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Orchestrating cell morphology from the inside out – using polarized cell expansion in plants as a model

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Intracellular organization forms the basis of changes in the extracellular matrix. In walled cells, these changes are essential for morphogenesis and growth. The highly polarized cells of mosses and liverworts together with root hairs and pollen tubes are geometrically simple cells that develop in the absence of complex tissue-scale signaling, providing an excellent model to study cell polarity. Recent advances present a unifying theme where the cytoskeleton and its associated motors work in coordination with vesicle trafficking. This coordination results in a recycling system near the cell tip, where endocytosed molecules are sorted and combined with exocytic cargo driving growth. Interestingly, functional similarities between filamentous fungi and plants promise to advance our understanding of cell polarization and growth across kingdoms.

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Introduction

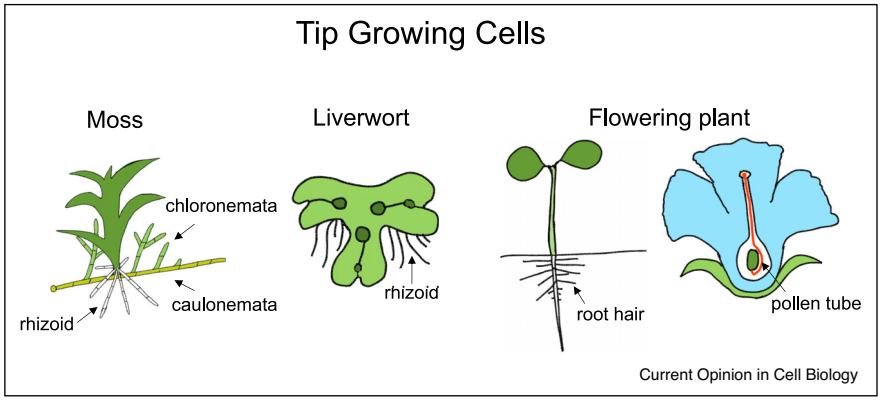
Cells polarize in response to a myriad of extracellular cues. For example, in multicellular organisms, orientation within tissues is critical for proper tissue and organ function. Plant cells are encased by their extracellular matrix, and thus fixed within the plant body. Consequently, to respond to polarizing cues, plant cells alter their intracellular organization, often resulting in changes to the extracellular matrix impacting cell and tissue morphology.

Polarity in plants can be discussed on a number of levels, ranging from orientation of aerial and underground tissues to individual cells as they divide within a tissue to generate structures such as stomata — pores in leaves driving gas exchange without which plants would not be able to utilize environmental carbon dioxide to build their body. Deciphering the molecular mechanisms controlling cell polarity within the context of a whole tissue can be a daunting task, as many cell non-autonomous factors contribute to polarity outcomes. However, all land plants have cell types that are highly polarized and protrude from the plant body by polarized cell expansion, or tip growth, responding more to environmental cues, rather than neighboring cellular cues. These cell types are found throughout the plant kingdom. Early diverging lineages, such as bryophytes, have chloronemata and caulonemata in mosses and rhizoids in liverworts and mosses (Figure 1). Vascular plants have root hairs, single-cell protrusions off the epidermis of the root, as well as pollen tubes that emerge from a pollen grain that has landed on a receptive stigma (Figure 1) in the case of flowering plants or cones for gymnosperms.

Flowering plant pollen tubes, which are by far the most studied tip-growing cell type, have a short life-span, growing for at most a day. Furthermore, their signaling system solely responds to unique cues emitted from the ovule in the flower. In contrast, the other common model systems, root hairs and moss caulonemal cells, live relatively longer, ranging from weeks to months and both cell types respond to multiple extracellular signals, such as nutrient and water availability, gravity, and light. Thus, plants provide a unique system to study the evolution of both the initiation and maintenance of cell polarity in cell types that are separated by hundreds of millions of years of evolution, but respond to similar extracellular cues (root hairs versus rhizoids/caulonemata), and within a single plant where cells emerge in response to distinct extracellular cues (root hairs versus pollen tubes).

