. The activated ROP11 (GTP-ROP11) recruits MIDD1 to the plasma membrane, which in turn binds cortical microtubules. MIDD1 subsequently recruits AtKinsein-13A (Kin13A), which depolymerizes the cortical microtubule. **(B)** Locally activated ROP11 recruits MIDD1 to the plasma membrane domains. MIDD1 subsequently induces microtubule disassembly by recruiting AtKinesin-13A, resulting in the formation of secondary wall pits.

and promotes depolymerization of cortical microtubules. In the absence of MIDD1, however, AtKinsein-13A neither localizes to nor affects cortical microtubules (Oda and Fukuda, 2013a). Therefore, it is likely that MIDD1 is a scaffold protein that links the plasma membrane domains to AtKinesin-13A and therefore facilitates specific localized cortical microtubule depolymerization (**Figure 1A**).

ROP GTPase REGULATES CORTICAL MICROTUBULE DYNAMICS THROUGH THE MIDD1-AtKinesin-13A COMPLEX

Microtubule depletion domain 1 belongs to the RIP/ICR family, members of which interact with the active forms of ROP GTPases via their conserved C-terminal motif (Lavy et al., 2007; Li et al., 2008). ROP11 distributes broadly at the plasma membrane and, together with MIDD1, accumulates specifically on cortical microtubules in the secondary wall pits (Oda and Fukuda, 2012a). Further cellular analysis revealed that the active form of ROP11 recruits MIDD1 to the plasma membrane. Introduction of a constitutively activated mutant ROP11 strongly inhibits formation of secondary wall pits in metaxylem vessels by affecting pit-specific localization of AtKinesin-13A (Oda and Fukuda, 2012a, 2013a). These results suggest that locally activated ROP11 is essential for formation of secondary wall pits through the recruitment of the MIDD1-AtKinesin-13A complex (**Figure 1B**).

ROP GTPases GENERATE MICROTUBULE-DEPOLYMERIZING PLASMA MEMBRANE DOMAINS

ROP11 is specifically active at the secondary wall pits, posing the question of how regulation of this local activation is achieved. ROP GTPases are activated by plant-specific guanine nucleotide

exchange factors (ROPGEFs) and are inactivated by ROP GTPase-activating proteins (ROPGAPs; Wu et al., 2000; Berken et al., 2005; Gu et al., 2006). ROPGAP3 and ROPGEF4 mediate the local activation and inactivation, respectively, of ROP11 in differentiating xylem cells (Oda and Fukuda, 2012a). Both ROPGEF4 and ROPGAP3 localize to plasma membrane domains in secondary wall pits. Interestingly, ROPGEF4 is concentrated at the center of the domains, while ROPGAP3 localizes much more broadly in the secondary wall pits. Knockout or knockdown of *ROPGEF4* causes reduced density of secondary wall pits in metaxylem vessels, suggesting that ROPGEF4 promotes formation of secondary wall pits through local activation of ROP11 (**Figure 1A**; Oda and Fukuda, 2012a). Co-expression of ROP11, ROPGEF4, and ROPGAP3 in non-xylem cells causes evenly spaced patches of ROPGEF4 at the plasma membrane, which in turn activates ROP11 around the patches. This locally activated ROP11 recruits MIDD1 and finally causes local disassembly of cortical microtubules (Oda and Fukuda, 2012a).

The next question that arises is how such spontaneously activated ROP11 domains might be formed. Loss of either ROPGEF4 or ROPGAP3 abolishes the activated ROP11 domains. Furthermore, constitutively active or negative mutants of ROP11 cannot mediate this event (Oda and Fukuda, 2012a). Therefore, GTP-GDP cycling of ROP11 by ROPGEF4 and ROPGAP3 is essential for the self-organization of the activated ROP11 domains. Neither microtubules nor actin microfilaments are required for domain formation. A possible mechanism to explain this spontaneous formation of active ROP11 patches is positive feedback from active ROP11 via ROPGEF4 as some receptor-like kinases are known to interact with ROPGEFs (Zhang and McCormick, 2007; Duan et al., 2010; Chang et al., 2012; Akamatsu et al., 2013). For example, AtPRK2, a receptor-like kinase, which functions in polarized growth of pollen tubes, interacts with both ROPGEF1 and ROP GTPases, and phosphorylates ROPGEF1 to activate ROP GTPase (Chang et al., 2012). Similarly, an unknown receptor-like kinase(s) might interact with ROPGEF4 and ROP11 to initiate positive feedback and consequently form activated ROP11 patches. A negative feedback mechanism between ROPGAP3 and ROPGEF4 via ROP11 may also contribute to the self-organization of activated ROP11 patches.

CORTICAL MICROTUBULES ACT AS A FENCE INHIBITING THE MOVEMENT OF THE ROP-MIDD1 COMPLEX

Another important finding with respect to the formation of localized secondary wall pits is that cortical microtubules restrict the localization of active ROP11. Treatment with taxol, which stabilizes microtubules, elongates active ROP11 domains, suggesting that cortical microtubules can affect the localization of active ROP11 (Oda and Fukuda, 2012a). Further reconstruction experiments in non-xylem cells revealed that active ROP11 domains are enclosed by cortical microtubules to form polygonal structures. However, disruption of cortical microtubules by chemical treatment with oryzalin or co-expression of AtKinesin-13A resulted in round active ROP11 domains. These results strongly suggest that cortical microtubules act as a fence preventing outward diffusion of active ROP11 (Oda and Fukuda, 2012a).

How does MIDD1 contribute to this phenomenon? In non-xylem cells, cortical microtubules restricted active ROP11-MIDD1 domains when truncated MIDD1, which lacks its microtubule-binding domain, was introduced with ROP11, ROPGEF4, and ROPGAP3 (**Figure 2B**). In the absence of MIDD1, however, cortical microtubules did not affect the localization of ROP11 (**Figure 2A**). In the absence of ROP11, truncated MIDD1 was distributed broadly in the cytoplasm and its distribution was unaffected by cortical microtubules (Oda and Fukuda, 2012a). By contrast, cortical microtubules could eliminate truncated MIDD1 that was artificially anchored to the plasma membrane by fusion with a membrane-binding domain, even in the absence of ROP11 (**Figure 2C**). These observations strongly suggest that MIDD1 mediates the restriction of active ROP11 by cortical microtubules (Oda and Fukuda, 2012a). The precise mechanism by which cortical microtubules restrict the ROP11-MIDD1 complex at the plasma membrane remains to be determined. Cortical microtubules are closely anchored to the plasma membrane by unknown MAPs, and it is thus likely that the active ROP11 protein complex, which includes MIDD1 and AtKinesin-13A, is restricted in its ability to diffuse through the space between the cortical microtubules and the plasma membrane. Consistent with this hypothesis is the elimination of an *Arabidopsis* formin, AtFH1, which has a large cytoplasmic domain, from the plasma membrane by cortical microtubules (Martiniere et al., 2011). Similarly, cortical actin microfilaments limit the diffusion of plasma membrane-anchored proteins in animal cells (Kusumi et al., 2005). Instead of actin filaments, plasma-membrane-associated cortical microtubules are used in plants as membrane fences to restrict localization of plasma membrane-anchored proteins.

INTERPLAY BETWEEN MICROTUBULES AND PLASMA MEMBRANE DOMAINS MAY ESTABLISH PATTERNS

Evenly distributed active ROP11 domains are spontaneously generated by ROPGEF4, ROPGAP3, and ROP11, and recruit the MIDD1-AtKinesin-13A complex to the domains, which in turn depolymerizes cortical microtubules at the domains. The surrounding cortical microtubules limit the localization of ROP11-MIDD1 complex to the domains, probably by inhibiting the outward lateral diffusion of the ROP11-MIDD1 complex (**Figure 2D**). These two interactions cause a mutual inhibition between cortical microtubules and plasma membrane domains underlain by active ROP11, resulting in spatial restriction of cortical microtubules and the plasma membrane domains (**Figure 2E**). Variability in the balance between these two pathways may produce a range of secondary wall pit shapes. For example, if the restriction of plasma membrane domains by cortical microtubules is dominant, reticulate secondary walls with oval pits will be formed. Conversely, if the microtubule depolymerization activity of the plasma membrane domain becomes dominant, pitted secondary walls with round pits will be formed. Therefore, this interplay between the plasma membrane domains and cortical microtubules may be a key determining factor leading to different secondary wall patterns.

Similar interplay between plasma membrane domains and cortical microtubules is reported in leaf epidermal morphogenesis.

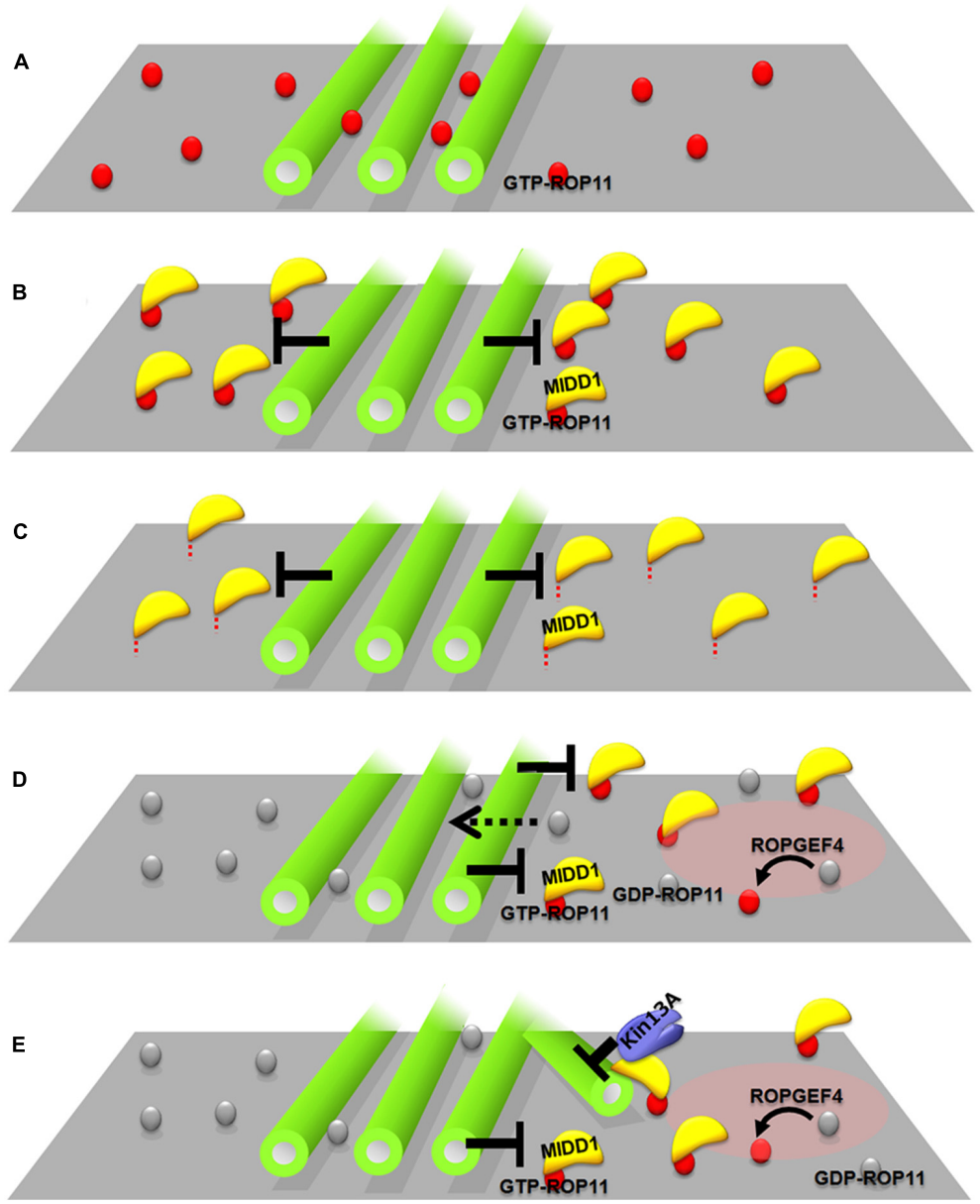

FIGURE 2 | Interplay between cortical microtubules and plasma membrane domains via the ROP11-MIDD1 complex. (A–C) MIDD1 mediates elimination of active ROP11 by cortical microtubules. **(A)** Cortical microtubules do not affect the localization of active (GTP-) ROP11. **(B)** Cortical microtubules eliminate active ROP11-truncated MIDD1 complex from the plasma membrane (truncated mutant lacking microtubule-binding domain). **(C)** Cortical microtubules eliminate the membrane–anchored truncated MIDD1. **(D,E)** Interplay between cortical microtubules and plasma membrane domains in xylem cells. **(D)** Cortical microtubules restrict the plasma membrane domain of active ROP11 generated by ROPGEF4. Inactive ROP11 is not affected by cortical microtubules, because there is no interaction with MIDD1. **(E)** Active ROP11-MIDD1-AtKinesin-13A complex associates with the microtubule growing into the domain and induces depolymerization.

In leaf epidermis, pavement cells grow to form an interdigitating structure where lobes and indentations develop side-by-side. ROP6 and its effector RIC1 recruit microtubule-severing katanin protein to facilitate the ordering of a parallel cortical microtubule array that restricts cell growth in indentations, while ROP2/4 and their effector RIC4 enhance cortical actin microfilaments to promote local growth of lobes (Fu et al., 2005, 2009; Lin et al., 2013). The cortical actin microfilaments inhibit endocytosis of an auxin efflux carrier PIN1, promoting accumulation of PIN1 protein at the lobe forming region, which in turn promotes accumulation of auxin, which then activates the ROP6-RIC1 pathway in its neighbor cell via auxin binding protein ABP1 (Xu et al., 2010; Nagawa et al., 2012). These two signaling pathways appear to spatially restrict one another: the ROP2/4-RIC4 pathway eliminates RIC1 from the lobe forming regions and inhibits parallel ordering of cortical microtubules, while the ROP6-RIC1 pathway inhibits the

ROP2/4-RIC4 pathway, preventing co-existence of these two pathways in the same area. Although the precise mechanism by which these two pathways spatially restrict each other is still unclear, one possibility is that the parallel cortical microtubules in the indentation inhibit lateral diffusion of ROP2/4-RIC4 complex on the plasma membrane, in a similar manner to the restriction of the ROP11-MIDD1 complex by cortical microtubules. It is also possible that the ROP-MIDD-AtKinesin-13 pathway contributes to the elimination of cortical microtubules from lobes and that the dynamic interplay between cortical microtubules and plasma membrane domains of ROP GTPase might be widely utilized in plant cell morphogenesis.

CONCLUDING REMARKS

Recent studies on xylem vessel differentiation and leaf epidermis morphogenesis revealed dynamic interplay between cortical microtubules and plasma membrane domains. ROP GTPases play critical roles in generating *de novo* plasma membrane domains and in regulating the function of the domains by recruiting different effector proteins and MAPs. In xylem cells, MIDD1 plays a central role in this interplay. Considering the diversity of the ROP GTPases and MIDD/RIP/ICR family members, the ROP-MIDD/RIP/ICR pathway may be involved in various cellular events by mediating interplay between plasma membrane domains and cortical microtubules. Recent proteomic approaches suggest that MIDD1/RIP/ICR members are also MAPs (Hamada et al., 2013). Further analysis of the pathways will provide novel insights regarding the molecular mechanisms underlying local control of plasma membrane domains coupled to microtubule dynamics in various plant cells.

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Overlapping and divergent signaling pathways for ARK1 and AGD1 in the control of root hair polarity in *Arabidopsis thaliana*

Cheol-Min Yoo and Elison B. Blancaflor*

Plant Biology Division, The Samuel Roberts Noble Foundation, Ardmore, OK, USA

Edited by:
Clément Thomas, Public Research Centre for Health, Luxembourg

Reviewed by:
Ingo Heilmann, Martin-Luther-University Halle-Wittenberg, Germany
Fatima Cvrckova, Charles University in Prague, Czech Republic

****Correspondence:***
Elison B. Blancaflor, Plant Biology Division, The Samuel Roberts Noble Foundation, 2510 Sam Noble Parkway, Ardmore, OK 73401, USA
e-mail: eblancaflor@noble.org

We previously showed that seedlings harboring mutations in genes encoding ARK1, an armadillo repeat-containing kinesin, or AGD1, a class 1 ARF-GAP, have root hairs that exhibit wavy/spiral growth and two tips originating from one initiation site. These root hair defects were accompanied by bundling of endoplasmic microtubules and filamentous actin (F-actin) that extended to the extreme root hair apex. The similar phenotypes of *ark1* and *agd1* mutants suggest a tight coordination between the cytoskeleton and membrane trafficking in the control of root hair polarity. Indeed, cell biological and genetic studies of the *agd1* mutant provided evidence that AGD1's involvement in root hair development involves cross-talk among phosphoinositides (PIs), the actin cytoskeleton and other small GTPases such as ROP2 and RABA4b. Here we show that *ark1* root hairs mirror those of *agd1* with regard to altered targeting of ROP2 and RABA4b, as well as abnormal tonoplast organization. Furthermore, like *agd1*, enhanced root hair defects in double mutants in *ARK1* and genes encoding a type B phosphatidylinositol-4-phosphate 5-kinase 3 (*PIP5K3*), a phosphatidylinositol-4-phosphate (*PI-4P*) phosphatase (*RHD4*), a phosphatidylinositol transfer protein (*COW1*), and a vegetative actin isoform (*ACT2*), were observed. However, root hair shape of some *ark1* double mutant combinations, particularly those with *act2, pip5k3* and *rhd4* (*ark1 act2*, *ark1 pip5k3*, *ark1 rhd4*), differed in some respects from *agd1 act2, agd1 pip5k3,* and *agd1 rhd4*. Taken together our results continue to point to commonalities between ARK1 and AGD1 in specifying root hair polarity, but that these two modulators of tip-growth can also regulate root hair development through divergent signaling routes with AGD1 acting predominantly during root hair initiation and ARK1 functioning primarily in sustained tip growth.

Keywords: Arabidopsis, ARF-GAP, cytoskeleton, kinesin, membrane trafficking, root hairs, tip growth

INTRODUCTION

Root hairs are long, tubular extensions of specialized root epidermal cells called trichoblasts. Their formation is typically triggered by the pH-dependent loosening of the trichoblast cell wall, which is visually manifested as slight bulging at a specific site on the trichoblast surface (Bibikova et al., 1998). This so called "initiation stage" is followed by a period of cell elongation where growth is confined to the extreme tip of the root hair cell, a process known as tip growth. As a result, a fully expanded root hair cell assumes the shape of a straight tube with a consistent diameter. The highly predictable growth of a root hair has made it a good model system to identify molecular components of polarity establishment in plant cells (Rounds and Bezanilla, 2013).

An essential component of the root hair growth machinery is the trafficking of vesicles containing cell wall and membrane precursors that must be directed to the very tip of the cell to sustain growth. This process is known to be mediated by the actin cytoskeleton, actin binding proteins, calcium gradients and small GTP binding proteins (small GTPases) (Pei et al., 2012; Gu and Nielsen, 2013). Also pivotal for root hair development are the phosphoinositide (PI) group of signaling lipids, which together with their respective metabolic enzymes could function as site-specific signals on the cell membrane that direct elements of the cytoskeleton and the vesicle trafficking complex, such as the exocyst, to defined regions of the cell to maintain tip growth (Heilmann, 2009; Žárský et al., 2009).

Through our previous forward genetic work in *Arabidopsis*, we identified an Armadillo Repeat-containing Kinesin 1 (ARK1) and an Adenosine Diphosphate Ribosylation Factor (ARF)-GTPase Activating Protein (GAP) Domain-containing protein (AGD1) as additional components that specify root hair polarity. Both the *ark1* and *agd1* mutants exhibited wavy and bifurcated root hair growth instead of the straight growth, single growth point phenotype typical of wild-type root hairs (Yoo et al., 2008). ARK1 together with its homologs, ARK2 and ARK3, belongs to a plant specific group of kinesin microtubule motor proteins due to its C-terminal armadillo repeat-containing domain (Richardson et al., 2006). Consistent with its predicted function, the N-terminal kinesin motor domain of ARK1 was shown to bind polymerized microtubules *in vitro* and a green fluorescent protein

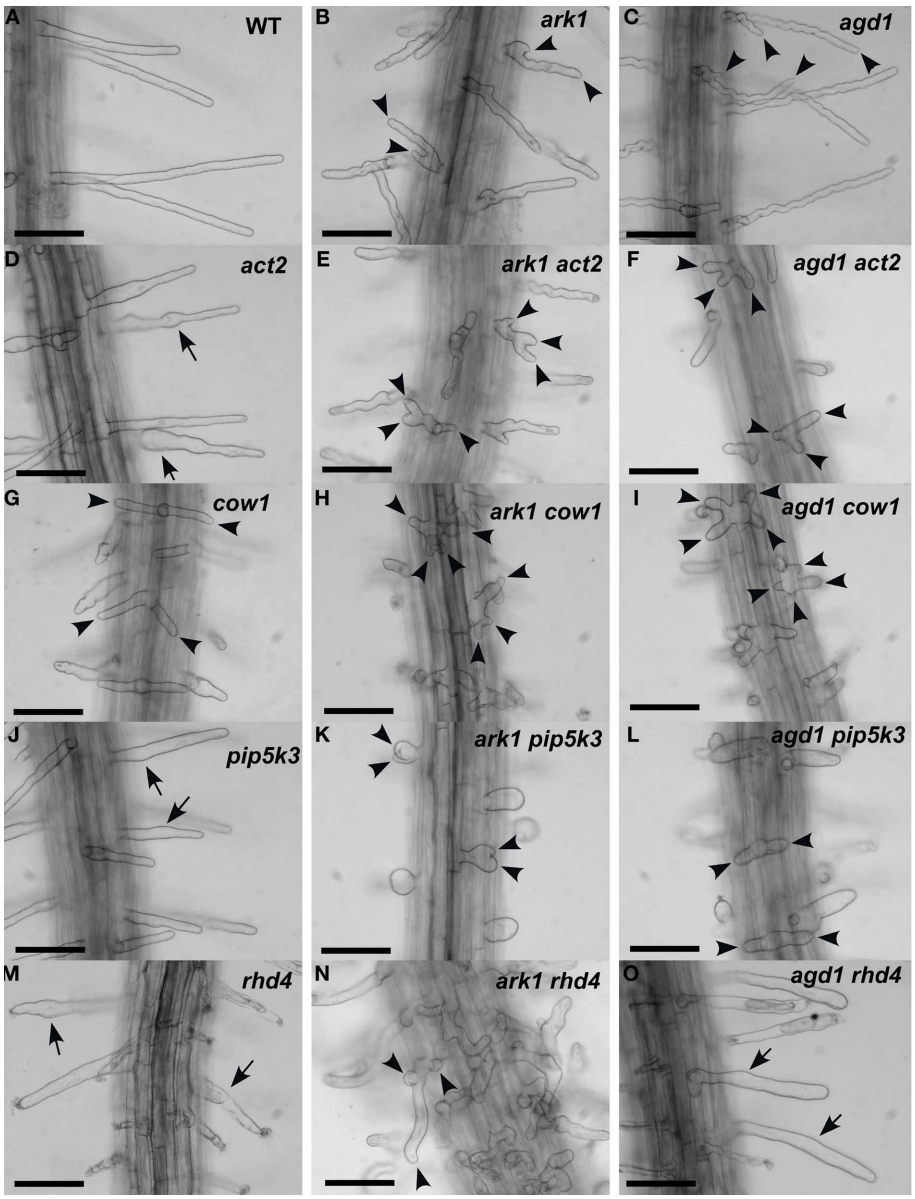

FIGURE 1 | Root hair phenotypes of *ark1, agd1* and their respective double mutants. (A–C) Wild-type and *ark1*, and *agd1* root hairs. **(D,G,J,M)** Root hairs of single *act2*, *cow1*, *pip5k3,* and *rhd4* mutants. **(E,H,K,N)** Root hairs of double mutants with *ark1*. **(F,I,L,O)** Root hairs of double mutants with *agd1*. Multiple tips from a single root hair initiation point are indicated by arrowheads. Regions of the root hair that swell or bulge are indicated by arrows. Bars = 100 μm.