In plants, tip growth results from the coupling of precise cell wall remodeling at a particular site and turgor-driven cell expansion such that cells grow only at their apex. The apical domain, therefore, is a region of extensive membrane remodeling requiring exocytic delivery of vesicles carrying extensible cell wall material and endocytic retrieval of excess membranes and signaling components marking the apical domain. Here we discuss recent advances linking the cytoskeleton to directional

Figure 1



Drawings depicting tip-growing cells in mosses, liverworts and flowering plants. In plants, the products of meiosis can replicate mitotically and generate distinct tissues. The number of divisions and kinds of tissues generated vary throughout the plant kingdom. In early diverging land plants, such as mosses and liverworts, the majority of tissues are haploid having derived from these mitotic divisions. In contrast, flowering plants only undergo a few mitotic divisions after meiosis generating the ovule and pollen. Thus, the majority of flowering plant tissues develop from a fertilized embryo and are diploid. Mosses have three distinct haploid tip-growing cell types: chloronemata, caulonemata, and rhizoids. All three are multicellular. Chloronemal cells are the first to emerge from the spore and have transverse cell plates. Caulonemal cells develop from chloronemal cells and have oblique cell plates. Together these two cell types comprise branching filamentous protonemata, the juvenile tissues of moss that colonize and establish the plant. The adult tissues develop from protonemata and are depicted as a leaf-like structure in the drawing. Protruding from the base of the adult tissue are tip-growing rhizoids, which in general do not branch. Rhizoids serve to anchor the adult tissues in the soil. The moss *P. patens* is particularly amenable to genetic manipulation, regenerates quickly, is easy to propagate in the lab, and is especially suited for live-cell imaging. As a result, *P. patens* has become a powerful model for studying tip growth. Liverworts germinate from a spore into a thallus that is anchored into the soil by single-celled rhizoids. The recent sequencing of the *Marchantia polymorpha* genome [50] together with rapid advances in genome editing [51], has propelled this system for the study of evolutionary development questions in plants. While relatively fewer studies have focused on rhizoid development [52,53], *M. polymorpha* promises to be an excellent comparative system to study tip growth. Flowering plants have two tip-growing cell types: root hairs (diploid) and pollen tubes (haploid). Root hairs are single cell protrusions that emerge from the root epidermis. The pollen tube, also a single cell, grows out from the pollen when a pollen grain lands on a receptive stigma. A number of different plants have been used to study root hairs and pollen tubes, including *A. thaliana*, lily, tobacco, and rice. However, as more molecular tools emerge for diverse plant species, tip growth studies will expand to a larger number of species.