(GFP)-ARK1 fusion decorated microtubules in transient expression studies (Yang et al., 2007; Yoo et al., 2008). The C-terminal armadillo repeat-containing domain of ARK1 was also demonstrated to bind polymerized actin *in vitro*, leading to the proposal that it coordinates microtubule and F-actin cross-talk during root hair growth (Yang et al., 2007). AGD1 on the other hand is an ARF-GAP, a protein that modulates the activity of the ARF family of small GTPases, which are known regulators of membrane and organelle trafficking. The activity of ARF-GTPases, like other small GTPases, is regulated through a cycle of GTP binding and hydrolysis, which activate and inactivate the ARF-GTPase, respectively. The latter process is mediated by the action of ARF-GAPs (Donaldson and Jackson, 2011). In *Arabidopsis*, there are 15 AGD proteins divided into four classes with AGD1 belonging to the multi-domain class1 ARF-GAPs (Vernoud et al., 2003). AGD1 was shown to localize to punctate bodies reminiscent of the endomembrane system, which support its predicted role as a modulator of vesicle trafficking (Yoo et al., 2008). Recently, we showed that AGD1 impacts root hair polarity by maintaining the correct targeting of various root hair tip growth including Rho Of Plants2 (ROP2) and RabA4B small GTPases, calcium gradients, and PI-4P domains (Yoo et al., 2012).

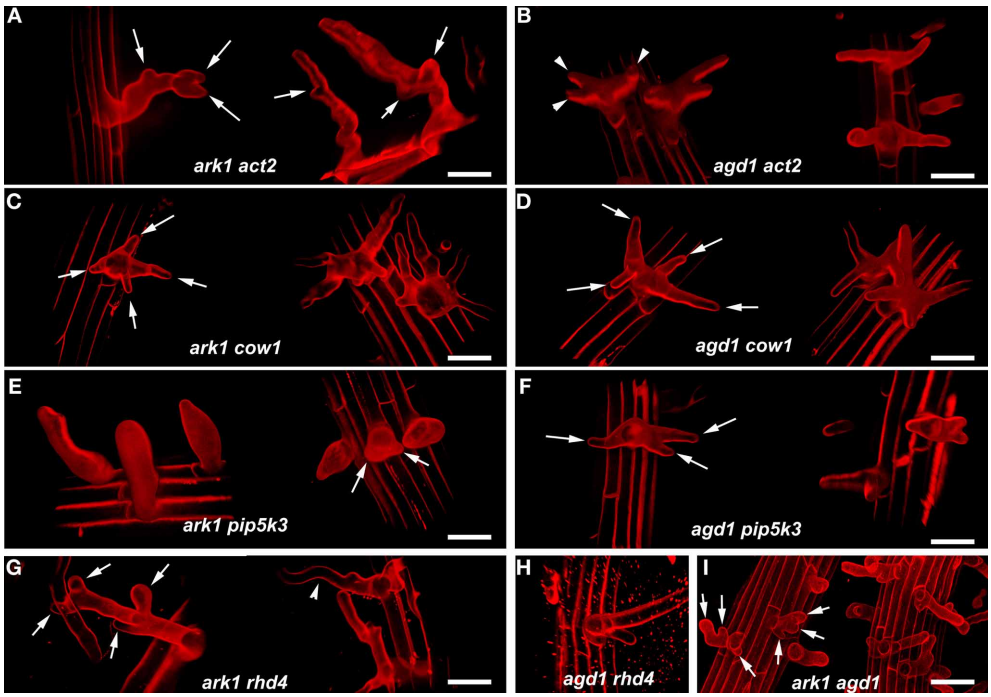

FIGURE 2 | Representative confocal microscope images of various *ark1* and *agd1* double mutants. Roots were stained with propidum iodide and a series of optical sections were projected and rendered using Volocity 6.3 software to more accurately visualize the resulting shapes of the double mutants. **(A,B)** Note that multiple root hair tips appear to emerge at various points in elongating *ark1 act2* root hairs (arrows) while multiple tips in *agd1 act2* are restricted to early initiation (arrowheads). **(C,D)** In *ark1 cow* and *agd1 cow1*, multiple root hairs tips are prevalent at a single initiation point (arrows). **(E,F)** Bulbous root hairs are a typical feature of *ark1 pip5k3*. Some bulbous root hairs appear to have two tips (arrows). Like *agd1 act2* and *agd1 cow1*, *agd1 pip5k3* root hairs have multiple root hairs emerging from one initiation point (arrows). **(G)** *ark1 rhd4* root hairs have new tips emerging throughout root hair elongation (arrows) and exhibit wavy growth typical of *ark1* single mutants (arrowhead). **(H)** *agd1 rhd4* root hairs resemble *rhd4* single mutants. **(I)** Root hairs of *ark1 agd1* root hairs are mostly similar to the multiple tip phenotype (arrows) exhibited by *agd1 act2*, *agd1 cow1*, *ark1 cow1*, and *agd1 pip5k3*. Bars = 50 μm.

The *ark1* and *agd1* mutants exhibited disrupted root hair microtubules and F-actin (Yang et al., 2007; Sakai et al., 2008; Yoo et al., 2008). This together with the fact that root hairs of *agd1* and *ark1* resembled wild-type root hairs treated with actin and microtubule inhibitors (Bibikova et al., 1999), has led us to suggest that ARK1 and AGD1 might have overlapping signaling functions in specifying cytoskeletal organization during root hair tip growth (Yoo et al., 2008). However, the observation that low concentrations of brefeldin A (BFA), a fungal macrolide inhibitor of ARF-GTPase activation, causes *agd1*, but not *ark1* root hairs to revert to straight growth, have also pointed to the possibility that AGD1 and ARK1 modulate root hair development through distinct molecular pathways (Yoo et al., 2008).

To further clarify the functional relationship between ARK1 and AGD1 in the control of tip growth, we generated double mutants in *ARK1* and other genes known to affect root hair development (e.g., *ACT2*, *COW1*, *PIP5K3*, and *RHD4*). The resulting double mutants were compared to corresponding double mutants of *agd1* described previously (Yoo et al., 2012). In addition, dynamic imaging of the small GTPases, RABA4b, and ROP2, in root hairs of *ark1* was conducted and compared to *agd1*. Here, we show that like AGD1, ARK1 is involved in maintaining the stability of small GTPases that direct root hair tip growth. However, subtle differences in root hair shape between double mutant combinations to *ark1* and *agd1* continue to point to divergent signaling pathways by which ARK1 and AGD1 mediate polar root hair growth.

MATERIALS AND METHODS

GENERATION OF DOUBLE MUTANTS

All of the *Arabidopsis* lines used in this study are of the Col-0 ecotype. We used *ark1-1* (Salk_035063, a T-DNA mutant of the *At3g56870* gene; Yoo et al., 2008), *agd1-1* (a deletion mutant of the *At5g61980* gene; (Yoo et al., 2008, 2012), *act2-3* (Salk_048987, a T-DNA mutant of the *At3g18780* gene; Nishimura et al., 2003), *cow1* (Salk_002124, a T-DNA mutant of the *At4g34580* gene; Yoo et al., 2012), *pip5k3-4* (Salk_026683, a T-DNA mutant of the *At2g26420* gene; Stenzel et al., 2008), and *rhd4-1* (a point mutant of the *At3g51460* gene; Thole et al., 2008). The *agd1-1* mutant was isolated from a forward genetic screen and described previously (Yoo et al., 2008, 2012) while other single mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). Double mutants were identified by polymerase chain reaction (PCR)-based genotyping or DNA sequencing.

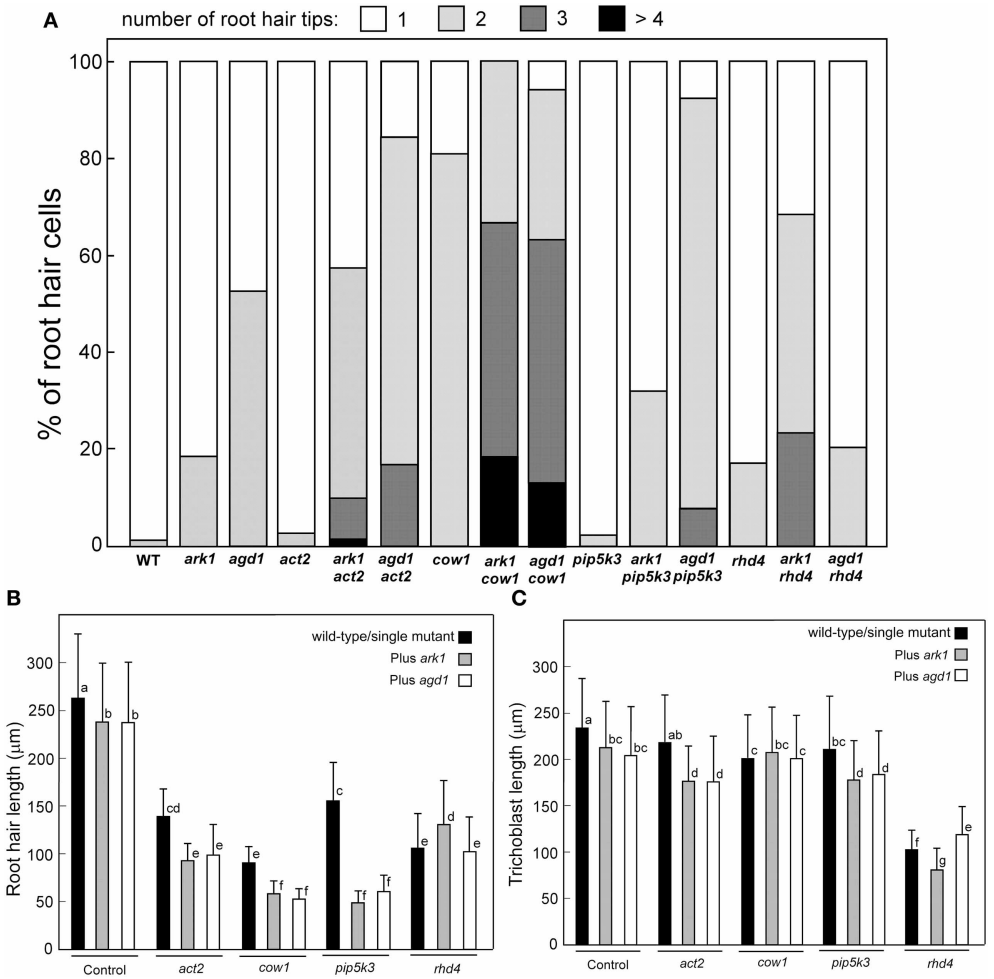

FIGURE 3 | Quantification of root hair defects in *agd1* and *ark1* single and various mutant combinations. (A) Frequency of multiple tips. Root hair tips from each root hair or initiation site were counted, and numbers of the root hair tips are presented as a percentage of the number of root hairs sampled. More than 100 trichoblasts were sampled for each single mutant/double mutant. Quantification of root hair **(B)** and trichoblast **(C)** length. Black bar indicates the average length of wild-type root hair cells or single *act2, cow1, pip5k3*, and *rhd4* (leftmost black bars). The rightmost gray and unfilled bars represent average root hair cell or trichoblast length of *ark1* and *agd1*, respectively. All other bars show the average root hair length of different double mutant combinations. Data are means (±*SD*) from >120 root hairs or trichoblasts. Means with different letters are significantly different as determined by Tukey's HSD test ($P < 0.005$).

GROWTH CONDITIONS AND EVALUATION OF ROOT HAIR PHENOTYPES

Seeds of wild-type and mutants were surface sterilized and planted in a half strength of Murashige and Skoog (MS) media containing 0.5% agar layered on 48 × 64 mm coverslips, as described in Dyachok et al. (2009). To analyze root hair phenotypes, 4-day-old seedlings were examined with Nikon Eclipse TE300 stereo-microscope equipped with a 10× Plan Fluor DLL objective and photographed with a Nikon DXM1200 camera (Nikon Corporation, Melville, NY, USA). For measurement of trichoblast length, root tissues were stained with 10 μM of propidium iodide and examined using the 20× Plan Fluor objective of a Nikon Optiphot-2 microscope equipped with epi-fluorescence. Images of propidium iodide-stained roots were captured with a Nikon DS-Ri1 camera. Root hair and trichoblast length from the digital images were measured using ImageJ ver. 1.46r software (http://rsbweb.nih.gov). The data were analyzed by One-way Analysis of Variance (ANOVA) to test statistical significance, and Tukey's honestly significant difference (HSD) test for multiple comparisons of means. Statistical analysis was conducted using SPSS ver. 19 software (IBM). For 3D rendered images of double mutant root hairs, root tissues were stained with 10 μM propidium iodide and imaged with an UltraView ERS spinning-disc confocal microscope (Perkin-Elmer Life and Analytical Sciences, Waltham, MA, USA) equipped with a 40× objective. Propidium iodide was excited using the 561-nm line of the argon-krypton laser and emission was detected at 615 nm. More than 200 optical sections of a root hair were taken at 0.2 μm intervals, and the image data were projected using Volocity software version 6.3 (Improvision).

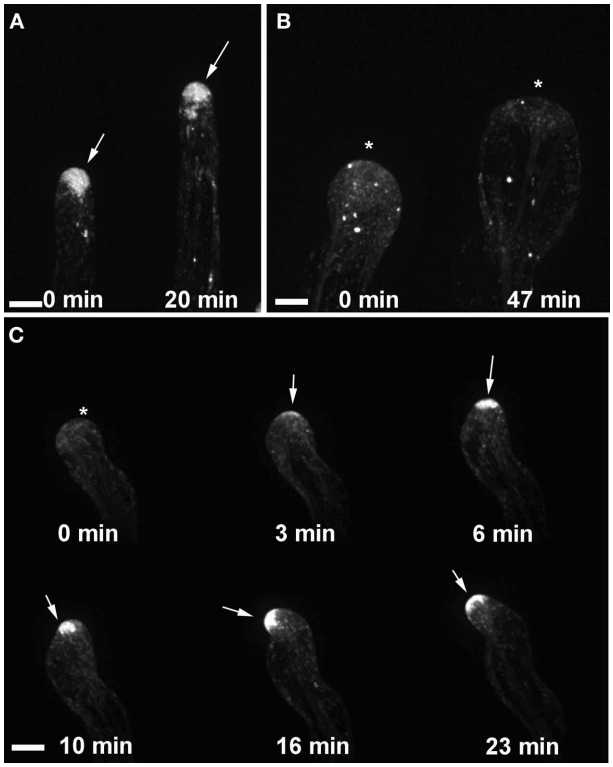

FIGURE 4 | Targeting of RABA4b secretory vesicles to the root hair tip is disrupted in *ark1*. (A) EYFP-RABA4b is preferentially enriched at the tips of wild-type seedlings during root hair elongation (arrows). **(B)** In *ark1* root hairs, there is no enrichment of the EYFP-RABA4b signal in a swelling root hair (asterisks). **(C)** In a waving root hair, the EYFP-RABA4b signal often dissipates (asterisk) but eventually reforms when tip growth resumes (arrows). The EYFP-RABA4b typically follows the change in root hair growth direction. Time-lapse movie sequences of corresponding still images are presented as **Movies S1** (for wild-type), **S2**, and **S3** (for *ark1*). Bars = 20 μm.

IMAGING ROP2, RABA4b AND A TONOPLAST MARKER IN LIVING ROOT HAIRS

For imaging the small GTPases, wild-type plants expressing Enhanced Yellow Fluorescent Protein (EYFP)-ROP2 (Xu and Scheres, 2005) and EYFP-RABA4b (Preuss et al., 2004) were crossed with *ark1-4* plants (Yoo et al., 2008). For imaging vacuolar membrane dynamics, wild-type plants expressing GFP-tonoplast intrinsic protein (TIP; GenBank acc. no: U39485; Cutler et al., 2000) were crossed with *agd1-1 and ark1-4* plants (Yoo et al., 2008). Root hairs were imaged with an UltraView ERS spinning-disc confocal microscope (Perkin-Elmer Life and Analytical Sciences, Waltham, MA, USA) equipped with a ×63 water-immersion objective (Numerical aperture 1.40). EYFP and GFP were excited using the 488-nm line of the argon-krypton laser, and emission was detected at 510 nm. Root hairs were imaged by collecting optical sections at 1 μm intervals. Analyses of EYFP-ROP2 and EYFP-RABA4b localization in growing root hairs were conducted on images of projected stacks of optical sections acquired every 30 s–1 min over a period of 1–2 h. For the analyses of GFP-TIP localization optical sections were acquired every 1 s over a period of 2 min.

RESULTS

ENHANCED ROOT HAIR PHENOTYPES IN ark1 DOUBLE MUTANTS

We compared root hairs of *ark1 act2, ark1 pip5k3, ark1 cow1,* and *ark1 rhd4* with *agd1 act2, agd1 pip5k3, agd1 cow1*, and *agd1 rhd4* using bright field and confocal microscopy (**Figures 1**, **2**). Representative bright field images of wild-type and single *agd1* and *ark1* mutant root hairs are shown in **Figures 1A–C** while representative bright field images of single *act2, cow1, pip5k3,* and *rhd4* mutants are shown in **Figures 1D,G,J,M**. Single *act2* mutants, which are disrupted in the gene encoding *ACTIN2*, often had irregular root hair diameters with thicker bases and root hairs that were shorter than wild-type (Ringli et al., 2002; **Figure 1D**). Quantitative analysis showed that the percentage of *act2* root hairs with two tips and was similar to wild-type and significantly less than *ark1* and *agd1* single mutants (**Figure 3A**). The average root hair length of *act2* was significantly less than wild-type, and *ark1* and *agd1* single mutants (**Figure 3B**). Root hairs of *ark1 act2* double mutants showed additive morphological defects (i.e., double mutants had shorter root hairs than their respective single mutant and displayed the wavy and branched phenotypes characteristic of single *ark1* mutants; compare **Figures 1E** with **1B**; **Figure 3B**). However, unlike *agd1 act2*, which often had two or three tips restricted to the initiation site (**Figures 1F**, **2B**), *ark1 act2* produced additional tips not only at initiation but also during the root hair elongation stage (**Figures 1E**, **2A**).

The *COW1*mutant, which is disrupted in the gene encoding a sec14p domain phosphatidyl inositol (PtdIns) transfer protein (PITP) (Böhme et al., 2004; Vincent et al., 2005) was previously identified as an enhancer of *agd1*(Yoo et al., 2012). Single mutants of *cow1* typically had two tips originating from one initiation site (**Figures 1G**, **3A**), while *agd1 cow1* had root hairs with up to five tips originating from one initiation site (**Figures 1I**, **2D**, **3A**). Double mutants of *ark1 cow1* also had a large percentage of root hairs with up to four to five tips (**Figures 1H**, **2C**, **3A**) indicating that *cow1* enhances *ark1* defects similar to *agd1*. Root hairs of *ark1 cow1* and *agd1 cow1* were significantly shorter than root hairs of their respective single mutants (**Figure 3B**).

PIP5K3 encodes a type B phosphatidylinositol-4-phosphate 5-kinase 3 that catalyzes the formation of phosphatidylinositol 4,5-bisphosphate (PI-4,5P2) from PI-4P. Consistent with previous studies, a mutation in *PIP5K3* resulted in plants with root hairs that were shorter and slightly thicker than wild-type (Kusano et al., 2008; Stenzel et al., 2008; **Figures 1J**, **3B**). The *ark1 pip5k3* double mutants had stunted root hair growth manifested visually by the formation of short bulbous structures. Although a majority of root hairs of *ark1 pip5k3* formed bulbous structures, some root hairs displayed rudiments of what appeared to be two tips (**Figures 1K**, **2E**). On the other hand, the multiple tips that formed in root hairs of *agd1 pip5k3* double mutants were very distinct and quantitative analysis showed that these were more numerous than *ark1 pip5k3* (**Figures 1L**, **2F**, **3A**). The root hair length of the double mutants of *pip5k3* to either *ark1* or *agd1* was synergistically reduced (**Figures 1J–L**, **3B**). Although overall reduction in root hair length was similar between *ark1 pip5k3* and *agd1 pip5k3* (**Figure 3B**), the overall shape of *ark1 pip5k3* was clearly different from that of *agd1 pip5k3* (compare **Figures 1K,L** and **Figures 2E,F**).