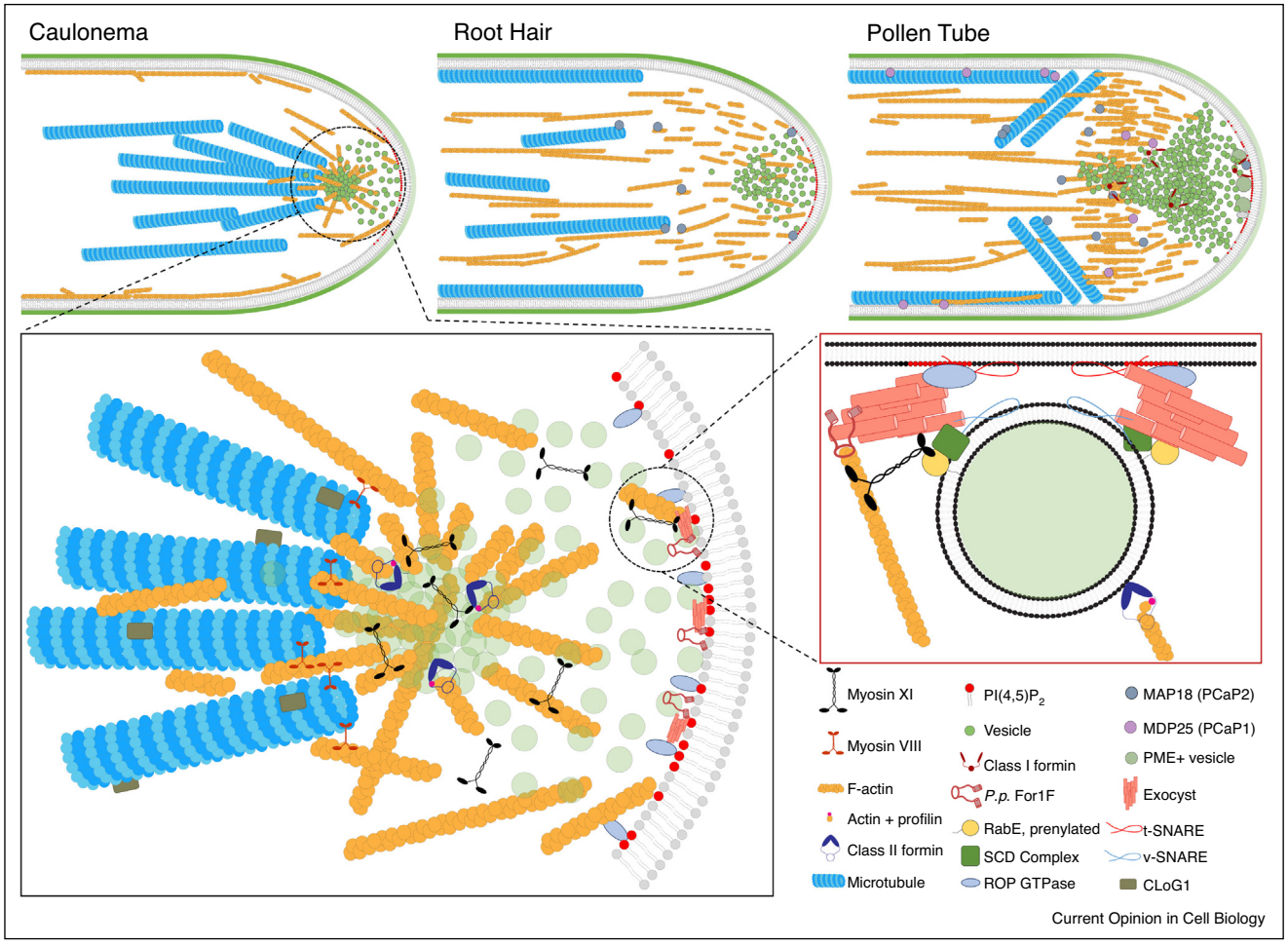
persistence during growth and possible links to membrane remodeling.

Cytoskeletal coordination

Despite differences in ultrastructural organization (Figure 2), all tip-growing systems are thought to employ the actin cytoskeleton to spatially regulate membrane trafficking to maintain unidirectional expansion. Our antiquated understanding of actin's function in tip growth was based upon the simple definition of active transport whereby molecular motors transport cargo to exocytic sites along established tracks to overcome diffusion limitations. However, F-actin is not a passive track, but rather is a dynamic entity that undergoes rapid, stochastic reorganization [1]. Furthermore, the most dynamic F-actin structures that are required for tip growth (Figure 1), such as the cortical fringe in pollen and the intense F-actin spot in caulonemal cells, demonstrate a level of organization that defies point-to-point transport. Recent studies in *Physcomitrella patens* demonstrated that the F-actin spot predicts the site of expansion [2••] and explored possible explanations of F-actin's function, discovering that while F-actin directionally transports vesicles, diffusion alone is theoretically sufficient for growth, albeit at a fraction of the normal rate [3••]. However, depletion of F-actin results in sudden growth arrest, implicating that F-actin actively clusters/focuses membranes, particularly vesicles, in a small area at the growing tip [3••,4].

The three most well-studied plant tip-growing cells types share one fundamental characteristic at the growing apex: fine, likely single, actin filaments coincident with a dynamic cloud of vesicles (Figure 2). Importantly, this organization appears interdependent, suggesting an underlying mechanism that generates and couples the polarized vesicle distribution with F-actin. In *P. patens*, the actin motor myosin XI and secretory vesicles predict the arrival of F-actin at the cell apex; and ectopically generated vesicle clusters also contain myosin XI and precede F-actin formation in the shank region of a tip cell [5]. Intriguingly, the class II formin, which also localizes with the apical actin accumulation, also precedes F-actin accumulation in similar ectopic clusters [6] and at the cell apex [2••]. Recent work investigating class I formins in the pollen tube converged upon a direct association between tip-localized vesicles, F-actin nucleators, and the emergence of actin filaments [7,8•]. As myosin XI is a relatively low-abundance protein in *P. patens*, we

Figure 2



A stylized perspective of three tip growing plant systems and a working model of exocytosis (red box). Based on published data as summarized in the text, all systems display an apically localized and interdependent pool of vesicles, myosins, and formins. This polarized organization is maintained by a dynamic F-actin network that is spatially stabilized by microtubules. In addition to a polarized cytoplasm, the apical plasma membrane is enriched in $PI(4,5)P_2$ [54], ROP GTPases, and subunits of the exocyst that facilitate actin polymerization and exocytosis. Our exocytosis model is conceptually similar to the process found in yeast. Tip-localized $PI(4,5)P_2$ and active ROPs capture the exocyst at discrete membrane sites, thereby functionally restricting the exocyst. Additionally, given the expansion of exocyst subunits in plants, it is likely different subunit composition could regulate the exocyst. The exocyst then coordinates with the secretory vesicle through vesicle-localized effectors, such as myosin XI, Rab GTPases, and their regulators, such as the putative GEF, the SCD complex.

speculate the mechanism described above in concert with homotypic interactions between vesicle-localized Rabs [9] and other unknown processes could promote clustering resulting in a liquid–liquid phase separation and the characteristic vesicle cluster that drives tip growth. This hypothesis is inspired by the recently proposed diffusion capture paradigm by Bracha *et al.* and offers a fascinating avenue of future investigation [10].

Traditionally, the study of tip growth in plants disproportionally focused on the actin cytoskeletal network as it is indispensable for tip growth. However, recent studies demonstrate that microtubules are critical for growth directionality and implicate microtubules and F-actin as an increasingly interconnected system for maintenance of polarity. In *P. patens* microtubules function in polarity fidelity by spatially restricting actin polymerization and vesicle clustering [2••,11••] (Figure 2). One possible mechanism that could explain the observed restrictive effect of microtubules on F-actin is through direct microtubule-to-actin interactions. Microtubule and actin cross talk appears to be a consistent feature of tip-growing systems given direct observations in multiple cell types. In *P. patens*, myosin VIII interacts with microtubules and actin to promote persistent polarized growth [2••] (Figure 2). Recent evidence using a conditional forward genetics screen in moss, identified CLoG1, a novel microtubule depolymerizing end

tracking protein, which is involved in tip growth and localizes to zones of microtubule-F-actin overlaps at the tip [12•] (Figure 2). In angiosperms, the microtubule-associated proteins MDP25/PCaP1 and MAP18/PCaP2 associate with actin and demonstrate calcium-dependent severing activity [13,14] (Figure 2). In addition to direct crosslinking of microtubules to actin, studies have revealed interactions between microtubules and F-actin nucleators, such as formins [15–18] and a subunit of the ARP2/3 complex [19]. This raises the intriguing possibility of microtubules orchestrating F-actin organization through assisting the clustering of vesicles rich in actin nucleators.