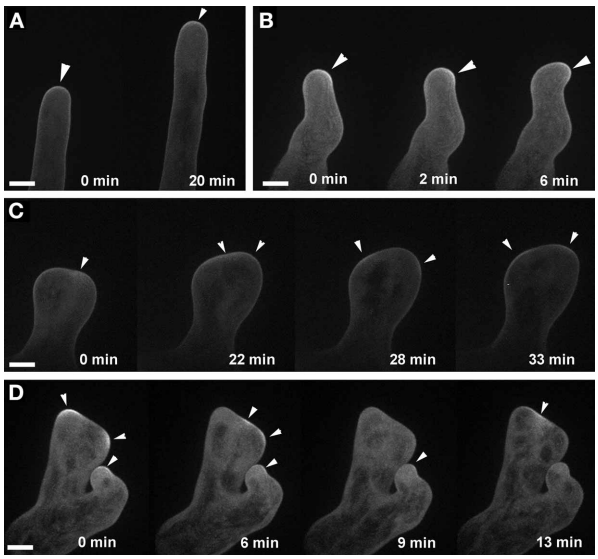

FIGURE 5 | ROP2 localization to the apical cell membrane of root hairs is altered in *ark1*. (A) A wild-type root hair shows enhancement of EYFP-ROP2 at the apical plasma membrane (arrow heads). **(B)** In a wavy root hair of *ark1*, plasma membrane localized EYFP-ROP2 shifts position prior to the change in root hair growth direction (arrowheads). **(C,D)** In *ark1* root hairs that swell and form branches, EYFP-ROP2 signal appears to become enriched at more than one location within the cell. Also, the EYFP-ROP2 signal dissipates then shifts to a different plasma membrane domain (arrowheads). Time-lapse movie sequences of still images in panels **(A**,**C)** are presented as **Movies S4** (for wild-type) and **S5** (for *ark1*), respectively. Bars = 20 μm.

As reported previously, *RHD4* encodes a PI-4P phosphatase. Root hairs of *rhd4* mutants were short, branched and randomly formed bulges along their length (Thole et al., 2008; **Figure 1M**). Previously, we found that *agd1 rhd4* root hairs showed similar root hair phenotypes as the single *rhd4* mutant suggesting that *rhd4* is epistatic to *agd1* (**Figures 1M,O, 2H**; Yoo et al., 2012). Here we found that the *ark1 rhd4* double mutant exhibited additive effects. For example, *ark1 rhd4* had swollen root hairs that bulged in random positions, which was a typical phenotype of *rhd4* single mutants. Root hairs of *ark1 rhd4* also showed curling and branching along their length, a feature characteristic of *ark1* single mutants (**Figures 1B,M,N, 2G**). Quantitative analysis of the percentage of root hairs with multiple tips and average root hair length confirmed that *rhd4* is epistatic to *agd1* but not to *ark1* (**Figures 3A,B**). In agreement with our previous report, *ark1 agd1* root hairs were shorter than their respective single mutants and exhibited multiple tips from one initiation point (**Figure 2I**).

We also measured trichoblast length in the various single and double mutant combinations. Based on this analysis, we found that *agd1* and *ark1* had slight but statistically significant reduction in trichoblast length compared to wild-type (**Figure 3C**). The reduction in trichoblast length was enhanced in *ark1 act2*, *agd1 act2*, *ark1 pip5k3,* and *agd1 pip5k3* double mutants but not in *ark1 cow1* and *agd1 cow1*. It was also found that *rhd4* single mutants had dramatically reduced trichoblast length compared to wild-type and all other single mutants examined. However, when combined with a mutation in the *ARK1* or *AGD1* gene, opposite effects on trichoblast length were observed. It was found that *ark1 rhd4* had shorter trichoblasts than *rhd4* while those on *agd1 rhd4* were longer (**Figure 3C**). No differences in the location of root hairs along the trichoblasts were observed.

We then measured primary root length of single and double mutants under conditions used for examining root hair and trichoblast length to determine if differences in trichoblast length correlated with altered primary root length. We found that the shorter trichoblast in *rhd4*, *ark1 rhd4,* and *agd1 rhd4* translated into shorter primary roots compared to all other single or double mutant combinations. However, the longer trichoblasts in *agd1 rhd4* compared to *rhd4* or *ark1 rhd4* were not correlated with longer primary roots. Interestingly, we also found that *cow1* primary roots were shorter than wild-type but was restored to wild-type lengths in *ark1 cow1* (**Figure S1**).

RABA4b- AND ROP2-GTPase TARGETING TO THE ROOT HAIR TIP IS DISRUPTED IN ark1

We showed previously that the wavy root hair growth of *agd1* was accompanied by mistargeting of tip-localized RabA4b trans-Golgi compartments (Yoo et al., 2012). Given the similar root hair defects of *agd1* and *ark1*, we asked whether root hairs of *ark1* also show aberrant RabA4b dynamics. Using spinning disc confocal microscopy, we found that EYFP-RabA4b signal often dissipated as the root hair elongated in contrast to the consistent signal observed in wild-type (**Figures 4A–C**; **Movies S1–S3**). Furthermore, swelling root hairs of *ark1* did not show any preferential EYFP-RabA4b accumulation (**Figure 4B**). As shown in **Movie S2**, EYFP-RabA4b accumulated at the root hair apex of swelling *ark1* root hairs but the signal would quickly dissipate. Like in the previously reported *agd1* root hairs, EYFP-RabA4b signal in waving root hairs of *ark1* constantly shifted at the growing apex such that the relocalization of the signal was followed by a change in the direction of root hair growth (**Figure 4C**; **Movie S3**).

Targeting of plasma-membrane ROP2 was altered in *ark1* root hairs similar to what was observed in *agd1* (Yoo et al., 2012). In root hairs of *ark1*, EYFP-ROP2 localized to the apical plasma membrane (**Figure 5A**). However, unlike wild-type root hairs, the EYFP-ROP2 signal in wavy root hairs of *ark1* shifted to the side where tip growth changed direction (**Figure 5B**). In *ark1* root hairs that showed extreme polarity defects, intense plasma-membrane EYFP-ROP2 signal would alternately shift from one defined site to another. As such, the root hair was never able to attain the tubular shape typical of wild-type root hairs (**Figures 5C,D**; **Movies S4**, **S5**).

VACUOLAR MEMBRANE DYNAMICS IS ALTERED IN TIPS OF agd1 AND ark1 ROOT HAIRS

We next sought to determine whether *agd1* and *ark1* root hairs exhibited other defects in membrane organization that could be linked to cellular tip abnormalities reported previously (Yoo et al., 2008, 2012). We looked closely at the dynamics of the vacuolar membrane because of its dependence on the organization of

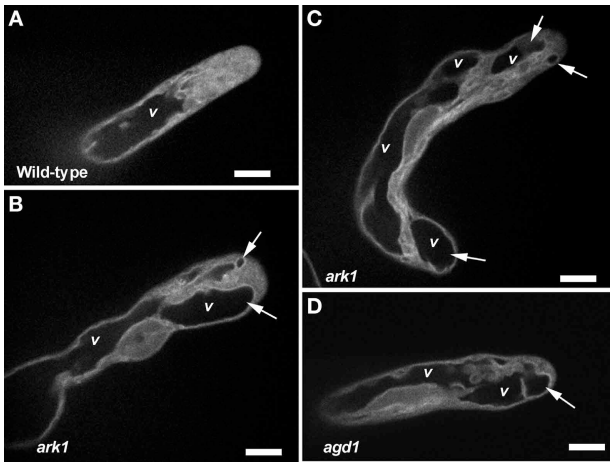

FIGURE 6 | Abnormal tonoplast organization at the growing apex was observed in *ark1* and *agd1* root hair cells. Tonoplast was visualized by expressing GFP-TIP in the plants. **(A)** In wild-type, the vacuole (*v*) is maintained at a distance from the extreme root hair apex. **(B–D)** In the *ark1* and *agd1* root hairs the vacuolar membranes occasionally protrude into the extreme apex (arrows). Time-lapse movie sequences of corresponding still images are presented as **Movies S6** (for wild-type), **S7** (*ark1*), and **S8** (for *agd1*). Bars = 20 μm.

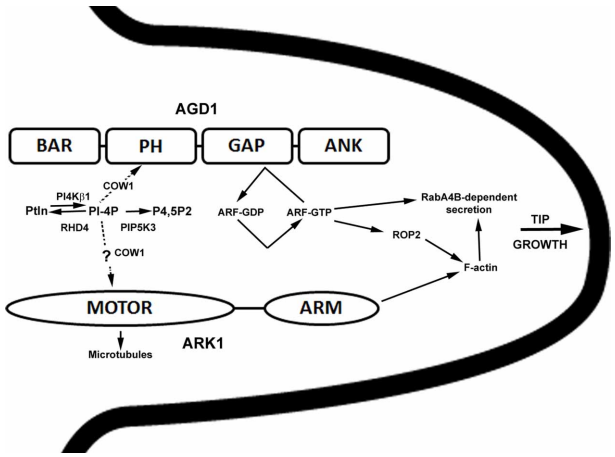

FIGURE 7 | Simplified and speculative model for ARK1- and AGD1-mediated control of root hair tip growth. The various domains of AGD1 and ARK1 proteins are shown. Bin1-Amphiphysin-Rvs167p/Rvs161p (BAR), Pleckstrin Homology (PH), GAP, and Ankyrin repeat (ANK) for AGD1 (Vernoud et al., 2003) and kinesin MOTOR and armadillo repeat-containing (ARM) domains for ARK1 (Yang et al., 2007). In this model, RHD4, PI4Kβ1, and PIP5K3 influence AGD1 activity by their timely depletion and synthesis of PI monophosphates (e.g., PI-4P; Preuss et al., 2006; Kusano et al., 2008; Stenzel et al., 2008; Thole et al., 2008). COW1 is involved in the transfer of PIs (dashed arrow; Phillips et al., 2006) to facilitate binding to PH domain of AGD1 (Yoo et al., 2012) or possible interaction with ARK1. PI binding to AGD1 would in turn modify the activity of a yet to be determined ARF-GTPase. Because ARF-GTPase has been shown to control ROP2 targeting (Xu and Scheres, 2005), AGD1 could influence F-actin organization indirectly and as a result mediate RabA4B and vacuolar dynamics (Preuss et al., 2004; Yoo et al., 2008, 2012). AGD1 and ARK1 could act in parallel pathways where ARK1 mediates crosstalk between microtubules (via the kinesin motor domain) and F-actin (via the ARM domain) (Yang et al., 2007). Although genetic interaction studies presented here implicate PIs in the ARK1 function, possibly through COW1 (dashed arrow), the precise mechanisms by which this is accomplished is unclear.

actin and microtubules (Higaki et al., 2006; Oda et al., 2009), which are bundled in tips of *agd1* and *ark1* root hairs (Yoo et al., 2008). To image the vacuolar membrane, we expressed GFP-TIP in *agd1* and *ark1* (Cutler et al., 2000). In actively elongating wild type root hairs, GFP-TIP was highly dynamic and the membrane delineating the tonoplast was restricted to a subapical region of the root hair (**Figure 6A**; **Movie S6**). On the other hand, GFP-TIP delineated vacuolar membranes occasionally protruded into the extreme apex (**Figures 6B–D**; **Movies S7**, **S8**).

DISCUSSION

Previously, we reported that *agd1* and *ark1* had similar root hair and cytoskeletal defects. This suggested that despite predicted differences in the functions of the AGD1 and ARK1 proteins, they likely share common molecular targets in defining root hair growth directionality and polarity (Yoo et al., 2008). It was shown, however, that the vesicle trafficking inhibitor, BFA, completely rescued *agd1* root hair defects but not those of *ark1*. Furthermore, analysis of the root hair phenotypes of double *ark1 agd1* mutants revealed that *agd1* was not epistatic to *ark1* (**Figure 2I**; Yoo et al., 2008). Taken together these results indicate that the pathways where AGD1 and ARK1 function may diverge at certain points along the root hair developmental program (Yoo et al., 2008). To further tease apart the stages of root hair development where ARK1 and AGD1 might share common molecular targets and where they might diverge, we conducted genetic interaction studies and live cell imaging of root hair polarity markers in the *ark1* mutant similar to previous studies with *agd1* (Yoo et al., 2012).

The resulting root hair phenotypes of various *ark1* double mutants and how they compared with the *agd1* double mutants reported previously (Yoo et al., 2012) provided some clues as to points in the root hair developmental network where *ARK1* might diverge from *AGD1*. For example, the predominant morphological defect of *ark1 act2* double mutants was the formation of branches/multiple tips along the entire length of their root hairs. In contrast, *agd1 act2* defects were expressed primarily as the formation of multiple tips at the initiation site (**Figures 1**, **2**; Yoo et al., 2012). These results indicate that ARK1 in conjunction with actin might be involved in the maintenance of polarity throughout root hair development (i.e., from root hair initiation to tip growth). On the other hand, AGD1 is likely to exert its predominant effects on actin-dependent root hair developmental processes during root hair initiation. The notion that *ARK1* might have a broader function in root hair development than *AGD1* is further supported by the observation that like *ark1 act2* double mutants, *ark1 rhd4* root hairs had the tendency to produce an excessive number of tips and bulges that formed along the entire root hair as it elongated. With regard to how *ARK1* might function in concert with *COW1*, the multiple initiation sites of the *ark1 cow1* double mutant were very similar to those observed in *agd1 cow1*. This indicates that ARK1 and AGD1 converge on the Sec14p-like phosphatidylinositol

transfer protein encoded by *COW1* with such interaction impacting mostly the events that occur during root hair initiation.

It is noteworthy that double mutants of *ark1 rhd4* had longer root hairs than *rhd4* single mutants (**Figure 3B**). The longer root hairs could be explained by the obvious absence of ruptured root hairs in *ark1 rhd4* compared to *rhd4* single mutants (**Figures 1M,N**). Earlier reports showed that *rhd4* over-accumulated PI-4P on internal membrane compartments rather than the plasma membrane (Thole et al., 2008), which might contribute to increased delivery of cell wall materials to the tip resulting in bulging and bursting of the root hairs (Galway et al., 1999). *ARK1* could be functioning as a suppressor of *RHD4* in regard to root hair length by redirecting vesicles containing PI-4P that typically over-accumulate in *rhd4* mutants to the newly developing tips of *ark1 rhd4*. As a result excess PI-4P is diluted or retargeted to their correct location on the plasma membrane, preventing premature root hair rupture. It would be interesting to see using PI-4P biosensors (Vermeer et al., 2009) whether the dynamics of PI-4P in *ark1 rhd4* differs from that of *rhd4*. Interestingly, whereas *ARK1* appears to be a suppressor of *RHD4* with regard to root hair length, a lesion in *AGD1* seems to suppress the shorter trichoblast length resulting from a mutation in *RHD4* (**Figure 3C**). Although the mechanisms on how this is accomplished are unknown, our data point to another level of divergence between ARK1 and AGD1 where AGD1 might function in both RHD4-mediated tip and diffuse growth processes.

Whereas AGD1 contains a pleckstrin homology (PH) domain that binds to PIs (Vernoud et al., 2003; Yoo et al., 2012), ARK1 has not been biochemically characterized for PI interactions. However, in a recent study of neuronal axons, which partly mirror root hair tip growth, it was reported that PIPKα is not only involved in PI metabolism but directly promotes microtubule depolymerizing activity of the kinesin, KIF2A (Noda et al., 2012). Although the mechanisms by which ARK1 regulates microtubule organization remain to be elucidated (Zhu and Dixit, 2012), it is tempting to speculate that its activity might also be influenced by components of PI metabolism similar to what has been demonstrated for neurite development. It is possible that actin binding to the armadillo repeat-containing domain of ARK1 (Yang et al., 2007) might somehow be linked to ARK1 crosstalk with PIs. As noted earlier, the similar root hair phenotypes of *ark1 cow1* and *agd1 cow1* indicate that such cross-talk might be accomplished via the PI transfer protein, COW1.

Similar patterns of RabA4B and ROP2 mistargeting in *ark1* root hairs to that shown for *agd1* were observed (**Figures 4**, **5**; **Movies S2**, **S3**, **S5**; Yoo et al., 2012). Such defects could be attributed to common cytoskeletal defects observed in both mutants (Yoo et al., 2008). Furthermore, we found that both *ark1* and *agd1* root hairs mirrored each other in terms of abnormalities in vacuolar membrane organization. It has been reported that the cytoskeleton is important in regulating plant vacuolar structure and dynamics (Higaki et al., 2006; Oda et al., 2009). The spatially close localization of F-actin and vacuolar membrane, and the movement of F-actin along cytoplasmic strands adjacent to large vacuoles, is likely responsible for actin-dependent regulation of vacuolar dynamics. Moreover, it has been demonstrated that actin or microtubule inhibitors induced smaller vacuolar compartments that detached from the larger vacuoles (Higaki et al., 2006; Oda et al., 2009). In *ark1* and *agd1* mutants, the vacuolar membranes continuously formed small compartments at the tip (**Movies S7**, **S8**) suggesting that the disruption in their cytoskeleton parallels that induced by cytoskeletal inhibitors. Taken together, our live cell imaging studies identify additional downstream molecular targets common to ARK1 and AGD1 in the maintenance of root hair polarity.

In summary, our studies revealed that ARK1, an unconventional plant kinesin, is an important component that ties membrane organization to the cytoskeleton during root hair development. Genetic interaction and cell biological data presented here continue to point to an interaction between ARK1 and AGD1 in molecular pathways that modulate tip growth, and such cross-talk occurs in defined steps within the root hair developmental program that involve PI metabolism. A proposed model speculating on how ARK1 and AGD1 might function in root hair development is presented in **Figure 7**. Whereas AGD1 exerts its greatest impact at the early stages of root hair initiation and tip growth, ARK1 has a broader role that covers the entire root hair developmental program.

AUTHOR CONTRIBUTIONS

Cheol-Min Yoo generated double mutants and mutant plants expressing membrane polarity and tonoplast markers. Elison B. Blancaflor conducted live cell imaging of *ark1* root hairs. Cheol-Min Yoo and Elison B. Blancaflor analyzed the data and wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fpls.2013.00528/abstract

Figure S1 | Primary root length of various single and double mutants. Data are means (±*SD*) from primary roots of 24 seedlings. Means with different letters are significantly different as determined by Tukey's HSD test ($P < 0.005$).

Movie S1 | EYFP-RABA4b localization in an elongating wild-type root hair. Total elapsed time of the movie is 1 h.

Movie S2 | EYFP-RABA4b localization in a swelling *ark1* root hair. Total elapsed time of the movie is 1 h.

Movie S3 | EYFP-RABA4b localization in an *ark1* root hair that constantly shifts direction. Total elapsed time of the movie is 1 h.

Movie S4 | EYFP-ROP2 localization in an elongating wild-type root hair. Total elapsed time of the movie is 1 h.

Movie S5 | EYFP-ROP2 localization in a swelling *ark1* root hair. Total elapsed time of movie is 1 h.

Movie S6 | Vacuolar membrane dynamics visualized by GFP-TIP expression in a wild-type root hair. Total elapsed time of movie is 2 min.

Movie S7 | Vacuolar membrane dynamics visualized by GFP-TIP expression in an *ark1* root hair. Total elapsed time of movie is 2 min.

Movie S8 | Vacuolar membrane dynamics visualized by GFP-TIP expression in an *agd1* root hair. Total elapsed time of movie is 2 min.

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The GIP gamma-tubulin complex-associated proteins are involved in nuclear architecture in *Arabidopsis thaliana*

Morgane Batzenschlager[1], Kinda Masoud[1], Natacha Janski[1], Guy Houlné[1], Etienne Herzog[1], Jean-Luc Evrard[1], Nicolas Baumberger[1], Mathieu Erhardt[1], Yves Nominé[2], Bruno Kieffer[3], Anne-Catherine Schmit[1]* and Marie-Edith Chabouté[1]

[1] Centre National de la Recherche Scientifique, Institut de Biologie Moléculaire des Plantes, UPR 2357, Conventionné avec l'Université de Strasbourg, Strasbourg, France
[2] Biotechnologie et Signalisation cellulaire, Institut de Recherche de l'Ecole de Biotechnologie de Strasbourg, UMR 7242, Université de Strasbourg, Illkirch, France
[3] Institut de Génétique et Biologie Moléculaire et Cellulaire, Ecole Supérieure de Biotechnologie de Strasbourg, Illkirch, France

Edited by:
Clément Thomas, Public Research Centre for Health, Luxembourg

Reviewed by:
Susana Moreno Diaz De La Espina, Consejo Superior de Investigaciones Científicas, Spain
Megan C. King, Yale University, USA

***Correspondence:**
Anne-Catherine Schmit, Institut de Biologie Moléculaire des Plantes, UPR2357 du CNRS, 12, rue du Gl Zimmer, 67084 Strasbourg-Cedex, France
e-mail: schmit@unistra.fr

During interphase, the microtubular cytoskeleton of cycling plant cells is organized in both cortical and perinuclear arrays. Perinuclear microtubules (MTs) are nucleated from γ-Tubulin Complexes (γ-TuCs) located at the surface of the nucleus. The molecular mechanisms of γ-TuC association to the nuclear envelope (NE) are currently unknown. The γ-TuC Protein 3 (GCP3)-Interacting Protein 1 (GIP1) is the smallest γ-TuC component identified so far. AtGIP1 and its homologous protein AtGIP2 participate in the localization of active γ-TuCs at interphasic and mitotic MT nucleation sites. Arabidopsis *gip1gip2* mutants are impaired in establishing a fully functional mitotic spindle and exhibit severe developmental defects. In this study, *gip1gip2* knock down mutants were further characterized at the cellular level. In addition to defects in both the localization of γ-TuC core proteins and MT fiber robustness, *gip1gip2* mutants exhibited a severe alteration of the nuclear shape associated with an abnormal distribution of the nuclear pore complexes. Simultaneously, they showed a misorganization of the inner nuclear membrane protein AtSUN1. Furthermore, AtGIP1 was identified as an interacting partner of AtTSA1 which was detected, like the AtGIP proteins, at the NE. These results provide the first evidence for the involvement of a γ-TuC component in both nuclear shaping and NE organization. Functional hypotheses are discussed in order to propose a model for a GIP-dependent nucleo-cytoplasmic continuum.

Keywords: gamma-tubulin complex, AtGIP1/MOZART1, AtTSA1, nuclear envelope, *Arabidopsis thaliana*

INTRODUCTION

The nuclear envelope (NE) is a very specialized structure of eukaryotic cells separating the cytoplasm from the nucleoplasm. It consists of an outer and an inner nuclear membrane (INM) which join together at the sites where protein channels, corresponding to nuclear pore complexes (NPCs), mediate the traffic of macromolecules (Dultz and Ellenberg, 2007). In interphase cells, the ONM forms a continuous membrane system with the rough endoplasmic reticulum (ER), while the INM contains a subset of specific membrane-associated proteins (Dreger et al., 2001; Burke and Ellenberg, 2002). The NE regulates selective exchanges between the nucleoplasm and the cytosol but also provides anchoring sites for chromatin and the cytoskeleton (Hetzer et al., 2005). Indeed, recent data revealed a rather complex interplay between both compartments, not only through NPCs, but also through the establishment of a nucleo-cytoplasmic continuum, linking the cytoskeleton to the nucleoskeleton (Crisp et al., 2006; Ostlund et al., 2009). This continuum is based on the formation of specialized LINC (LInkers of Nucleoskeleton and Cytoskeleton) complexes. In various animal and fungi models, the core units of LINC complexes consist of KASH domain-containing proteins located in the outer nuclear membrane (ONM) and SUN domain proteins located in the INM, interacting with each other in the perinuclear space to form bridges across the NE. On the cytoplasmic side, the KASH domain proteins contact either directly actin filaments or indirectly microtubules (MTs) through motor proteins, such as dyneins and kinesins (Méjat and Misteli, 2010). On the nucleoplasmic side, SUN domain proteins interact with chromatin through lamins, lamin-binding proteins (except in plants and fungi) or specific INM associated proteins, thus organizing the nuclear content (Stuurman et al., 1998; Mekhail and Moazed, 2010).

In animals, interactions between SUN-KASH complexes and the cytoskeleton are largely involved in nuclear positioning, migration and anchorage (Crisp et al., 2006; Méjat and Misteli, 2010). They are also involved in tethering MT-Organizing Centers (MTOCs) to the NE. However, the molecular adaptors linking MTOCs to the ONM are poorly characterized so far. From the zygote stage to the early steps of *C. elegans* embryogenesis, the ZYG-12 KASH protein is recruited at the NE by SUN1/MTF-1, and mutations in the *ZYG-12* gene perturb the coupling between the nucleus and the centrosome (Malone et al., 2003). A connection between ZYG-12, dynein and MTs has been demonstrated, but remains hypothetical for the nuclear attachment of

the centrosome/γ-TuCs (Malone et al., 2003; Zhou et al., 2009). In human fibroblasts, the emerin type II INM protein has also been found located at the ONM, where it plays a crucial role in the association of the centrosome to the outer NE through a MT-dependent mechanism. However, neither the nesprin-1 and nesprin-2 KASH domain proteins nor the SUN1 and SUN2 INM proteins seemed to be involved in this process (Salpingidou et al., 2007). To date, the role of SUN-KASH bridges linking the MTOC/centrosome to the NE remains unknown in mammals.

In plants, besides the NPC components (Tamura et al., 2010), the molecular characterization of NE proteins and their functional importance in a nucleo-cytoplasmic continuum have emerged. The first SUN proteins were characterized in rice, Arabidopsis and maize, respectively, (Moriguchi et al., 2005; Graumann et al., 2010; Murphy et al., 2010). More recently, the first plant KASH proteins were characterized in Arabidopsis and named WIPs (Zhao et al., 2008; Zhou et al., 2012). Arabidopsis WPP domain-interacting proteins (AtWIPs) are three tail-anchored ONM proteins which were shown to interact with Arabidopsis SUN1 and SUN2, thus defining the first LINC complex identified in plants (Zhou et al., 2012). AtWIPs redundantly anchor Arabidopsis RanGTPase-activating protein 1 (AtRanGAP1) to the outer NE (Xu et al., 2007), while mammalian RanGAP is anchored by the nucleoporin RanBP2 to control the trafficking of soluble proteins between the nucleoplasm and the cytoplasm (Mahajan et al., 1997). Interestingly, the lack of either AtSUN (Oda and Fukuda, 2011) or AtWIP proteins (Zhou et al., 2012) has only led to moderate phenotypes at the level of nuclear morphology in specific cell types, and no developmental or fertility defects have been observed. The first characterization of SUN-KASH interactions in plants indicates that, although LINC complexes are conserved among eukaryotes, they may have, at least partly, functionally diverged.

Until now, no relationship - neither with chromatin nor with γ-TuCs-has been established at the NE for a plant LINC complex. One of the functions of the NE is to act as an MTOC in the plant acentrosomal nucleation system (Stoppin et al., 1994; Canaday et al., 2000). Although γ-TuC components were shown to be recruited at the nuclear surface (Seltzer et al., 2007), their anchoring partners at the NE remain completely unknown. GIP proteins (AtGIP1 and AtGIP2) were discovered in Arabidopsis as partners of GCP3, one of the γ-TuC core subunits (Janski et al., 2008, 2012; Nakamura et al., 2012). AtGIPs localize to both cortical (Nakamura et al., 2012) and mitotic MT arrays and are required for proper γ-TuC localization, spindle integrity, and chromosomal segregation (Janski et al., 2012). The phenotype of *gip1gip2* knock down Arabidopsis mutants is severe and the complete KO is lethal (Nakamura et al., 2012), highlighting the functional importance of GIP proteins in Arabidopsis. These small proteins (around 7-8 kDa) are conserved among eukaryotes but have only been characterized in human (Hutchins et al., 2010) and more recently in *S. pombe* (Dhani et al., 2013; Masuda et al., 2013).

Here we present an exhaustive comparison of GIP proteins in unicellular and multicellular organisms and propose a structural model for Arabidopsis GIPs, partly validated by circular dichroism (CD). Beside their localization at MT mitotic arrays in dividing cells, AtGIPs are also present at the NE during interphase as a dotted pattern (Janski et al., 2012). At the subcellular level, we observed enlarged and deformed nuclei in the *gip1gip2* root tips. Such alterations of the nuclear shape were also observed in other tissues (in undifferentiated and differentiated cells) and were corroborated by a mislocalization of the AtSUN1 INM marker and a misdistribution of NPCs. A novel AtGIP1-interacting partner, AtTSA1, was identified through a yeast two-hybrid screen. Both AtGIP1 and AtTSA1 localized at the NE in Arabidopsis root tip cells. These results point out an important role played by AtGIP1, and possibly its associated proteins, in the nucleo-cytoplasmic interface organization and functions.

MATERIALS AND METHODS

PLANT MATERIAL AND GROWTH CONDITIONS

Arabidopsis transformation was performed using the floral dipping method (Clough and Bent, 1998) and the *Agrobacterium tumefaciens* strain GV3101 to produce GIP1::AtGIP1-GFP, GIP2::AtGIP2-GFP, 35S::AtSUN1-YFP, and 35S::AtTSA1-RFP transgenic lines. 35S::AtGIP1-GFP and 35S::AtGIP2-GFP Arabidopsis lines have been described previously (Janski et al., 2012). 35S::EYFP-AtCENH3 lines were described by Lermontova et al. (2006).

Wild-type and transgenic Arabidopsis lines were grown *in vitro* on Murashige and Skoog medium (SERVA Electrophoresis GmbH, Heidelberg, Germany) at 20°C in 12 h per day of 70 μmol m^{-2} s^{-1} fluorescent lighting, subcultured on soil and transferred to a growth chamber with light/dark cycles of 16h/8h for 2 weeks and then to the greenhouse.

CONSTRUCTION OF RECOMBINANT PLASMIDS

Arabidopsis GIP1 and TSA1 cDNA and genomic fragments were generated by PCR using primers derived from the gene sequence (**Table S1**).

To express AtGIP1 in *Escherichia coli*, the AtGIP1 cDNA was cloned into the pET102D vector (*Invitrogen*™ Life Technologies, Saint Aubin, France). The sequences encoding thioredoxin and the 8 N-terminal AtGIP1 amino acids were deleted, and a sequence corresponding to a hexa-histine tag was introduced at the 5′ of the AtGIP1 coding sequence, by PCR-based directed mutagenesis, to obtain pET(His)6AtGIP1.

The AtGIP1 cDNA was fused in-frame to the Gal4 DNA binding domain in the yeast vector pAS2ΔΔ (Institut Pasteur, Paris, France). Other yeast recombinant vectors, used in this study, have been described by Janski et al. (2008; 2012).

AtGIP1 or AtGIP2 promoter and coding sequences were cloned into pDONR™207 and transferred into the plant expression vector pGWB604 or pMDC107, respectively, by performing an LR reaction according to the manufacturer's protocol (Gateway® Technology Manual - *Invitrogen*™), to express GFP fusion proteins.

AtTSA1 (*At1g52410.1*) cDNA in pDTSA1 (derived from pDONR™201-kindly provided by Pr. Takamasa Suzuki, Nagoya University, Japan) was introduced into the binary vector pH7RWG2 (Karimi et al., 2002), by performing an LR reaction according to the manufacturer's protocol (Gateway® Technology

Manual-*Invitrogen*™), to express the AtTSA1-mRFP fusion. 35S::AtSUN1-YFP was cloned into pCAMBIA1300 as described previously by Zhou et al. (2012).

EXPRESSION AND PURIFICATION OF RECOMBINANT PROTEINS

pET(His)6AtGIP1 was transformed into BL21(DE3) *E. coli* strain and expression was performed in an auto-inducing medium (Studier, 2005) at 20°C for 65 h. Cells were harvested by centrifugation and lysed in Tris 50 mM pH 8, NaCl 300 mM, glycerol 5%, urea 8 M, DTT 5 mM, Tween-20 0.1% by sonication. Lysate was clarified by centrifugation at 15000 *g* for 20 min and the protein purified by binding onto Ni-agarose resin (Protino NiNTA Macherey-Nagel, Hoerd, France). The purified protein was refolded by pulse dilution in Na phosphate buffer 10 mM pH 7.4, NaCl 25 mM, and 1 mg/ml of NVoy polymer (Expedeon, Harston, UK), followed by an incubation at 4°C for 16 h and 5 h at 25°C. Aggregated proteins were removed by centrifugation at 100 000 *g*, 1 h at 4°C. Refolded proteins were analyzed on SDS-PAGE gel and quantified by Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as standard.

CIRCULAR DICHROISM

CD experiments were recorded on a Jasco J-815 spectropolarimeter (Easton, MD, USA) equipped with an automatic 6-position Peltier thermostated cell holder. The instrument was calibrated with 10-camphorsulphonic acid. Far-UV CD data were collected in the 182-260 nm range using a 0.1 mm pathlength cell (Quartz-Suprasil, Hellma UK Ltd, Southend on Sea, UK) at 25°C ± 0.1°C. Spectra were acquired using a continuous scan rate of 100 nm/min and were presented as an average of 25 successive scans. The response time and the bandwidth were 0.5 sec and 1 nm, respectively. The absorbances of the sample (at a concentration of 28 μM) and buffer were kept as low as possible. Spectra were obtained in 10 mM sodium phosphate buffer at pH 7.4 with 25 mM NaCl and 1mg/ml of Nvoy polymer. The spectrum was corrected by subtracting the solvent spectrum obtained under identical conditions. The secondary structure was estimated with the CDPro suite software (Sreerama and Woody, 2000). A far UV circular dichroism spectrum was used as described (Kelly et al., 2005). The spectrum of the AtGIP1 construct has been deposited in the Protein Circular Dichroism Data Bank (Whitmore et al., 2011) (http://pcddb.cryst.bbk.ac.uk) with the accession code CD0004243000 for release upon publication.

YEAST TWO-HYBRID ASSAYS

The CG1945 haploid Mat a yeast strain was transformed with the pAS2ΔΔ-AtGIP1 recombinant plasmid. The screening of an *A. thaliana* cDNA library (Clontech, Saint-Germain-en-Laye, France) was carried out as described by Janski et al. (2008). Candidate yeast colonies were selected on a minimal medium lacking histidine, tryptophan and leucine, and supplemented with 20 mM of 3-amino-1, 2, 4-triazole (3-AT). Prey plasmids were isolated from positive colonies, reintroduced into the AH109 yeast strain and re-assayed for growth on selective media, using various stringency, according to the Clontech Matchmaker user manual.

TEM AND IMMUNOLOCALIZATION

Root tissue samples were taken from 9-day old Arabidopsis seedlings, fixed overnight in 1.5% glutaraldehyde, 2 h post-fixed with 0.1% (v/v) osmium tetroxyde in 150 mM phosphate buffer and stained overnight with 2% uranyl acetate (modified Seltzer et al., 2007). Samples were dehydrated through an ethanol series, then infiltrated with EPON 812 medium grade resin (Polysciences Inc, Eppelheim, Germany) and polymerized for 48 h at 60°C. Ultrathin sections (70 nm) were cut using an ultracut E microtome (Reichert, Wien, Austria) and collected on grids coated with formvar (Electron Microscopy Science, Fort Washington, PA, USA). Immunolocalization of AtGIP-GFP fusion proteins was performed by incubating the sections with a rabbit anti-GFP antibody (A11122, *Invitrogen*™) diluted 1/500 in 1% bovine serum albumin in phosphate buffer saline (PBS) for 2 h at room temperature. After repeated washing with PBS, the sections were then incubated for 2 h with goat-anti-rabbit antibodies coupled to 15 nm colloidal gold particles (Aurion EM Reagents, Wageningen, The Netherlands) followed by washing with PBS and H_2O. Samples were visualized with a Hitachi H-600 electron microscope operating at 75 kV and Images were captured with a CCD Advantage HR Hamamatsu camera and AMT software (Advanced Microscopy Techniques, Danvers, MA, USA).

IMMUNOLOCALIZATION ON ROOT TIP CELLS

Arabidopsis seedlings were fixed for 40min in 1.5% paraformaldehyde and 0.5% glutaraldehyde in PEMT buffer (50 mM PIPES, 2 mM EGTA, 2 mM MgSO4, 0.05% Triton X-100, pH 7.2), and then treated as described by Erhardt et al. (2002). Unspecific binding was blocked by incubation in PBS containing 2% Bovine serum albumin and 0.1% Triton X-100 (IF buffer) for 10 min. Primary and secondary antibodies were diluted in IF buffer and incubated for 1h at room temperature. The primary antibodies used in this study were the rabbit polyclonal anti-AtGIP1 (1/1000) (Janski et al., 2012), monoclonal anti-α-tubulin (clone DM1A, Sigma-Aldrich, St Louis, MO, USA) (1/6000), and polyclonal anti-AtSUN1 (1/1000) (kindly provided by D. Evans). Alexa 488- and Alexa 568-conjugated goat anti-rabbit IgG and goat anti-mouse IgG secondary antibodies (1:300) were purchased from Molecular Probes® (Life Technologies, Saint Aubin, France). DNA was stained using 0.1 μg/mL 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI).

FLUORESCENCE MICROSCOPY

The fluorescence of AtGIP1-GFP, AtSUN1-YFP, and AtTSA1-RFP was visualized on living seedlings mounted in water. Seedlings and immunostained cells were observed with a Zeiss LSM 780 confocal microscope in multitracking mode which is able to specifically discriminate each fluorochrome signature (Carl Zeiss AG, Le Pecq, France).

ACCESSION NUMBERS

Sequence data from this study can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: Arabidopsis (At) GIP1: At4g09550;

AtGIP2: At1g73790; AtTSA1: At1g52410; AtNAI2: At3g15950; At3g15960; AtSUN1: At5g04990. Accession numbers of the sequences used for establishing the phylogenetic tree of GIP peptidic sequences (**Figure 1**) are presented in **Table S2**. CD data deposit on the accession number CD0004243000.

RESULTS

AtGIPs ARE α-HELIX PROTEINS CONSERVED IN UNICELLULAR AND MULTICELLULAR ORGANISMS

AtGIPs were previously identified as partners of AtGCP3, a member of the γ-TuC (Janski et al., 2008). These small proteins have

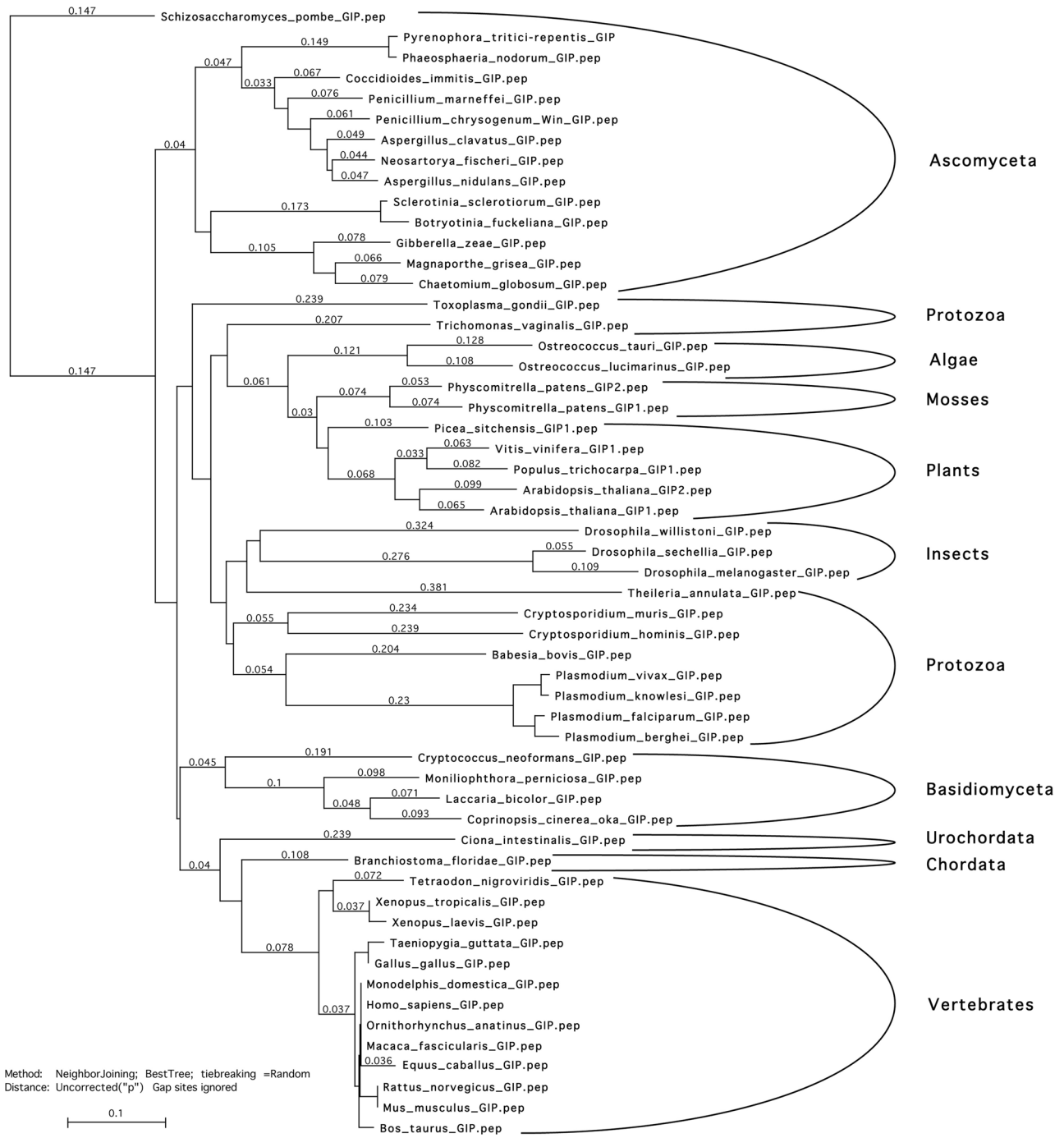

FIGURE 1 | Phylogenetic tree of GIP peptidic sequences. Sequences were aligned using *T*-coffee and Myers-Miller matrix. A tree was constructed using the neighbor joining algorithm. Analyses were performed using the MacVector software. GIP sequences segregate accordingly to the genus of life evolution. This suggests that GIPs probably originate from one and the same ancestor and that they are not the products of a convergent evolution that would result in a mix of the sequences. Accessions numbers of the used sequences are presented in **Table S2**.

been identified in numerous organisms, as shown in **Figure 1** and **Table S2**. The following sequence-[MILVT] xxx[LV] xxx [LIVT]-[DTS] xxx [LIFVM]xx[CLIAVF]-[ILVME]-x [LMFVA]xxxGxx [PGA], xx [LIV]-[VIAS]x [VILAM]-[VIFLG]-has been established as a GIP signature. Used in database screens, it allowed us to identify GIPs in a large variety of eukaryote species, except *S. cerevisae*. In the plant kingdom, a GIP gene duplication occurrence was linked to the conquest of terrestrial habitats. AtGIP1, used as a reference, shares 41 to 78% identity with the 115 plant GIP sequences analyzed so far. Aminoacid similarities were mapped between AtGIP1 and AtGIP2 or human GIP (**Figure 2A**). GIPs do not harbor any characterized consensus functional domains, except a glycine (**Figure 2A**, in bold) and repeated leucines, mostly conserved throughout evolution (**Figure 2A**, stars).

In order to determine the AtGIP1 secondary structure content, we recorded a far UV circular dichroism spectrum. The secondary structure was estimated with the CDPro suite software using CD data of the SP37 reference database (a base of 37 soluble proteins of known secondary structure elements) and the CONTIN method. CONTIN fits the CD of the GIP protein by comparison with a linear combination of the spectra from a large database of proteins with known conformations. This led to 82% for α-helices, 4% for β-sheets and 14% for other secondary structure contents. These results indicate that α-helices mainly constitute AtGIP1 (**Figure 2B**). Accordingly, computer modeling predicts that the AtGIP1 3D structure is composed of three α-helices (**Figure 2C**). Interestingly, the conserved glycine residue (aa 42 in AtGIPs), found in all organisms except *S. pombe*, may constitute a glycine elbow needed for flexibility in the relative positioning of the three α-helices within the predicted 3D structure (**Figure 2C**).