Exocytosis

The rapidly expanding cell requires faithful intracellular trafficking of secretory vesicles containing cargo that facilitates the extension of the cell wall. Our mechanistic understanding of polarized trafficking and exocytosis is surprisingly lacking, which we suggest is a consequence of insufficient tools to mark and observe active areas of exocytosis. A quantification method, based on relative levels of exocytosis, corrected for endocytosis, is a welcome development for this problem [20].

As a first approximation, by analyzing the localization and loss of function phenotypes of various exocyst subunits, which is a tethering complex for exocytic vesicles, there is evidence in caulonemata, root hairs, and some pollen tubes that exocytosis is enriched at the apical plasma membrane [21–23,24•,25•] (Figure 2). We know from yeast and mammalian studies that the exocyst serves as a multivalent platform orchestrating exocytosis through interactions of plasma membrane and vesicle-localized effectors, such as the molecular motor myosin V and the Rab GTPase, Sec4 [26]. An emerging hypothesis suggests that in plants myosin XI associates with its vesicular cargo through an interaction with a Rab GTPase on the vesicle membrane and then with the exocyst for vesicle tethering at the plasma membrane (Figure 2). This transport model is supported by preliminary evidence demonstrating a myosin XI interaction with the RabE subfamily, which is homologous with the yeast Rab Sec4 [27]. Furthermore, biochemical and proteomic data places RabE, its putative GEF (Figure 2, SCD complex), and the exocyst complex at sites of polarized exocytosis [28•]. The remarkable degree of similarity between two evolutionarily distant clades should empower us to not only use insights from yeast and mammals to inform our future experiments but opens the possibility that other fundamental findings in plants are shared between all polarized growth cell types.

Despite these advancements, it is likely that the seemingly homogenous vesicle cluster we observe at the mesoscale is an amalgamation of different vesicle subpopulations that are decorated by a unique complement of proteins and internal cargo. This is evidenced by a variety of unconventional secretion pathways [29], such as pectin methylesterase bypassing the TGN in pollen tubes [30]. As a result, while the highest vesicle flux may occur at the tip, exocytosis occurs across the entire surface of the cell, as is observed for the cellulose synthase complex [31]. Thus, identifying cargo that is specifically secreted at sites of polarized expansion will improve our ability to confidently detect specific exocytic events that regulate polarized growth.

The apical plasma membrane

Among proteins that are enriched at the apical plasma membrane, ROP GTPases are critical signaling molecules. In *P. patens*, as in other plants, ROPs are essential for polarized growth [32,33]. Systematic silencing of all the ROP regulators in moss revealed that of the GTPase activating proteins (GAPs) the ROPGAP family, not the REN family, are critical for polarized growth. Silencing of the guanine nucleotide exchange factors (GEFs) demonstrated that the SPIKE type of GEFs, known activators of ROP activity [34], were more important for cell polarization than the ROPGEFs. Moreover, the guanine dissociation inhibitors (GDIs), which localized throughout the cytoplasm, were also essential for cell polarity [35]. Using both endogenous knock-in of sequences encoding for fluorescent proteins coupled with inducible expression strategies, another recent study revealed apical localization of PpROP1 and PpROPGEF4, with PpROPGEF4 populating a more confined region at the extreme tip [36]. However, validating function of the fluorescent fusion proteins can be challenging, particularly for ROP, for which genomic alterations including fusions to fluorescent proteins were unable to support normal polarized growth in the absence of all other endogenous ROPs [33]. Thus, it will be critical to identify additional polarity markers amenable to tagging and live-cell imaging.