These sequence analyses emphasize the wide conservation of GIP proteins from fungi to humans, with the presence of a specific sequence signature, which very likely reveals their biological relevance in eukaryotic cells.

FUNCTIONS OF AtGIPs AT THE NE

Previous localization studies have shown that AtGIP1-GFP is present in the cytoplasm, the nucleoplasm and at the NE in interphase cells (Janski et al., 2012).

To further investigate the role of AtGIPs at the NE, morphological modifications of the nucleus were analyzed in the *gip1gip2* Arabidopsis mutants. Our observations revealed a drastic discrepancy in the size and shape of the mutant nuclei compared to WT (**Figures 3A,F**). Differences in size are likely due to the increase of ploidy previously described by FACS analyses (Janski et al., 2012). The difference in shape corresponds to a dramatic shift from a roundish shape to lobulated and highly dented nuclei. Using transmission electron microscopy (TEM) analyses, the NE of *gip1gip2* nuclei was shown to be deeply invaginated with protrusion compared to WT (**Figures 3K,L**). This phenotype was not limited to dividing cells (meristematic root cells, young leaves) but was also observed in various differentiated cells (mature cotyledon, petal), (**Figures 3C–E**, **H–J**). The quantification of these abnormalities was determined in root tips, where more than 70 % of the nuclei showed irregular shaping (**Figure 3G**). As NE defects may also affect NPC density, we investigated the NPC distribution using TEM in WT Arabidopsis and *gip1gip2* mutant root tips. In the NE of WT nuclei, the NPCs were evenly distributed and circular (**Figure 4A**). In comparison to WT nuclei in which the mean distance between two adjacent NPCs is of about 90 nm, this distance drops below 60 nm in the mutants. In about 33% of

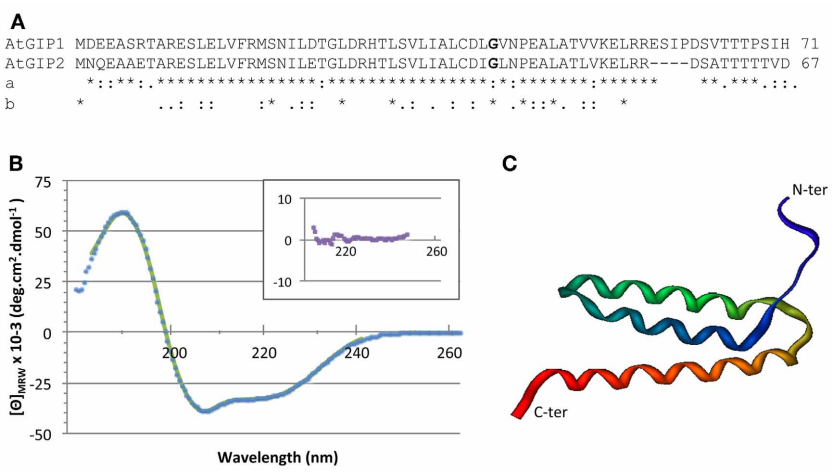

FIGURE 2 | GIP sequences and three dimensional structure.
(A) Arabidopsis GIP1 and GIP2 sequence alignment. Comparison **(a)** between both Arabidopsis sequences and **(b)** between AtGIP1 and other eukaryotes. (∗) identical aminoacids, (:) similar aa, (.) semi-conserved aa. The conserved glycine residue (aa 42) is shown in bold print. **(B)** The structural integrity of AtGIP1 protein was analyzed using circular dichroism spectroscopy. Spectra were recorded at 25° C using recombinant AtGIP1 solution at 28 μM in 10 mM sodium phosphate buffer (pH 7.4), complemented with 25 mM NaCl and 1 mg/ml of Nvoy polymer. The signal is expressed in mean residue ellipticity (deg.cm^2.dmol^{-1}). Experimental data and fitting results are shown in dots and solid line, respectively. Inset shows the residuals using an expanded y-axis to better display the random distribution. **(C)** An AtGIP protein model generated with the LOMETS online server (http://zhanglab.ccmb.med.umich.edu/LOMETS/) and the methods of LOMETS and MODELLER v9.3 (Wu and Zhang, 2007) in a fully automated procedure. The predicted AtGIP1 3D structure is composed of 3 α-helices (aa 9–20; aa 25–40; aa 45–61).

gip1gip2 nuclei ($n = 400$ p-value < 0.01) the NPC shape was altered (**Figures 4B,E**). In mutants, 17% of NPCs were separated by 0 to 30 nm only (**Figure 4C**) and some of them were fused together, indicating spacing abnormalities (white arrows in **Figure 4B**). The number of NPCs was evaluated in an total area of 2 μm^2 (**Figure 4D**). It shows an increase of 1.75 times in the mutant. This raises the question of AtGIP location at the NP. We therefore investigated the localization of AtGIP1-GFP and AtGIP2-GFP in transgenic Arabidopsis lines, using TEM and immunogold labeling using anti-GFP antibodies. Both fusion proteins were found at the NE, within and /or closely associated with the NPC (17% and 7% of gold particles for

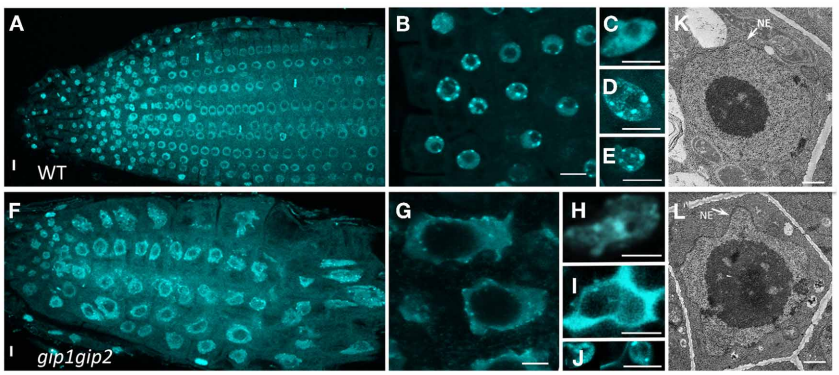

FIGURE 3 | Nuclear shape and DNA labeling in WT (A–E) and *gip1gip2* mutants (F–J) using DAPI staining. (A,F) General view of a root tip. **(B,G)** Enlarged view of root tip meristematic cells. **(C,H)** Cotyledon nuclei. **(D,I)** Leaf nuclei. **(E,J)** Petal nuclei. The nuclei of *gip1gip2* mutant cells exhibit an increased size and are highly deformed. Bars = 5 μm. **(K,L)** TEM performed on WT and *gip1gip2* mutant root tip seedlings, respectively, showing NE deformations in the mutant **(L,** arrow**)**. Bars = 500 nm.

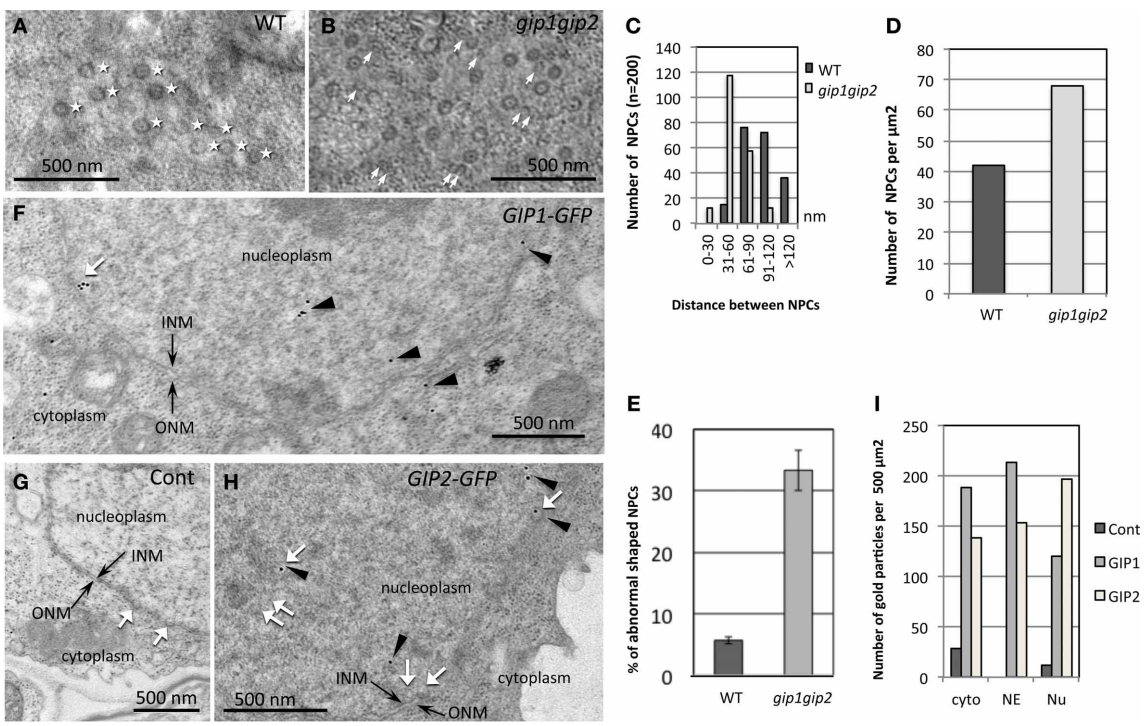

FIGURE 4 | Modification of NPC distribution in *gip1gip2* mutants and AtGIP distribution in cells expressing AtGIP-GFP constructs. (A,B) Tangential views of the nuclear surface in TEM with NPC repartition (white stars) which is regular in WT **(A)**. Heterogenous distribution of NPCs and abnormal shaped NPCs (white arrows) in *gip1gip2* mutants **(B)**. **(C)** Analysis of the distances between NPCs in WT and mutants (p-value < 0.001). **(D)** Quantification of NPC number per μm^2 in WT and mutants. **(E)** Quantification of abnormal NPCs. **(F–H)** Immunolabeling using anti-GFP antibodies (arrowheads) on cells expressing AtGIP1-GFP **(F)** or AtGIP2-GFP **(H)** and WT control cells **(G)**. **(I)** The quantification of gold particles was performed on 50 images corresponding to 3 independent experiments. Control cells which do not express a GFP fusion protein were labeled in parallel. Counting was performed in different subcellular compartments (Cytoplasm: cyto, NE and Nucleoplasm: Nu). Bars = 500 nm.

AtGIP1 and AtGIP2, respectively), and were also located in the nucleoplasm and the cytoplasm (**Figures 4F,H**, arrowheads and **I**). As expected, very few aspecific signals were observed in control cells (**Figures 4G,I**). These results confirm our previous observation of GIP-GFP distribution in fluorescence (Janski et al., 2012).

Alterations of the NE may be correlated to nuclear membrane protein reorganization. We have investigated whether the INM marker AtSUN1 was distributed differently in *gip1gip2* mutants and in WT nuclei. To this end, we introgressed the 35S::AtSUN1-YFP construct into a *gip1gip2* background. As expected, AtSUN1-YFP labeling first confirmed dramatic abnormally shaped nuclei in the mutants, compared to those observed in WT (**Figures 5A,B**). It secondly revealed an uneven AtSUN1 distribution through variability of fluorescence density at the membrane. Using an immunocytochemical approach with an antibody directed against AtSUN1, a similar phenotype on root tip nuclei was revealed in *gip1gip2* mutants, where AtSUN1 clusters appeared accumulated irregularly at the NE (**Figure 5G**, arrows). In WT cells, on the contrary, AtSUN1 was distributed uniformly at the INM, often with highly fluorescent dots where MTs emerged (**Figures 5C–E**).

AtGIP1-GFP localization as a spotty pattern at the NE (Janski et al., 2012) may be correlated to the MT nucleation sites dispersed at the nuclear surface. To further investigate the localization of AtGIP at the nucleo-cytoplasmic interface, the root tips of AtGIP1-GFP Arabidopsis seedlings were immunolabeled with anti-α-tubulin antibodies and incubated with DAPI. The observations of interphase cells revealed an AtGIP1-GFP dotted localization at the minus ends of some perinuclear MTs (**Figure S1**). Such dotted structures were, in certain cases, located close to bright DAPI stained heterochromatin. Interestingly, in root tip cells of an Arabidopsis line expressing the CENH3 centromeric histone fused to EYFP, some MT minus ends were also close to the EYFP-AtCENH3 signals observed at the nuclear periphery on chromocenters.

Our observations converge to a significant role of AtGIPs in both NE shaping and architecture, in addition to their function as γ-TuC components.

AtGIP1 INTERACTS WITH AtTSA1, A NE AND ER-LOCATED PROTEIN

The full-length AtGIP1 protein was used as a bait to screen a three-week-old Arabidopsis cDNA library in a yeast two-hybrid approach. Twenty-eight clones, positive for β-galactosidase activity, contained the coding sequence of *TonSoKu (TSK)-associating protein 1* (*TSA1*)/*At1g52410* gene with varying lengths toward the 5′-terminus. The *AtTSA1* (*At1g52410.1)* gene encodes a 755-amino acid protein (**Figures 6A,B**). Two homologous genes were found in Arabidopsis, NAI2 *(At3g15950)* and the At3g15960 protein (**Figure S2**). Yeast co-transformation was performed with rescued library plasmids to confirm the interaction between AtGIP1 and AtTSA1 under nutritional selection. The growth of yeast cells harboring the positive clones, coding for various AtTSA1 C-terminal domains (D1 to D4, **Figures 6A,B**), is shown in **Figure 6C**.

Yeast cells expressing the D1 to D4 C-terminal regions of AtTSA1 grew efficiently in the presence of AtGIP1 under nutritional selection, confirming the interaction between the two proteins in a yeast two-hybrid system (**Figure 6C**). The minimal domain of AtTSA1 interacting with AtGIP1 corresponds to the C-terminal part (123 last aminoacids) of the protein (D4, **Figures 6A,B**). This domain partially overlaps the AtTSA1 domain interacting with AtTSK (aa 616-707; Suzuki et al., 2005). The D4 minimal AtTSA1 domain was also shown to strongly interact with AtGIP2 in a yeast growth assay carried out on selective media of increasing stringency (**Figure S3**).

In order to study the relationship between AtGIP1 and AtTSA1 *in vivo*, the subcellular localization of AtTSA1 was investigated using stable Arabidopsis transgenic lines expressing the 35S::AtTSA1-RFP construct. In root interphase cells displaying a moderate fluorescence signal, AtTSA1-RFP was found at the NE and in the form of ER body-like structures (**Figures 7A,B,E,H**).

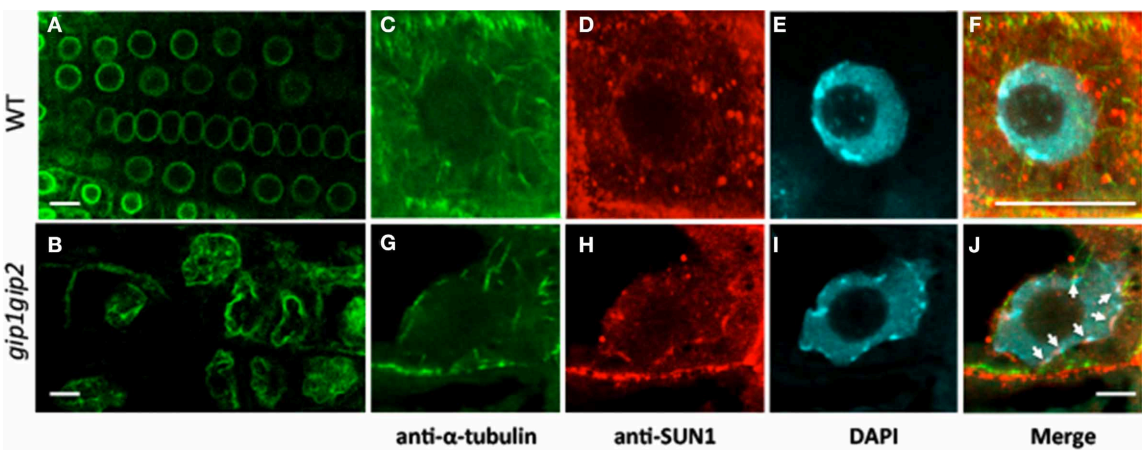

FIGURE 5 | Modification of the NE shape and organization. AtSUN1-YFP reveals the INM when expressed in WT Arabidopsis **(A)** and *gip1gip2* mutants **(B)**. Note the altered nuclear shape with lobulations, and mislocalization of AtSUN1 in the nucleoplasm of mutant cells. Immunostaining of endogenous AtSUN1 **(D,H)** compared with MTs **(C,G)** and DAPI-stained DNA **(E,I)** in WT and *gip1gip2* root nuclei, respectively. Corresponding merged pictures **(F,J)** show a disrupted perinuclear localization of AtSUN1 in *gip1gip2* mutants **(J**, arrows**)** compared to WT **(F)**. Bars = 10 μm.

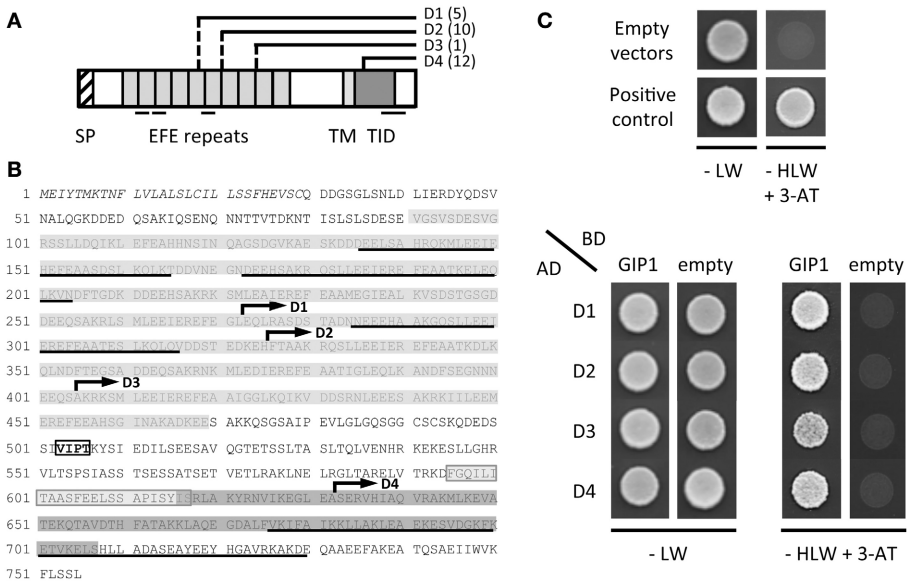

FIGURE 6 | AtGIP1 interacts with C-terminal domains of AtTSA1 in a yeast two-hybrid system. (A) Schematic representation of AtTSA1 protein and corresponding C-terminal domains identified by yeast two-hybrid screening. The features of AtTSA1 protein have been initially described in Suzuki et al. (2005). SP, signal peptide; EFE repeats, multimerization and calcium-binding repeat sequence; TM, putative transmembrane domain; TID, TSK-interacting domain. Several coiled-coil motifs predicted by the Paircoil2 software (McDonnell et al., 2006) are also underlined. D1 to D4 regions correspond to the C-terminal portion of AtTSA1 with varying lengths toward the N-terminus (depicted by solid and dashed lines). Corresponding cDNAs have been repeatedly identified by yeast two-hybrid screening using AtGIP1 as a bait (the number of positive clones identified for each domain is indicated in brackets). **(B)** AtTSA1 amino acid sequence. Features of the protein represented in **(A)** are reported on AtTSA1 primary sequence as follows: SP (italics), EFE repeats (gray), TM (light gray), TID (dark gray), coiled-coil motifs (underlined). D1 to D4 domains are indicated by an arrow starting from the first corresponding amino acid (D1: 273 to 755; D2: 326 to 755; D3: 405 to 755; D4: 633 to 755). VIPT motif (boxed text): putative NE localization signal (Zhou et al., 2012; see text). **(C)** Interaction of AtGIP1 with AtTSA1 domains in a yeast two-hybrid assay. AH109 cells co-transformed with bait (Gal4 Binding Domain, BD) and prey (Gal4 Activation Domain, AD) recombinant plasmids were spotted directly on control (−LW) and selection (−HLW + 3-AminoTriazole, 3-AT) plates and grown for 2 days at 30°C. Upper panel: negative (empty vectors) and positive (pBWRepA/pGADRb1; Xie et al., 1996) interaction controls. Lower panel: interaction test. Transformants coding for D1 to D4 C-terminal regions of AtTSA1 grow in the presence of AtGIP1 on selective medium, indicating an interaction between the different proteins.

Interestingly, AtTSA1-RFP showed a similar pattern of NE localization compared to that of AtGIP1-GFP (**Figures 7C,D,G**). We then introgressed the 35S::AtTSA1-RFP construct in AtGIP1-GFP lines. Such transgenic plants expressing AtTSA1-RFP and AtGIP1-GFP displayed a colocalization of both proteins at specific areas of the NE in interphase root cells. (**Figures 7F,I**).

In conclusion, AtTSA1 was identified as a possible AtGIP-interacting partner at the NE, suggesting the participation of both proteins in structural links between the NE and the cytoskeleton.

DISCUSSION

AtGIP STRUCTURE AND FUNCTIONAL MODELING

Analyses using CD (**Figure 2**) indicate that AtGIP1 is mainly formed by α-helices. Furthermore, computer modeling predicts that AtGIP1 may adopt a 3D structure composed of three central α-helices, leaving the N- and C-terminal domains less structured. Such a conformation could be assumed by other eukaryotic GIPs, considering the high degree of conservation of their central domain. In addition, a conserved putative glycine elbow in AtGIP1 may be of particular importance in modulating the 3D positioning of α-helices and in establishing interactions with partners such as AtGCP3, AtTSA1, and/or AtGIPs themselves. It must be noted that, AtGIP1-AtGIP1 and AtGIP1-AtGIP2 interactions were detected using a sensitive β-galactosidase lift assay, indicating possible homo- and heterotypic interactions between AtGIP proteins (**Figure S4**). AtGIP1 multimers were also detected during recombinant protein purification (data not shown), data which are in agreement with the oligomeric species described for MZT1/Tam4, the GIP1 homolog of *S. pombe* (Dhani et al., 2013). In fission yeast, it was speculated that the oligomerization of MZT1 might regulate the γ-TuC activity. Interestingly, AtGIPs (Janski et al., 2012) and MZT1/Tam4 (Dhani et al., 2013) interact with the N-terminal region of GCP3, arguing in favor of a role of GIPs as modulators of GCP3 activity. Due to their small size and multimerization propensity, GIPs could act as efficient adaptors of γ-TuCs.

FUNCTION OF MICROTUBULAR/MTOC-ASSOCIATED AtGIP1

AtGIPs exhibit a punctate localization at the NE (**Figure 7**), resembling that of AtGCP3, a core component of the γ-TuC (Seltzer et al., 2007). NE localization of GIP/MZT1/Tam4 was also observed in *S. pombe*, with remnant MTs often found at the outer nuclear periphery (Masuda et al., 2013). This suggests that GIPs may be recruited at the NE as part of γ-TuCs. As fewer γ-TuC components (GCP4, GCP3 and γ-tubulin) were recruited to MT nucleation sites (nuclear surface, spindle) in

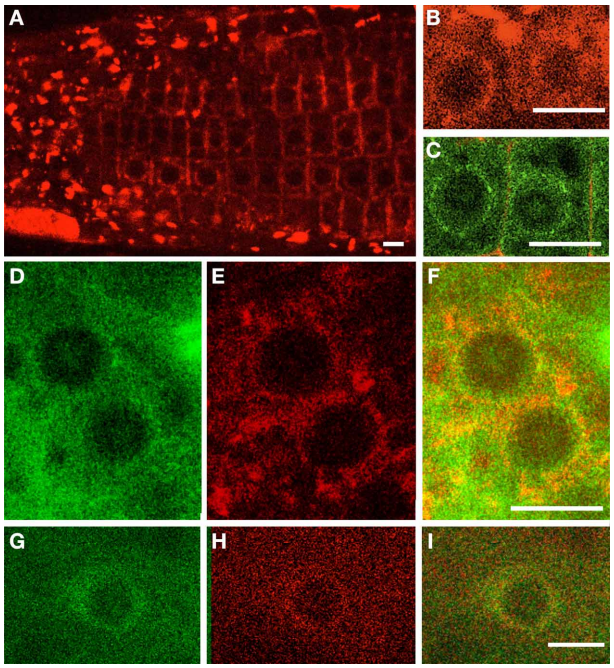

FIGURE 7 | Distribution of AtGIP and AtTSA1 in Arabidopsis root cells. 35S::AtTSA1-RFP **(A,B)** and 35S::AtGIP1-GFP **(C)** constructs were expressed independently or simultaneously **(D–I)** in Arabidopsis rootlets. Individual pictures of AtGIP1-GFP **(D,G)**, AtTSA1-RFP **(E,H)** localization are merged **(F,I)**. AtTSA1 localizes in ER body-like structures and at the nuclear periphery **(A,B,E,H)**. AtGIPs and AtTSA1 proteins localize at the NE. Colocalization is observed on the merged images **(F,I)**. Bars = 10 μm.

gip1gip2 mutants (Janski et al., 2012), AtGIPs may be crucial for anchoring γ-TuCs at MTOC sites, as was recently suggested in *S. pombe* (Masuda et al., 2013). The observations of AtGIP1-GFP at the minus end of perinuclear MTs reinforce such a hypothesis (**Figure S1**). In addition, AtGIP1 localization was close to NE underlying chromocenters. In Arabidopsis, chromocenters mainly consist of heterochromatin, previously described as associated with centromeres (Fransz et al., 2002; Soppe et al., 2002). Such observations suggest a possible mechanism in which a redistribution of MT forces could be involved in centromere mobility in the nucleoplasm, until they reach the nuclear periphery as observed in **Figure S1**. This would anticipate the further access of kinetochores/centromeres to MTs after NE breakdown, allowing rapid spindle building. Indeed, in higher plant prophase cells, pro-spindle MTs exert pushing forces which often leads to MT-loaded polar NE invaginations (Bajer and Mole-Bajer, 1969; Dixit and Cyr, 2002). These MTs often directly catch kinetochores at prometaphase onset, building kinetochore fibers. The intranuclear centromere mobility may therefore be linked to MT reorganization during G2 phase through the establishment of a GIP-dependent nucleo-cytoplasmic continuum.

INVOLVEMENT OF AtGIPs IN NUCLEAR SHAPING AND ORGANIZATION

Our understanding of the mechanisms determining the nuclear shape and size is still unclear. Intriguingly, *gip1gip2* mutant cells showed abnormal shaped nuclei (**Figure 3**) as observed in human diseases, such as envelopathies and cancers (Worman et al., 2010; Starr, 2012), In plants, the nuclear morphology varies from circular or slightly oval to spindle shaped, according to cell type and tissue specificities. Recently, the myosin XI-i motor protein was identified as a molecular adaptor between the actin cytoskeleton and the WIT-WIP-SUN bridge in Arabidopsis (Tamura et al., 2013). Contrary to the dramatic nuclear phenotypes observed in all tissues of *gip1gip2* mutants, *sun1, wip*, and *myosin XI-i* mutants led to only mild changes in the nuclear morphology of differentiated tissues, switching from the elongated shape to more roundish nuclei (Zhou et al., 2012; Tamura et al., 2013). These data suggest that AtGIPs are involved in specific complexes which may connect MT nucleation complexes to integral NE components. However, as the altered nuclear phenotypes cannot exclusively be explained by disrupted MT connections, plant GIPs may be involved in other functions, directly or indirectly linked to the NE environment. Indeed, the chromocenter size was disturbed in the *gip1gip2* mutant (see **Figure 3**) and GIPs were also found in the nucleoplasm close to heterochromatin (**Figure 4**).

Therefore, despite a role of AtGIPs at the ONM for helping γ-TuC anchoring, we cannot exclude a role of AtGIPs at the INM after diffusion through the nuclear pores, considering their small size. At the INM of human nuclei, SUN proteins connect chromatin through interactions with lamins and/or lamin-associated proteins of the lamina (Méjat and Misteli, 2010). In plants, despite the existence of a lamina-like structure (plamina) with NMCP/LINC components described as lamin-like proteins (Dittmer et al., 2007; Fiserova et al., 2009; Moreno Diaz de la Espina, 2009; Ciska and Moreno Diaz de la Espina, 2013), the interaction of NMCP/LINC with chromatin has not been established so far. Even though preliminary results suggest SUN/NMCP interactions, a SUN link with chromatin remains to be investigated (Graumann et al., 2013). However, NMCP/LINC, SUN, and WIP functional analyses reveal their involvement in maintaining the nuclear size and shape in differentiated cells (Dittmer et al., 2007; Oda and Fukuda, 2011; Zhou et al., 2012).

As AtGIPs are found at the NE close to NPCs (**Figure 5**), and considering the altered NPC and AtSUN1 distribution in *gip1gip2* mutants, a structural relationship between the plant SUN1, nucleoporins and GIPs probably exists. Indeed, *gip1gip2* mutants share developmental phenotypes (Janski et al., 2012) with nucleoporin mutants, such as dwarfism, short roots and sterility (Parry, 2013), while *sun1* mutants remain fertile (Zhou et al., 2012).

Altogether our data suggest the implication of AtGIPs in nuclear shaping and size control. The pleiotropic developmental defects observed in *gip1gip2* mutants (Janski et al., 2012) may reflect deep changes in developmental programmes, probably linked to modifications in the spatial regulation of the nuclear architecture.

AtGIPs-AtTSA1 COMPLEXES

AtTSA1 is structurally related to two proteins, At*NAI2 (At3g15950)*, an ER body component and the At3g15960 protein, predicted to be involved in DNA mismatch repair (Yamada et al., 2008). Interestingly, even though the three proteins share a highly conserved C-terminal domain (**Figure S2**), only AtTSA1 was identified in the conditions of our yeast

two-hybrid screen. The N-terminal half of AtTSA1 is composed of 10 repeats of an EFE motif, as described by Suzuki et al. (2005). This sequence is unique to plants and has been shown to mediate AtTSA1 multimerization. Consistently, 3 out of the 10 EFE repeats are predicted to promote coiled-coil structures (**Figure 6A**, underlined sequences), which are frequently involved in protein-protein interactions (Rose et al., 2004; Wang et al., 2012). The C-terminal sequence of AtTSA1 also displays a coiled-coil motif, which could play a role in its interaction with AtGIPs. In our yeast two-hybrid growth assay, both AtGIPs can interact with AtTSA1 domains. However, under high stringent conditions, the largest AtTSA1 domain (D1) is only able to interact with AtGIP1 and no longer with AtGIP2 (**Figure S3**). This suggests that AtGIPs may be part of different subcellular complexes including AtTSA1. However, we cannot exclude the implication of other actors in promoting and/or stabilizing the AtGIP-AtTSA1 association.

AtTSA1 also possesses a putative transmembrane (TM) domain close to its C-terminal region (Suzuki et al., 2005), as well as an adjacent VIPT motif resembling the highly conserved φ-VPT motif found in the WIP KASH domain proteins (φ, hydrophobic amino acid). The φ-VPT motif of AtWIP1 is essential for its NE localization, and a VIPT motif is also found at the C-terminal tail of AtWIP3 located in the perinuclear space (Xu et al., 2007). It must be noted that this motif is absent from both AtNAI2 and At3g15960 proteins. Altogether, these data suggest that AtTSA1 may be addressed to and/or retained at the NE. Consistently, in plants co-expressing AtTSA1-RFP and AtGIP1-GFP, AtTSA1 was found at the NE in interphase cells with a similar punctate localization pattern as that observed for AtGIP1 (**Figure 7**).

MODELING A NUCLEO-CYTOPLASMIC CONTINUUM

The NE has for long solely been considered as a selective barrier between the nucleoplasm and the cytoplasm of eukaryotic cells. However, the recent discovery of evolutionarily conserved LINC complexes, spanning the double membrane and involved in essential cellular processes, confers unsuspected versatility to the NE. The ONM is continuous with the ER and shares many of its proteins. AtTSA1 harbors an N-terminal signal peptide, indicating that the protein is initially targeted to the ER (Martoglio and Dobberstein, 1998) and as shown in this study, located at the NE. According to known cotranslational translocation processes (Hegde and Kang, 2008), AtTSA1 may be integrated in the ONM through its transmembrane domain, with its C-terminal part protruding on the cytoplasmic side. An AtGIP-AtTSA1 interaction may then be involved in the association of the γ-TuCs to the outer NE. The above described φ-VPT motif found in AtWIP1 is furthermore required for its interaction with the AtSUNs (Zhou et al., 2012; Tamura et al., 2013). Similarly, the VIPT motif of AtTSA1 may be important for its NE localization and retention, through a possible interaction with AtSUN1 and/or AtSUN2 proteins in the perinuclear space.

The AtTSA1 interacting partner TSK, also named BRU1/MGO3 in subsequent studies, is an epigenetic nuclear factor which plays an important role in genome and chromatin maintenance (Suzuki et al., 2004; Takeda et al., 2004; Guyomarc'h et al., 2006; Ohno et al., 2011). The AtTSK interaction with AtTSA1 in interphase would imply that AtTSA1 could also be located at the INM. As the domains of AtTSA1 interacting with AtTSK and AtGIPs are partly overlapping, there is the possibility that AtTSA1, AtTSK, and/or AtGIPs may be part of specific protein complexes, located close to the INM and involved in chromatin regulation. This is in agreement with the observed nuclear localization of a fraction of AtGIPs in interphase nuclei (Janski et al., 2012).

More recently, AtTSA1 was also described to interact with the N-terminal domain of CSN1, a subunit of the COP9 signalosome in Arabidopsis (Li et al., 2011). The CSN1-NTD is specifically required for the nuclear localization of the E3-ubiquitin ligase COP1 (Wang et al., 2009) which plays a major role in controlling light-induced chromatin decondensation (van Zanten et al., 2012). This is an additional indication for a role of AtTSA1 within the nuclear compartment.

Altogether, this study provides a first insight into the role of AtGIPs in determining the NE morphology. Our findings, in addition to previous results (Janski et al., 2012; Nakamura et al., 2012), indicate that these small proteins may have a dual function, both as components of MT nucleation complexes and also as adaptors and/or modulators of NE associated proteins. Elucidating AtGIP functions may help to decipher the complex molecular interplay established at the nucleo-cytoplasmic interface.

AUTHOR CONTRIBUTIONS

Morgane Batzenschlager and Kinda Masoud carried out the main research stated in the paper, obtained the results and analyzed the data; Natacha Janski first characterized the GIP interactors; Marie-Edith Chabouté proposed the concept of GIP's role in a nucleo-cytoplasmic continuum and developed the tools used in this work; Etienne Herzog and Marie-Edith Chabouté supervised the obtaining of data and analyzed the results with the PhD students; Yves Nominé and Bruno Kieffer performed the physical approach (CD); Jean-Luc Evrard performed the sequence comparisons and computer modeling; Guy Houlné contributed to the part of establishing transgenic lines; Mathieu Erhardt carried out the electron microscopy approach; Nicolas Baumberger performed the biochemical approaches; Anne-Catherine Schmit performed and supervised the microscopy approaches; Marie-Edith Chabouté, Anne-Catherine Schmit, Morgane Batzenschlager, and Etienne Herzog wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Traffic_and_Transport/10.3389/fpls.2013.00480/abstract

Figure S1 | Detail of the distribution of AtGIP1-GFP, chromatin, EYFP-AtCENH3 and microtubules in an Arabidopsis root cell. (A) Fluorescent image after α-tubulin immunolabeling (red) and DAPI staining (blue) in a cell expressing AtGIP1-GFP (green). **(B)** Corresponding drawing showing a perinuclear AtGIP1-GFP dot at a MT minus end close to chromocenters. The yellow square points out the colocalization between the minus end of a percinuclear MT and GIP1-GFP. Bar = 1.5 μm. **(C–J)** Fluorescent images after α-tubulin immunolabeling (red), and DAPI staining (blue) in cells expressing EYFP-CENH3 (green) arrows in **(C–E)**. **(H,K)** Corresponding drawings showing MT minus ends close to EYFP-CENH3 signals and chromocenters.

Figure S2 | Multiple sequence alignment of AtTSA1 and its two homologs in Arabidopsis. The sequences were aligned using the ClustalW multiple alignment tool. (∗) identical amino acids, (.) similar amino acids. The numbers show the amino acid residue positions. Both the signal peptide (aa 1–29) and the C-terminal region (aa 513–755) of AtTSA1 show sequence homology with AtNAI2 and At3g15960 proteins. Large gaps in the alignment are due to the lack of EFE repeats (aa 91–469) in At3g15960 protein.

Figure S3 | Comparative analysis of AtGIP1 and AtGIP2 interaction with AtTSA1 domains in a yeast two-hybrid growth assay. Full-length AtGIP1 and AtGIP2 are fused to the Gal4 DNA Binding domain (BD, bait) while the longest (TSA1-D1) and minimal (TSA1-D4) domains of AtTSA1 identified by yeast two-hybrid screening are fused to the Gal4 Activation domain (AD, prey). AH109 cells co-transformed with the different recombinant vectors were spotted directly and after ten-fold serial dilutions onto control (-LW) and selection media (− HLW + 3-AminoTriazole, 3-AT and -AHTL) of increasing stringency. Growth results are shown after a 2-day incubation at 30°C and were obtained for 4 individual colonies per combination. The interaction of AtGIP1 with both D1 and D4 domains of AtTSA1 is confirmed by the growth of cells even on high-stringency (-AHTL) media. On the other hand, a slower growth of AH109 transformants carrying AtGIP2 and TSA1-D1 recombinant proteins compared to the AtGIP2/TSA1-D4 combination indicates a differential requirement for AtTSA1 domains to establish the interaction between AtTSA1 and AtGIP proteins.

Figure S4 | AtGIP1 interacts with itself and AtGIP2 in a yeast two-hybrid system. Yeast strain Y187 was co-transformed with plasmids encoding AtGIP1 fused to the Gal4 Activation domain (AD), and either AtGIP1 or AtGIP2 fused to the Gal4 DNA Binding domain (BD). Protein interactions were assessed in a β-galactosidase filter assay (Janski et al., 2008), with the appearance of a blue color indicating interaction between the proteins tested. The retinoblastoma protein (Rb1) of maize (*Zea mays*) and the RepA protein of wheat (*Triticum aestivum*) dwarf geminivirus (geminivirus of the genus *Mastrevirus* which infects monocotyledonous plants) were used as positive controls (Xie et al., 1996). Yeasts co-transformed with AtGIP recombinant plasmids and empty AD or BD two-hybrid vectors were used as negative controls. Both AtGIP1-AtGIP1 and AtGIP1-AtGIP2 interactions were detected.

Table S1 | List of primers used in cloning experiments.

Table S2 | Accession numbers of the sequences used for establishing the phylogenetic tree presented in Figure 1. Note: The previous study predicted fission yeast MOZART1 homolog MZT1 to be a 97 amino acid protein (Hutchins et al., 2010; Dhani et al., 2013; Masuda et al., 2013), have shown that the initiator methionine corresponds to the methionine 34 of the previously annotated MZT1 ORF. Consequently, MZT1 is a protein of 64 amino acids similar to MOZART1 homologs from other species.

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When fat is not bad: the regulation of actin dynamics by phospholipid signaling molecules

Roman Pleskot[1], Přemysl Pejchar[1], Christopher J. Staiger[2] and Martin Potocký[1]*

[1] *Institute of Experimental Botany, v. v. i., Academy of Sciences of the Czech Republic, Prague, Czech Republic*
[2] *Department of Biological Sciences, Purdue University, West Lafayette, IN, USA*

Edited by:
Clément Thomas, Public Research Centre for Health, Luxembourg

Reviewed by:
Joshua Blakeslee, The Ohio State University, USA
Clément Thomas, Public Research Centre for Health, Luxembourg

***Correspondence:**
Martin Potocký, Institute of Experimental Botany, v. v. i., Academy of Sciences of the Czech Republic, Rozvojová 263, 165 02 Prague 6, Czech Republic
e-mail: potocky@ueb.cas.cz

The actin cytoskeleton plays a key role in the plant morphogenesis and is involved in polar cell growth, movement of subcellular organelles, cell division, and plant defense. Organization of actin cytoskeleton undergoes dynamic remodeling in response to internal developmental cues and diverse environmental signals. This dynamic behavior is regulated by numerous actin-binding proteins (ABPs) that integrate various signaling pathways. Production of the signaling lipids phosphatidylinositol 4,5-bisphosphate and phosphatidic acid affects the activity and subcellular distribution of several ABPs, and typically correlates with increased actin polymerization. Here we review current knowledge of the inter-regulatory dynamics between signaling phospholipids and the actin cytoskeleton in plant cells.

Keywords: actin, actin-binding proteins, capping protein, cytoskeleton, phosphatidic acid, phosphatidylinositol 4,5-bisphosphate, phospholipase D, signaling

INTRODUCTION

The plant actin cytoskeleton is a molecular scaffold that controls many aspects of cytoarchitecture including cytoplasmic streaming, movement and positioning of diverse organelles, or individual proteins. It also plays a prominent, albeit incompletely understood, role in endocytic and exocytic processes and has been implicated in cytokinesis, polar growth, and defense responses to pathogens (Higaki et al., 2007). Actin filaments are generated from monomeric actin subunits (G-actin) and arrayed into dynamic networks in plant cells; actin turnover and the formation of higher-order structures is tightly regulated by dozens of actin-binding proteins (ABPs). These proteins can be divided into several groups according to their binding properties and activities, e.g., monomeric G-ABPs; capping and severing proteins; side-binding proteins; and actin-nucleating factors (Staiger and Blanchoin, 2006; Henty-Ridilla et al., 2013).

To ensure proper spatial and temporal regulation of actin dynamics, the activity and binding properties of ABPs are further modulated by upstream-signaling molecules (reviewed, e.g., in Thomas et al., 2009; Blanchoin et al., 2010; Fu, 2010). Here we review the role of minor signaling membrane components, phosphatidylinositol 4,5-bisphosphate (PIP2), and phosphatidic acid (PA), that have been discovered as important regulators of actin dynamics in plant cells. In particular, we address the following subjects: (i) characteristics of PIP_2 and PA that permit their function in cells; (ii) specific production of actin-regulating PIP_2 and PA pools; (iii) current knowledge on the regulation of different ABPs mediated by direct interaction with PIP_2 and/or PA; and (iv) putative crosstalk between PA and PIP_2 in the regulation of actin dynamics.

UNIQUE STRUCTURAL PROPERTIES OF PIP_2 AND PA DETERMINE THEIR BIOLOGICAL ACTIVITY

Although both PA and PIP_2 are negatively charged (i.e., acidic) in the physiological pH range, they markedly differ in their structural and biophysical properties. PIP_2 contains a bulky headgroup, with net charge ranging from –3 to –5 under physiological pH and an inverted conical shape that promotes positive curvature of membranes (**Figure 1**). Since total concentration of PIP_2 in the plant plasma membrane is less than 1% (Munnik and Nielsen, 2011), PIP_2 (together with other phosphoinositides, PPIs) is believed to function as an address label that defines membrane identity and as a landmark molecule for its protein partners, rather than having a general structural role in the lipid bilayer.

In contrast to the distinct structure of PIP_2 that makes it very distinguishable in the membrane for its interaction partners, PA represents the simplest glycerophospholipid, consisting of a hydrophobic diacylglycerol (DAG) body and a single phosphate as the polar hydrophilic headgroup (**Figure 1**). PA is more abundant than PIP_2 in the plant plasma membrane (usually between 5 and 10% of total phospholipids; Furt et al., 2010) and can change local properties of the lipid bilayer due to its cone-like shape, favoring negative membrane curvature (Kooijman et al., 2003; Testerink and Munnik, 2011). Interestingly, the specificity of PA interactions with its binding proteins is the result of a unique PA property called the electrostatic/hydrogen bond switch, where the negative charge of the PA headgroup is increased from –1 to –2 and stabilized upon formation of hydrogen bonds with arginine and lysine residues of effector proteins (Kooijman et al., 2007).