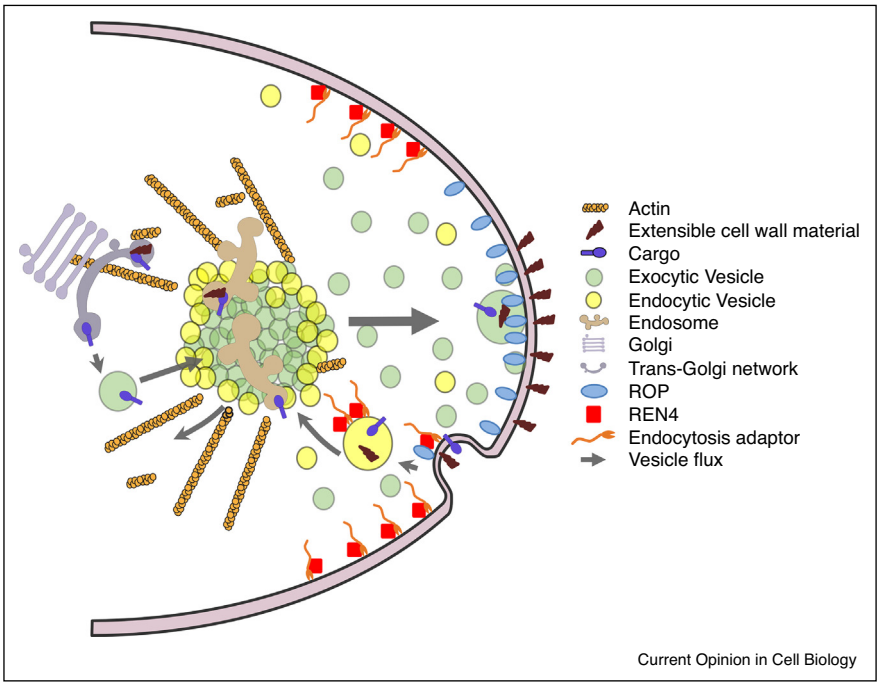
Recent publications are illuminating mechanistic details of ROP polarization. Denninger and collaborators demonstrated that successive polarization of distinct ROP-GEFs and subsequent ROP recruitment promotes root hair initiation and outgrowth [37•]. Furthermore, in root epidermal cells ROP6 partitions to discrete nanodomains through an interaction between ROP's intrinsic polybasic region and the anionic phospholipid phosphatidylserine [38••]. It remains an open question how ROP-GEFs, anion phospholipids, and other signals are integrated to drive the initial polarization event. Recent work in root hairs has also discovered that the microtubule-actin crosslinking protein MAP18 preferentially interacts with the GDP-bound state of ROP2 [39••]. This interaction, which may occur at the cytoskeleton, effectively promotes ROP activity by shielding ROP from extraction by the ROP-GDI, providing an exciting link between the cytoskeleton and ROP activity.

Endocytosis

Retrieval of membrane from the apex via endocytosis also plays a fundamental role in polarizing cells [40]. In root hairs endosome motility and FM4-64 uptake were both impaired in the absence of actin [41,42]. Molecular evidence linking endocytosis to polarized growth was provided by studies of the T-PLATE endocytic adaptor complex. T-PLATE was found to be enriched at the emerging tip of germinating pollen in Arabidopsis. Critically, mutations of subunits in the T-PLATE complex impair pollen tube germination in Arabidopsis, resulting in accumulation of callose at ectopic sites in the pollen grain [43]. More recently, inhibition of clathrin-dependent endocytosis in pollen tubes was shown to impair tip growth [44]. Thus, with impaired endocytosis, delivery of wall material is deregulated, suggesting that endocytosis contributes to regulating and or establishing the site of polarized growth during pollen tube germination and growth.