In addition to differences in polar headgroups, distinct membrane properties of PIP_2 and PA may also result from different acyl compositions. In tobacco leaves, PA is predominantly made of

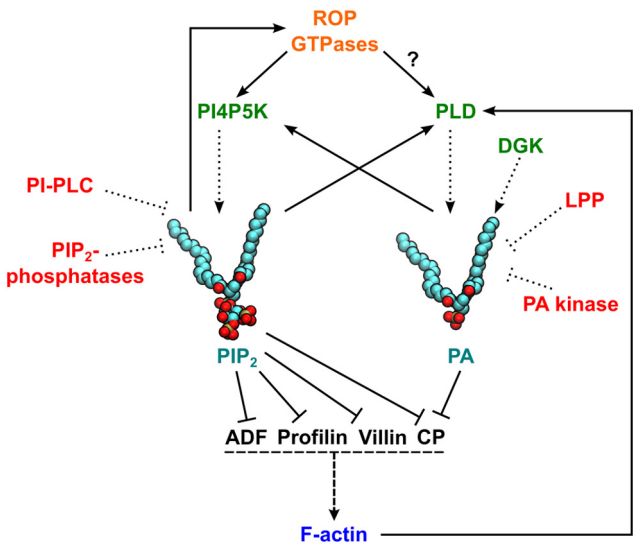

FIGURE 1 | Schematic representation of actin regulation by phosphatidylinositol 4,5-bisphosphate (PIP_2) and phosphatidic acid (PA). Solid lines represent pathways leading to activation (arrow ends) or inhibition (line ends). Dotted lines indicate pathways leading to PIP_2/PA production/degradation. Dashed lines represent an induction of actin polymerization. A question mark indicates that experimental data are available only for non-plant cells. Enzymes generating PIP_2 or PA are in green and proteins involved in phospholipid degradation or signaling attenuation are in red. In PIP_2 and PA structural models, red and brown balls represent the oxygen and phosphorus atoms in headgroups, respectively, and carbon atoms are shown in cyan. ADF, actin-depolymerizing factor; CP, capping protein; F-actin, filamentous actin; DGK, diacylglycerol kinase; LPP, lipid phosphate phosphatases; PI4P5K, phosphatidylinositol 4-phosphate 5-kinase; PI-PLC, phosphatidylinositol-specific phospholipase C; PLD, phospholipase D; ROP, Rho of plants.

palmitic and linoleic acid, whereas PIP_2 contains mainly palmitic, stearic, and oleic acids (Furt et al., 2010).

TIGHTLY REGULATED AND DISTINCT POOLS OF PIP_2 AND PA ARE INVOLVED IN ACTIN REGULATION

PIP_2 PRODUCTION

Phosphoinositides biosynthesis begins with the formation of phosphatidylinositol (PI), which is produced by the condensation of cytidine-diphosphodiacylglycerol and D-*myo*-inositol in the endoplasmic reticulum (ER) (Löfke et al., 2008). The inositol ring of PI can be further phosphorylated at D-3, D-4, and D-5 position by specific evolutionarily conserved lipid kinases (Brown and Auger, 2011). The key enzyme in PIP_2 synthesis is phosphatidylinositol 4-phosphate 5-kinase (PI4P5K). In *Arabidopsis*, 11 genes encoding PI4P5K isoforms were identified (Mueller-Roeber and Pical, 2002). These genes could be further divided into two subgroups based on their overall structure, one group containing *AtPI4P5K1–9* and the other formed by *AtPI4P5K10–11* (Ischebeck et al., 2010). PI4P5Ks have an essential role in root-hair growth, pollen development, and guard cell opening (Munnik and Nielsen, 2011). Intriguingly, a double mutant of *PI4P5K10* and *11* has increased sensitivity to actin-monomer binding drug latrunculin B, whereas overexpression of these isoforms causes aggregation of apical actin fringe in tobacco pollen tubes (Ischebeck et al., 2011), suggesting that PIP_2 produced by this group of PIP4P5Ks is specifically involved in the regulation of actin dynamics.

In addition to PPI formation, reduction in PPI levels is also likely to regulate the actin cytoskeleton. Phosphoinositide-specific phospholipase C (PI-PLC) is an enzyme that hydrolyzes PIP_2 into DAG and inositol trisphosphate (IP_3), and was shown to affect actin organization in *Petunia* pollen tubes by knockdown studies (Dowd et al., 2006). Moreover, two non-related families of phosphatases are present in plant genomes: inositol polyphosphate 5-phosphatases (5PTases), that can cleave both PIP_2 and inositol polyphosphates, and PPI phosphatases containing SAC domain that preferentially cleave membrane PPIs. Interestingly, the *fra3* mutant that has been identified as 5PTase15 implicated in controlling actin organization and secondary cell wall synthesis in fiber cells (Zhong et al., 2004). Actin disorganization was also shown in *fra7* mutant, coding for SAC-bearing PPI phosphatase (Zhong et al., 2005).

PA PRODUCTION

In addition to ER-localized biosynthesis of PA that serves as a precursor for structural phospholipids and triacylglycerols, two distinct pathways can lead to formation of PA with signaling properties. The most studied pathway involves hydrolysis of structural phospholipids by phospholipase D (PLD), directly yielding PA. In comparison to yeast and animal genomes, the PLD family is expanded in plants with 12 genes in *Arabidopsis* and even more in other dicot and monocot genomes (Eliáš et al., 2002; Pleskot et al., 2012a). Interestingly, the PLDβ1 isoform from *Arabidopsis* and tobacco was found to interact directly with actin and is implicated in the regulation of actin polymerization (Kusner et al., 2003; Pleskot et al., 2010).

In addition to the PLD pathway, PA can be also produced by phosphorylation of DAG from the activity of diacylglycerol kinase (DGK). Intriguingly, "signaling" DAG in plant cells can be generated either from PIP_2 via PI-PLC or from structural phospholipids via the activity of non-specific PLC (Munnik and Nielsen, 2011; Pokotylo et al., 2013), thus linking PPIs and PA signaling. The knowledge about plant DGKs is scarce and no molecular or genetic data are available that would support a role in actin regulation. However, several animal DGK isoforms have been implicated in actin regulation, and a plant DGK activity was found to be associated with F-actin in carrot cell cultures (Tan and Boss, 1992).

MULTIFACETED ROLE OF PIP_2 IN THE REGULATION OF ACTIN CYTOSKELETON

There are several different ways that PIP_2 can affect actin polymerization, dynamics, and association with the membrane: through direct binding and regulation of distinct ABPs, indirectly through regulation of the activity and localization of ROP (Rho of plants) GTPases, or via recruiting scaffolding proteins to the plasma membrane (Zhang et al., 2012).

Actin-binding proteins were among the first proteins whose biological activity was shown to be regulated by PIP_2 (reviewed in Zhang et al., 2012). There seems to be a clear distinction between inhibiting and activating properties of PIP_2 in

actin polymerization, such that all PIP_2-sensitive G-actin-binding and actin-severing proteins are inactivated by PIP_2, whereas for proteins acting in actin assembly or linking the filaments to the membrane, their interaction with PIP_2 leads to increased actin polymerization and/or membrane attachment (Saarikangas et al., 2010). In contrast to the majority of PPI-binding non-cytoskeletal proteins, which have structurally well-defined PPI-binding motifs, like pleckstrin homology (PH), Phox homology (PX) or Fab-1, YGL023, Vps27, and EEA1 (FYVE) domains, most ABPs do not possess obvious structural modules, but they instead use patches of basic/aromatic amino acids, e.g., heterodimeric capping protein (CP) contains such clusters on the C-terminal parts of both subunits (Kim et al., 2007; Pleskot et al., 2012b, see also below for details).

A number of PPI-regulated ABPs have been studied in animal cells including members of ADF (actin-depolymerizing factor)/cofilin, profilin, twinfilin, CP, gelsolin, villin, α-actinin, vinculin, talin, spectrin, ERM (ezrin/radixin/moesin), and actin nucleating protein families (Saarikangas et al., 2010). In plants, four distinct ABP classes (profilin, ADF/cofilin, CP, and villin) have been described to be regulated by PIP_2 to date (Gungabissoon et al., 1998; Braun et al., 1999; Dong et al., 2001; Xiang et al., 2007).

Profilin is a globular protein of low molecular mass, which forms a 1:1 complex with G-actin (Kovar et al., 2000). Profilin suppresses spontaneous nucleation of actin and prevents assembly at the slow-growing, pointed end of actin filaments (Staiger and Blanchoin, 2006). In contrast to non-plant counterparts, plant profilin does not catalyze nucleotide exchange on actin (reviewed in Day et al., 2011). Profilin colocalizes with PIP_2 at the tip of growing root hairs (Braun et al., 1999). Moreover, plant profilin directly binds PIP_2(Kovar et al., 2001) and it could be speculated that similar to its animal homologs, profilin can then dissociate from profilin-G-actin complexes releasing free G-actin (Witke, 2004). Interestingly, plant profilin also inhibits the activity of PIP_2-degrading enzyme, PI-PLC (Kovar et al., 2000).

Proteins of the ADF/cofilin family represent conserved ABPs across eukaryotes (Hussey et al., 2002). ADF/cofilin recycles actin monomers by severing and creating new filament ends (Andrianantoandro and Pollard, 2006; Henty et al., 2011). *Zea mays* (Zm) ADF3 directly binds and is inhibited by PIP_2. Moreover, similar to the profilin-PIP_2 interaction, the ZmADF3 binding of PIP_2 suppresses the activity of PI-PLC (Gungabissoon et al., 1998). Similar findings were reported for ADF1 from lily pollen (Allwood et al., 2002), suggesting that PPI regulation is a common feature of plant ADF/cofilin isoforms.

Villin belongs to the ABP protein superfamily gelsolin/villin/fragmin and is composed of six gelsolin-homology domains at its core and a villin headpiece domain at its C-terminus. *Arabidopsis* contains five *VILLIN* genes, however, genes coding for gelsolin and fragmin are not present in model plant genomes. Interestingly, actin-severing activity of ABP29, a probable splice variant of the 135-kDa villin from lily, was shown to be inhibited by PIP_2 (Xiang et al., 2007). However, the analogous regulation of full-length plant villin remains to be demonstrated.

Capping protein is a heterodimeric protein distributed across almost all eukaryotes (Pleskot et al., 2012b); it binds to the fast growing end of actin filaments, thus inhibiting polymerization. CP bound to actin filaments also protects against disassembly (Huang et al., 2003). Similar to animal cells, it was shown that the ability of *Arabidopsis* CP to bind actin fast-growing ends is inhibited PIP_2 *in vitro* (Huang et al., 2006). However, unlike animal and yeast CPs, the *Arabidopsis* CP homolog has been also identified as a direct target of PA both *in vitro* and *in vivo* [see below for more details; (Huang et al., 2006; Li et al., 2012a)].

Rho of plants small GTPases are a plant-specific subfamily and sole members of the Rho/Rac/Cdc42 family of Ras-related G-proteins in plants, where they serve as "master switches" involved in diverse signaling and developmental pathways. Activated ROP variants are associated with the plasma membrane, where they are thought to control cell growth by coordinating actin organization and membrane trafficking (Mucha et al., 2011). Importantly, PIP_2 was shown to colocalize with ROP GTPases at the apical plasma membrane of tobacco pollen tube and pollen ROP physically interacts with PI4P5K activity (Kost et al., 1999; Yalovsky et al., 2008). Importantly, type II plant ROP GTPases have a polybasic motif at the C-terminal part of the protein, which is necessary for plasma membrane localization (Lavy and Yalovsky, 2006). It is therefore tempting to speculate that this polybasic motif binds PIP_2 directly, as described for many members of the human small GTPase family (Heo et al., 2006). Furthermore, it was recently shown that PIP4P5K regulates actin dynamics in pollen tubes by counteracting Rho-GDI (Rho-guanine nucleotide dissociation inhibitor), thereby regulating the pool of membrane-localized ROP GTPases (Ischebeck et al., 2011).

PA REGULATES PLANT ACTIN CYTOSKELETON DYNAMICS THROUGH CP

In the last decade, several studies describe changes in signaling PA levels that generate a pronounced effect on plant actin cytoskeleton organization (Lee et al., 2003; Motes et al., 2005; Huang et al., 2006; Apostolakos et al., 2008; Pleskot et al., 2010, 2012a). Given the profound effect of PA production on actin polymerization in eukaryotes, it is surprising that no ABPs regulated by PA were described in animal or yeast cells. Indeed, the PA effect on actin in animals appears to be mainly indirect, by controlling production of PIP_2 through PI4P5Ks [(Roach et al., 2012); and see below]. In plant cells on the other hand, CP was found to be regulated by PA as well as PPIs *in vitro* (Huang et al., 2006). Furthermore, the critical role of PA in plant CP regulation was confirmed by utilizing *cp* knockdown mutants (Li et al., 2012a,b). Structural aspects of the AtCP inhibition by PA highlight a key role for the C-terminal part of CPα subunit, as demonstrated through molecular dynamics simulations (Pleskot et al., 2012b). The fact that a direct interaction between actin and PLDβ exists in plant cells (Pleskot et al., 2010) leads to the hypothesis of a positive feedback loop model for actin dynamics regulation by PLDβ and PA. Briefly, intracellular or intercellular signals cause activation of PLDβ and subsequently increase the local PA concentration. PA binds CP and prevents its binding to the fast growing end of actin filaments, thus promoting actin polymerization. Newly formed actin filaments promote PLDβ activity, leading to local enhancement of PA concentration

and further enhancement of actin assembly (Pleskot et al., 2010, 2013).

CONCLUDING REMARKS AND HYPOTHESES

During the last 20 years, multiple direct and indirect interactions between PIP_2- and PA-centered signaling pathways and the regulation of actin dynamics have been revealed. Despite the fact that the regulation of actin dynamics is a point of convergence for many signaling pathways and exhibits complex feedback regulation (**Figure 1**), general conclusions can be drawn: The elevation of PIP_2 and/or PA levels increases both density and complexity of the actin network and conversely the inhibition of PA/PIP_2 production leads to actin filament disruption. Although many similarities can be found in ABP–phospholipid regulation between plant and animal cells, there is one principal difference: in plants, PIP_2 levels are 10 times lower than PA levels (Drøbak, 1993; Zonia and Munnik, 2004). It is therefore tempting to speculate that many plant ABPs adapted to the distinct levels of PA and PIP_2. It might be expected that additional ABPs interact with PA and/or PIP_2 in plant cells, and this should be a topic for future exploration.

Many published reports on ABP–phospholipid regulation assume that the protein–lipid bilayer interaction is mono-specific, i.e., a single species of lipid is responsible for recruiting a given ABP to the membrane. However, work from animal and yeast cells has shown that a mono-specific reaction is the exception rather than the rule: for the majority of lipid effectors, membrane translocation probably depends both on a specific lipid but also on the surrounding lipid environment (Moravcevic et al., 2012). Indeed, several recent computational studies, albeit not on proteins involved in the regulation of the actin dynamics, show the involvement of other phospholipids for protein domains previously thought to function in a mono-specific way. Kooijman et al. (2007) experimentally described the positive effect of phosphatidylethanolamine on the PA binding by AtPDK1, AtCTR1, and Raf-1. Similar results were obtained for the binding of PPIs by PH, PX, and FYVE domains (Psachoulia and Sansom, 2008, 2009; Lumb et al., 2011). Several PA-binding proteins also have affinity for different PPIs. The binding of another signaling phospholipid could be mediated by the same domain, as in the case of AtPDK1 and p47phox PX domain, or through a completely distinct domain, for example the C1 and C2 domains of mammalian PKCε (Testerink and Munnik, 2011), but the molecular details are largely missing. Interestingly, the C1-domain, a canonical DAG-binding motif, binds more strongly to DAG embedded in the negatively charged membrane and DAG-mediated targeting of effector proteins thus seems to be also enhanced by synergistic binding to acidic phospholipids, such as PA and PIP_2 (Colón-González and Kazanietz, 2006). From this point of view, dual regulation of plant CP by both PA and PIP_2 (Huang et al., 2006) might represent just the tip of an iceberg.

A cooperative effect between PA and PIP_2 in the regulation of the actin dynamics could be also indirect. Recently, Roach et al. (2012) described the ability of PA to activate PI4P5K and the authors showed the crucial importance of membrane targeting of PI4P5K by PA in the regulation of actin reorganization in animal cells. The activation of kinase activity by PA was shown for AtPI4P5K1 (Perera et al., 2005). Given the fact that several PLD isoforms are activated by PIP_2 (Li et al., 2009), one can expect that a vivid crosstalk between PA and PIP_2 signaling to the actin cytoskeleton exists in all eukaryotic cells.

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The exocyst at the interface between cytoskeleton and membranes in eukaryotic cells

Lukáš Synek*[1], *Juraj Sekereš*[1,2] *and Viktor Žárský*[1,2] *

[1] *Laboratory of Cell Biology, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Prague, Czech Republic*
[2] *Laboratory of Plant Cell Biology, Department of Experimental Plant Biology, Faculty of Science, Charles University in Prague, Prague, Czech Republic*

Edited by:
Christopher J. Staiger, Purdue University, USA

Reviewed by:
Andreas Nebenführ, University of Tennessee, USA
Frantisek Baluska, University of Bonn, Germany

****Correspondence:***
Viktor Žárský, Laboratory of Plant Cell Biology, Department of Experimental Plant Biology, Faculty of Science, Charles University in Prague, Vinicna 5, 12844 Prague, Czech Republic
e-mail: zarsky@ueb.cas.cz

Delivery and final fusion of the secretory vesicles with the relevant target membrane are hierarchically organized and reciprocally interconnected multi-step processes involving not only specific protein–protein interactions, but also specific protein–phospholipid interactions. The exocyst was discovered as a tethering complex mediating initial encounter of arriving exocytic vesicles with the plasma membrane. The exocyst complex is regulated by Rab and Rho small GTPases, resulting in docking of exocytic vesicles to the plasma membrane (PM) and finally their fusion mediated by specific SNARE complexes. In model Opisthokont cells, the exocyst was shown to directly interact with both microtubule and microfilament cytoskeleton and related motor proteins as well as with the PM via phosphatidylinositol 4,5-bisphosphate specific binding, which directly affects cortical cytoskeleton and PM dynamics. Here we summarize the current knowledge on exocyst-cytoskeleton-PM interactions in order to open a perspective for future research in this area in plant cells.

Keywords: exocyst, actin cytoskeleton, microtubule cytoskeleton, phospholipids, myosin, small GTPases, Exo70, secretion

THE EXOCYST AS A REGULATORY HUB IN THE ACTIVE CELL CORTEX

Polarized surface growth in eukaryotic cells involves interactions between the cytoskeleton and membrane transport pathways. The last steps of the secretory pathway taking place in the vicinity of the plasma membrane (PM) are regulated by an array of small GTPases, the exocyst tethering complex, and SNARE proteins. The exocyst is a protein complex comprising eight subunits (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84) engaged in docking and tethering of secretory vesicles, providing a spatial and temporal regulation of exocytosis (Hsu et al., 1996; TerBush et al., 1996) and interacting directly or indirectly with membranes, cytoskeletal proteins, as well as with small GTPases from the Rab, Ral, and Rho subfamilies and many other proteins in the cell cortex (Wu et al., 2008). As such, the exocyst seems to act as an integrating hub in the cell cortex, mainly in the context of exocytosis. In general, proper exocyst function is essential for polar growth and cell morphogenesis, including invadopodia, lamellipodia, and neuronal dendrites formation in animal cells, bud growth in budding yeast, and cytokinesis in fission yeast (reviewed in Heider and Munson, 2012; Vaškovičová et al., 2013; **Figure 1**). A growing number of papers document functions of the plant exocyst in similar processes with high demand for exocytosis, including root hair growth, hypocotyl cell elongation, cytokinesis, seed coat formation and papilla formation after a pathogen attack in plants (Synek et al., 2006; Hála et al., 2008; Fendrych et al., 2010; Kulich et al., 2010; Pecenkova et al., 2011; Vaškovičová et al., 2013).

THE EXOCYST AND ACTIN CYTOSKELETON

Deep insight into exocyst functions and their mechanisms came from genetic studies on budding yeast, where the exocyst was originally discovered as a protein complex (Novick et al., 1980; TerBush et al., 1996). In budding yeast cells, secretory vesicles are transported along formin- and Arp2/3-generated actin cables. A common model of the exocyst action suggests that most exocyst subunits arrive to the PM in association with secretory vesicles and cannot localize properly after disruption of the actin cytoskeleton (Boyd et al., 2004; Bendezú et al., 2012). However, Sec3p and part of the Exo70p population can reach its destination, a newly forming bud, independently of the actin cytoskeleton probably via direct association with Rho GTPases (Boyd et al., 2004). Therefore, Sec3p and Exo70p are supposed to act as landmarks of sites for the exocyst localization and action (Finger et al., 1998; Boyd et al., 2004).

Mutations in several exocytosis-related genes cause actin cytoskeleton defects in budding yeast, leading subsequently to impaired cell growth and morphogenesis and also to an mRNA transport and polarization defect that is actin-dependent (Aronov and Gerst, 2004). The identified genes included those encoding *SEC10* and *SEC15* exocyst components and *CDC42* and *RHO3* GTPases regulating the exocyst polar targeting (Wu et al., 2008).

An interesting reciprocal relationship was observed during cell wounding response, where Sec3p and the Bni1p formin are degraded in order to eliminate competition for secretory vesicles required to repair the damaged membrane and cell wall, which are arriving along the pre-polarized cytoskeleton directing current polarized growth. The Bnr1p formin and the Exo70p exocyst subunit relocalize to the damage site followed by redistribution of the Myo2p myosin and delivery of new material (Kono et al., 2012).

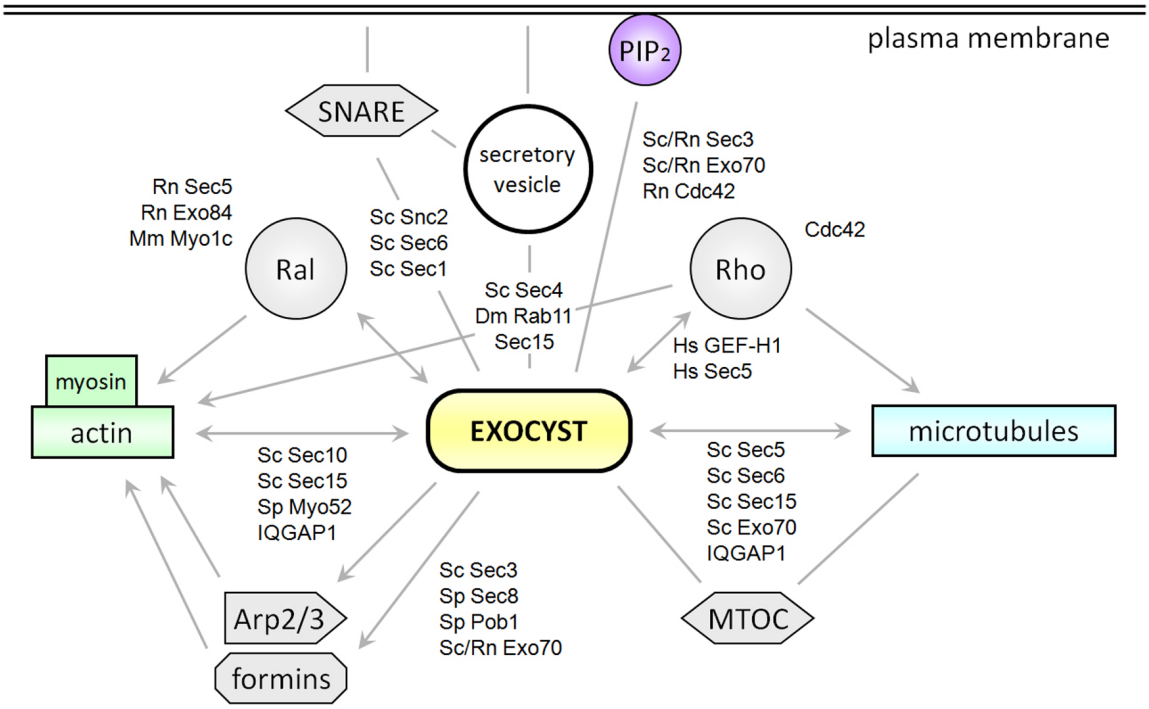

FIGURE 1 | Interactions of the exocyst complex with the cytoskeleton, plasma membrane, and associated proteins. Scheme of exocyst interactions described in the text, including the key players Ral and Rho GTPases, SNARE proteins, formins, and the Arp2/3 actin-nucleating complex, microtule-organizing center (MTOC) and phosphatidylinositol 4,5-bisphosphate (PIP_2) at the plasma membrane. Dm, *Drosophila melanogaster*; Mm, *Mus musculus*; Hs, *Homo sapiens* Rn, *Ratus norwegicus*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*.

In budding yeast, cell polarity and polarized exocytosis is coordinated also by the Rho3p GTPase (Adamo et al., 1999), which can regulate both actin polarity and transport of exocytic vesicles from mother cell to the bud, as well as vesicle docking to the PM. While the Rho3p vesicle delivery function is mediated by Myo2p, the docking requires Exo70p (Adamo et al., 1999).

In the fission yeast *Schizosaccharomyces pombe*, the actin cytoskeleton is dispensable for proper exocyst localization and polarized growth (Bendezú and Martin, 2011; Snaith et al., 2011). While actin-independent polar transport in budding yeasts might be constrained by the narrow bud neck, and bud growth requires motor-driven transport along actin cables, the open cylindrical shape of fission yeast cells may allow actin-independent vesicle transport (Bendezú and Martin, 2011). However, the exocyst and actin cytoskeleton share at least two common upstream regulators – Cdc42 (Estravís et al., 2011) and Pob1 (Nakano et al., 2011).

The polar exocyst localization and formation of actin cables are dependent on and mutually coupled by Pob1 via its interaction with the For3 formin and the Sec8 exocyst subunit, respectively. Simultaneous deletion of For3 and Sec8 results in isotropic growth, indicating a functional redundancy between microfilaments and the exocyst in cell polarization (Bendezú and Martin, 2011). In contrast, although unable to divide properly, *sec8 exo70* and *sec6 sec8* double mutants are still capable of polarized growth (Bendezú and Martin, 2011).

Although all fission yeast exocyst subunits can localize to cell poles largely independently of the actin cytoskeleton, at least Sec3, Sec5, and Exo70 (most probably as a part of the complete exocyst complex) are more efficiently transported to the cell apex by the Myo52 myosin V along microfilaments (Snaith et al., 2011; Bendezú et al., 2012). Either functional Sec3 or Exo70 is essential for viability and proper localization of other exocyst subunits, suggesting that, as in budding yeast, these two components act as exocyst tethers at the PM (Bendezú et al., 2012). A polarization pathway involving the exocyst relocalization and actin repolarization downstream of Cdc42 also participates in fission yeast mating (Bendezú and Martin, 2013).

Unexpectedly, the fission yeast Sec3 not only acts in exocytosis but also marks sites for actin recruitment and controls overall actin organization via direct binding of For3 (Jourdain et al., 2012). Mutants in Sec3 exhibit lack of microfilaments, depolarized actin patches, and disassembly of the cytokinetic actomyosin ring probably due to a failure in polarization of the For3 formin.

The Exo70 exocyst subunit also interacts both *in vitro* and *in vivo* with the yeast and rat Arpc1/Arc40 subunit of the Arp2/3 complex, a key regulator of actin polymerization. Inhibition of the Exo70 function in rat kidney cells blocks formation of actin-based membrane protrusions and affects cell migration (Zuo et al., 2006), pointing to yet unknown capacity of Exo70 to regulate the actin organization and coordinating thus actin cytoskeleton with membrane trafficking during cell migration. Exo70 was recently shown to promote Arp2/3-driven microfilament nucleation and branching (Liu et al., 2012). Because both the

exocyst and Arp2/3 complexes are well conserved across eukaryotes, including plants, their interaction is likely to be conserved as well.

In mammalian cells, actin organization, as well as membrane trafficking, cell growth and differentiation, is regulated by RalA and RalB, ubiquitous small GTPases from the Ras superfamily (Feig et al., 1996). Activated (GTP-bound) RalA forms a stable complex with the exocyst via binding to Sec5 (Brymora et al., 2001; Sugihara et al., 2002; Fukai et al., 2003) and Exo84 (Moskalenko et al., 2003; Jin et al., 2005) exocyst subunits in human and rat cells. Specific inhibition of the Sec5 activity blocks filopodia formation in 3T3 cells, a dynamic process that is highly dependent on actin reorganization and that can be normally induced by RalA or cytokines via Cdc42 (Sugihara et al., 2002). This inhibitory effect could not be attributed to disrupted secretion, since inhibition of secretion by brefeldin A did not affect filopodia formation (Sugihara et al., 2002), indicating that the exocyst-RalA complex may regulate actin reorganization independently of vesicle transport. Both RalA-Sec5 and RalA-Exo84 interactions are necessary for proper regulation of the actin cytoskeleton dynamics, as documented by different morphological consequences of uncoupling these interactions in PC-3 cells, such as defects in lamellipodia formation, rounder cells or extended spindles (Hazelett and Yeaman, 2012). RalA also interacts with the actin cytoskeleton via Myo1c, suggesting its function as a cargo receptor for the Myo1c motor (Chen et al., 2007). Taken together, the exocyst complex as an immediate effector of RalA obviously integrates the secretory pathway and actin cytoskeleton near the PM in mammalian cells (**Figure 1**).

Cells of mouse oocytes can use secretory (Rab11-positive) vesicles associated with the exocyst components via the Rab11–Sec15 interaction (Wu et al., 2005) as adaptable, motorized network nodes regulating the dynamics and density of microfilaments in a myosin Vb-dependent manner (Holubcová et al., 2013). Such an actin modulation is essential for asymmetric positioning of the meiotic spindle and thus for the development of a fertilizable egg in mammals.

Although we can find no dynamic membrane protrusions analogous to filopodia in plant cells, fine F-actin meshwork is essential for polar growth of root hairs, pollen tubes, or stigmatic papillae and this type of growth demanding precise regulation of exocytosis is also strongly dependent on the exocyst function (Yalovsky et al., 2008; Vaškovičová et al., 2013).

Ral GTPases are specific to animals – in plant cells, as in yeast, only homologs to Rho GTPases (called also Rac in animals) are present and due to some plant specific features they are called Rop (Rho of plant). Rop GTPases were clearly implied in the cortical cytoskeleton regulation mostly possibly via plant specific Rop-interacting adaptors (RICs; Fu et al., 2001; Yalovsky et al., 2008). Very significant for the speculations on plant exocyst-cytoskeleton links is a dominant land-plant specific way of Rop activation mediated by specific PRONE-GEF (plant-specific ROP nucleotide exchanger – GDP/GTP exchange factor) regulated by interacting receptor-like kinases (RLKs) that allow for very efficient cortical activation of Rop GTPases in response to plethora of different stimuli including changes in cell wall mechanics (Mucha et al., 2011). Moreover, the first Rop-exocyst interaction observed in plants is not direct – several GTP-bound Rops interact with the Sec3 exocyst subunit in *Arabidopsis* via a plant specific adaptor protein ICR1 which is implied in the regulation of auxin polar transport (Lavy et al., 2007; Hazak et al., 2010; see further). These features along with plant specific transmembrane anchorage of plant F-actin nucleating formins (Cvrčková, 2013; in this issue) indicate that the cortical wiring between actin cytoskeleton and exocytosis in plants will be quite specific.

THE EXOCYST AND TUBULIN CYTOSKELETON

Microtubules are not essential for exocytosis in budding yeast and no functional link with the exocyst complex has been documented so far (Hammer and Sellers, 2012). In rat kidney cells, however, Exo70 co-localizes with microtubules and the mitotic spindle, and *in vitro*, the exocyst complex reconstituted from recombinant subunits inhibits tubulin polymerization. However, deletions of any of Sec5, Sec6, Sec15, or Exo70 exocyst subunits diminished the inhibition activity. Surprisingly, Exo70 itself could inhibit tubulin polymerization, albeit the exocyst complex lacking the Exo70 subunit did not lose its activity completely. On the other hand, when Exo70 was overexpressed, the microtubule network became disrupted and filopodia-like PM protrusions were formed (Wang et al., 2004).

The protrusion formation is consistent with an observation in *Xenopus* neurons, where a local disassembly of microtubules by focal application of nocodazole induced an addition of a new membrane material at the affected site (Zakharenko and Popov, 1998).

In undifferentiated PC12 neuronal cells, the exocyst complex is associated with microtubules as well as microtubule organizing centers and can be co-immunoprecipitated with microtubules from the total rat brain lysate (Vega and Hsu, 2001). However, upon activation of neuronal differentiation, the exocyst redistributes from perinuclear localization to the growing neurite characterized by high exocytic activity at the PM. The subcellular exocyst localization was affected by treatment with microtubule-disrupting drugs, but not actin-disrupting drugs. These results support a possibility that the exocyst complex acts as a modulator of microtubules to mediate vesicle targeting in animal cells.

It is expected that also in respect to microtubular cytoskeleton-secretory pathway relationship the plant cells will have specific features due to the obvious dependence of the final steps of exocytosis and membrane recycling in plants on the actin cytoskeleton and very possibly exocytosis permissive feature of even dense cortical microtubuli (see below). However, both cytoskeletal systems in plant cells strongly interact (e.g., via specific actin nucleating formins) so that in the real biological context it will be challenging to separate their functions.

INTERPLAY BETWEEN THE EXOCYST AND BOTH TYPES OF CYTOSKELETON

In contrast to budding yeast, typical vertebral cells use microtubules for long-range cargo transport and microfilaments for short-range transport in cell cortex during later steps of vesicles traffic (Hammer and Sellers, 2012). Several studies pinpoint the potential importance of the exocyst in transition of cargo from microtubules to microfilaments.

Mammalian cell migration involves cooperative reorganization of the actin and microtubule cytoskeletons under the control of Rho GTPases (de Curtis and Meldolesi, 2012). Proper localization and activity of the exocyst is promoted by microtubule-associated GEF-H1, a GTP exchange factor for the RhoA actin activator, in HeLa cells (Pathak et al., 2012). Microtubule depolymerization results in the activation of GEF-H1, which further activates RhoA (Krendel et al., 2002). Importance of this regulation was documented experimentally on the cleavage furrow formation during cytokinesis (Birkenfeld et al., 2007) and on actin dynamics during cell migration (Nalbant et al., 2009). The depletion of GEF-H1 led to accumulation of Rab11-positive secretory vesicles within the cells and to mislocalization of Exo70 and Sec8 exocyst subunits (Pathak et al., 2012). GEF-H1 also directly binds the Sec5 exocyst subunit in a RalA GTPase-dependent manner; the interaction is stronger with free GEF-H1 than with its microtubule associated form (Pathak et al., 2012). The Sec5-GEF-H1 interaction promotes RhoA activation, which then regulates exocyst localization and possibly its assembly, as well as actin polymerization. Exocyst thus first helps to activate RhoA, which subsequently assists functioning of the exocyst, resulting in a positive feedback (Pathak et al., 2012).

Interestingly, despite the different mechanisms of cytokinesis between plants and animals/fission yeast (contraction versus building of a cell plate), the exocyst is involved in both types of cytokinesis (Fendrych et al., 2010).

IQGAP1 is another important regulator of both actin and microtubular cytoskeleton associated with the exocyst. The active RhoA and Cdc42 trigger association of Sec3 and Sec8 exocyst subunits with IQGAP1. This interaction is essential for MT1-MMP protease localization at invadopodia and thus for proper invadopodia functioning (Sakurai-Yageta et al., 2008). IQGAP1 stimulates actin bundling (White et al., 2012) and directly interacts with microtubule plus end binding protein CLIP-170 in neurons (Swiech et al., 2011).

EXOCYST INTERACTION WITH CELLULAR MEMBRANES

As mentioned earlier, in budding yeast, Sec3p and part of Exo70p population can reach newly forming bud also independently of microfilaments (Boyd et al., 2004). They bind the PM directly via phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) and indirectly by association with Rho GTPases (He et al., 2007; Zhang et al., 2008; Wu et al., 2010; **Figure 1**). Sec15p binds to the membrane of secretory vesicles via the Sec4p Rab GTPase (Guo et al., 1999) and Sec6p binds Snc2p, a vesicle-associated SNARE protein (Shen et al., 2013). Sec6p also contributes to anchor the exocyst complex at sites of secretion – possibly via interaction with PM-associated proteins (Songer and Munson, 2009). Besides facilitating exocytosis by interactions with Sec9p, a Qbc exocytic t-SNARE protein (Sivaram et al., 2005), and with Sec1, a protein from the Sec1/Munc18 family regulating SNARE functions (Morgera et al., 2012), the exocyst also interacts with the vesicles transporting myosin Myo2p (also a known Sec4p interactor) via the Sec15p subunit that directly binds the motor and allows for its release after vesicle tethering (Jin et al., 2011; Donovan and Bretscher, 2012).

In fission yeast, Sec6 and Sec8 exocyst subunits localize to cell tips largely independent of the actin cytoskeleton, but in a Cdc42 and PIP_2-dependent manner. Thus, the fission yeast long-range cytoskeletal transport and PIP_2-dependent exocyst represent parallel morphogenetic modules downstream of Cdc42, raising the possibility of similar mechanisms in other organisms (Bendezú and Martin, 2013). Bendezú et al. (2012) showed that Sec3 and Exo70 tether the exocyst complex arriving with secretory vesicles by direct binding to PIP_2 and Rho GTPases at the cell poles. In absence of the Myo52 motor protein, vesicles with the entire exocyst can still reach the cell pole by random movement, but less efficiently. In absence of both Sec3 and Exo70, vesicles and the rest of the exocyst fail in delivery and tethering and form aggregates. Also in plants Sec3 subunit of exocyst interacts with membrane lipids (Bloch et al., in preparation).

Very recently Zhao et al. (2013) discovered that Exo70 alone, through an oligomerization-based manner, can generate membrane curvatures *in vitro* independent of the exocyst function. This represents a mechanism creating protrusions even in the absence of actin, albeit it is not clear to what extent stimulated actin polymerization, membrane delivery, and membrane deformation contribute to cell shape changes *in vivo* including formation of membrane protrusions. Thus, Exo70 as a membrane-bending protein may couple the actin dynamics and PM remodeling in morphogenesis.

The exocyst is also essential for large-particle phagocytosis (Mohammadi and Isberg, 2013), Salmonella invasion into host cells (Nichols and Casanova, 2010) and formation of tunneling nanotubes – recently discovered structures connecting cytoplasm of animal cells (Ohno et al., 2010; Mukerji et al., 2012; Schiller et al., 2013). Each of these events could combine all three mechanisms mentioned above. Membrane-deforming ability of Exo70 could function well beyond the cell cortex-associated events, since the exocyst participates in many cellular processes (reviewed in Heider and Munson, 2012; Liu and Guo, 2012).

PERSPECTIVES ON THE EXOCYST–CYTOSKELETON INTERFACE IN ENDOMEMBRANE BIOGENESIS IN PLANTS

Regulation of the cytoskeleton structure and dynamics in plant cells is very much affected by the cell wall, implying close proximity between secretory pathway, cell wall biogenesis and cortical cytoskeleton. These cellular systems are regulated by small GTPases, especially from the ARF, RAB, and ROP families, major regulators of the cell polarity and morphogenesis closely related to their fungal or animal counterparts (Vaškovičová et al., 2013). Work in the laboratory of Shaul Yalovsky (Lavy et al., 2007; Hazak et al., 2010) showed that the SEC3 exocyst subunit interacts with an activated (GTP-bound) ROP at the PM via ICR1, a founding member of the ICR/RIP protein family (Li et al., 2008; Mucha et al., 2010). RIP3 (also known as MIDD1) interacts in a GTP-bound manner with ROPs and also with the Kinesin-13A to regulate the microtubular dynamics (Mucha et al., 2010). RIP3 is a crucial negative regulator of cortical microtubules in the patterning of secondary cell wall thickening directed by the ROP11 GTPase module (Oda and Fukuda, 2012). At PM sites, where cortical microtubules are locally destabilized, the localized exocytosis-dependent secondary cell wall thickening is blocked (Oda and Fukuda, 2012).

While local destabilization of cortical microtubules seems to stimulate exocytosis in animal cells (see above Zakharenko and Popov, 1998), dense microtubule cortical domains of somatic plant cells are often the cortical domains of highest secretory activity, as in xylem thickening or seed coat epidermal cells with a volcano-like cell wall thickening, where highly polarized delivery of pectins is targeted to extremely dense cortical microtubule domains (McFarlane et al., 2008; Oda and Fukuda, 2012). The exocytosis of pectins into pectin-accumulating pockets depends on exocyst function, implying a possibility that microtubule-rich domains might be a general cortical target recognized by EXO70s or other exocyst subunits, functioning as putative PM landmarks for exocytosis targeting (Žárský et al., 2009; Kulich et al., 2010). Extensive proliferation of the EXO70 gene in land plants (e.g., *Arabidopsis* in endowed with 23 EXO70 paralogs) possibly provides a potential for fine targeting into specific cortical areas (Synek et al., 2006; Cvrčková et al., 2012).

On the contrary, dense cortical microfilament meshwork might block exocytosis in both animal and plant cells (Valentijn et al., 1999; Žárský et al., 2009). For instance, a dense subapical F-actin fringe separating actively growing tip from the rest of the tobacco pollen tube might also be a mechanical obstacle for exocytosis (Lovy-Wheeler et al., 2005). The exocyst is also accumulated at the tip of growing pollen tubes and is obviously involved in exocytosis (Hála et al., 2008). The transport and delivery of secretory vesicles in plant cells is likely to depend on both microfilaments and an interaction of some exocyst subunits with the PM phosphoinositides, like in the case of yeast and animal cells (see above). Phosphoinositide binding was indeed predicted for several *Arabidopsis* EXO70 paralogs based on yeast and animal models (Žárský et al., 2009) and currently proved both biochemically and cytologically in our laboratory for the *Arabidopsis* SEC3 exocyst subunit (Bloch et al., in preparation).

The dynamics of several exocyst subunits at the PM, as monitored by TIRF microscopy in *Arabidopsis* epidermal cells, was unaffected by actin or microtubule cytoskeleton disruption after short (10 min) treatment with inhibitors, however, prolonged actin cytoskeleton disruption (1 h) resulted in exocyst redistribution and aggregation at the PM and impaired dynamics (Fendrych et al., 2013). This is consistent with microfilament involvement not only in the delivery but also in spatial distribution of secretory vesicles and endomembrane compartments (Staehelin and Moore, 1995).

Interestingly, exocyst complexes show almost no lateral movement within the PM in both plant and animal cells, as analyzed by the TIRF microscopy, and very similar time of persistence at the PM of about 10 s was recorded (Fendrych et al., 2013; Rivera-Molina and Toomre, 2013). Similarly, the KAT1 channel is localized inside the PM within positionally stable microdomains, which last, however, for 10s of minutes, in contrast to dynamics of the exocyst (Sutter et al., 2006). It is possible that some transmembrane proteins, e.g., plant-specific transmembrane formins (Martiniere et al., 2011; Cvrčková, 2013; in this issue) create, together with specific membrane lipids, functional clusters stabilized against the lateral movement in the PM. These transmembrane proteins might be immobilized by the binding extracellular domains in the cell wall matrix and provide landmarks for the delivery of secretory vesicles (Martinière et al., 2012).

CONCLUSION

Direct as well as a circumstantial evidence accumulated over the years concerning interactions and cooperation between the exocyst and cytoskeleton indicates that the exocyst, cytoskeleton, and membrane traffic meet at the active cellular cortex. The exocyst serves an important role in co-ordination of the vesicle trafficking with the cytoskeleton in eukaryotes, in addition to its canonical role in exocytosis. In plant cells, however, we have currently only limited and indirect evidence for this regulatory interplay, urging further research in this direction.

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