However, evidence of direct molecular links between endocytosis, the cytoskeleton and tip growth is surprisingly lacking. Of note, a recent study discovered that the Endocytosis Adaptor of Pollen Tube, EAP1, specifically binds to REN4, which is a GAP for the ROP GTPase. EAP1 participates in clathrin-mediated endocytosis and is involved in the active removal of REN4 from the cell apex [45••]. As GAPs, REN proteins activate the intrinsic GTPase activity of ROPs, converting them to a GDP-bound state, which is inactive for cellular signaling. Thus, RENs could limit ROP activity to particular membrane domains [32]. In particular loss of REN4 impairs pollen tube directionality, implicating that REN4 works to control a uniform site of polarized expansion [45••]. Interestingly, silencing RENs did not alter tip growth in moss caulonemata [35]. While the exact family of GAP is not conserved between pollen tubes and moss, proteins with similar functions still impact cell polarity. Nevertheless, a physical connection between a ROP regulator and endocytosis supports the idea that endocytosis actively ensures that the apical plasma membrane is a distinct membrane domain. Furthermore, based on the localization of EAP1, this leads to a model where endocytosis occurs at the collar region in pollen tubes [45••], which coincides with the highly dynamic actin fringe. It remains to be determined where endocytosis occurs in other tip-growing cells where the highly dynamic actin resides closer to the apical plasma membrane.

Figure 3



Model depicting the apex of a generic tip-growing plant cell as a cycling center coordinating exocytic and endocytic activity to optimally deliver growth material while simultaneously marking the apical plasma membrane as a distinct membrane domain. Grey arrows indicate vesicle trafficking pathways. ROP localizes to the apical plasma membrane, while REN4 localizes at the subapical plasma membrane, which in pollen tubes is in the collar region overlapping with the cortical actin fringe, to restrict ROP in a confined domain. In other tip growing cells the apical actin structure is closer to the tip, so we have depicted an endocytic event between the tip and the subapical domain. The removal of REN4 from the plasma membrane depends on its interaction with an endocytosis adaptor. Cargos for polarized growth are removed from the plasma membrane through endocytosis, then fused to the endosome. After briefly visiting the trans Golgi network, cargos are exocytosed back to the apical plasma membrane potentially through endosomes and exocytic vesicles. Actin organizes trans Golgi, endosomes and the cluster of vesicles near the growing tip. Cargoes, labeled in purple that are actively secreted at the cell tip, such as potential cell wall synthases or remodeling enzymes would be ideal targets for cellular imaging.

Conclusions

There are striking parallels between tip growth in plant cells and in filamentous fungi. For example, in *Aspergillus nidulans*, a dynamic cluster of membranes and actin forms near the cell apex [46]. Many of the same classes of molecules that are required in plants are also required in fungi [46,47]. Based on elegant studies, a current model posits that chitin synthase B, a cell wall-modifying enzyme essential for tip growth, is recycled from the apical plasma membrane to the trans Golgi network (TGN) via endosomes, with endocytosis of chitin synthase-dependent on actin. From the TGN, chitin synthase then gets rapidly exocytosed to the plasma membrane [48,49]. Even though tip growth likely evolved independently in plants and fungi, the striking similarities may help to inform studies in both systems.

Combining knowledge from both fungi and plants, we hypothesize that the apex of the cell is a cycling center for endosomes and the TGN, and that dynamic actin is central in coordinating endocytosis and exocytosis at the expanding tip (Figure 3). To test this, it will be essential to determine how exocytic cargo is sorted before reaching the apical domain. Thus, it is a priority to identify and image functional cargos that are specifically delivered to the tip (Figure 3) analogous to chitin synthase from filamentous fungi. Future work building upon the discovery of the physical link between ROP regulators and endocytosis [45••] (Figure 3) in additional plant cell types will provide the foundation for analyzing the dynamics and potential interdependence between endocytosis and cell polarity. Ultimately, determining the temporal and spatial polarization of exocytic, endocytic, and cytoskeletal factors during tip growth initiation will help to establish whether exocytosis, endocytosis, and/or the cytoskeleton generates the polarity cue. Then integrating cytoskeletal interactions and identifying molecular linkages that coordinate membrane trafficking will provide testable models for maintenance of polarized growth via possible feedback tying membrane trafficking and cytoskeletal organization.

Conflict of interest statement

Nothing declared.

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