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REVIEW PAPER

Exocytosis and endocytosis: coordinating and fine-tuning the polar tip growth domain in pollen tubes

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Abstract

Pollen tubes rapidly elongate, penetrate, and navigate through multiple female tissues to reach ovules for sperm delivery by utilizing a specialized form of polar growth known as tip growth. This process requires a battery of cellular activities differentially occurring at the apical growing region of the plasma membrane (PM), such as the differential cellular signaling involving calcium (Ca^{2+}), phospholipids, and ROP-type Rho GTPases, fluctuation of ions and pH, exocytosis and endocytosis, and cell wall construction and remodeling. There is an emerging understanding of how at least some of these activities are coordinated and/or interconnected. The apical active ROP modulates exocytosis to the cell apex for PM and cell wall expansion differentially occurring at the tip. The differentiation of the cell wall involves at least the preferential distribution of deformable pectin polymers to the apex and non-deformable pectin polymers to the shank of pollen tubes, facilitating the apical cell expansion driven by high internal turgor pressure. Recent studies have generated inroads into how the ROP GTPase-based intracellular signaling is coordinated spatiotemporally with the external wall mechanics to maintain the tubular cell shape and how the apical cell wall mechanics are regulated to allow rapid tip growth while maintaining the cell wall integrity under the turgor pressure. Evidence suggests that exocytosis and endocytosis play crucial but distinct roles in this spatiotemporal coordination. In this review, we summarize recent advances in the regulation and coordination of the differential pectin distribution and the apical domain of active ROP by exocytosis and endocytosis in pollen tubes.

Keywords: Cell wall, endocytosis, exocytosis, pollen tube, ROP GTPase, tip growth.

Introduction

Higher plants evolved a specialized male gametophyte, the pollen tube, which contains two sperm cells but expands as a single cell because pollen tube growth is governed by its haploid vegetative nuclear genome. Upon landing on the surface of the stigma, pollen grains rehydrate and germinate pollen tubes, which grow rapidly and directionally to the female reproductive cells (Higashiyama and Takeuchi, 2015; Higashiyama, 2018; Johnson *et al.*, 2019). Pollen tubes penetrate and navigate through a number of female sporophytic tissues and deliver the two sperm cells into the ovule for double fertilization. The directional growth toward the ovule is guided by attractants produced in the ovule (Higashiyama *et al.*, 2001; Okuda *et al.*, 2009; Lausser *et al.*, 2010; Kanaoka *et al.*, 2011; Palanivelu and Tsukamoto, 2012; Takeuchi and Higashiyama, 2012, 2016; Johnson *et al.*, 2019). Pollen grains also produce pollen tubes *in vitro* on pollen germination medium. Although the direction

of pollen tube elongation is random in the absence of attractants (Kim *et al.*, 2003), they expand unidirectionally with uniform shape, suggesting a self-organizing mechanism for precise maintenance of growth polarity. Easy manipulation and observation of the pollen tube have made it one of the most popular cell models to study the mechanism of polar cell expansion.

Pollen tubes elongate by tip growth, an extreme form of polar growth in which exocytosis is targeted to the apical region for apical cell surface expansion (Heslop-Harrison, 1987; Derksen *et al.*, 1995; Hepler *et al.*, 2001; Cheung and Wu, 2008; Kroeger *et al.*, 2009; Grebnev *et al.*, 2017; Luo *et al.*, 2017; Bibeau *et al.*, 2018). This tip-localized cell surface expansion needs to be tightly coordinated with the differential cell wall mechanics, because of high internal turgor pressure that inflates upon the cell wall (Vogler *et al.*, 2019). Therefore, the shape of pollen tubes, the rate of their elongation, and maintenance of their integrity are intimately linked to the differential stiffness and extensibility of the cell wall in the apex (Vogler *et al.*, 2019). Such an internal pressure-coordinated system begs two intriguing questions: (i) how is the deposition and modification of the cell surface materials coordinated with the cell wall mechanics that accommodate turgor pressure-driven cell expansion, and (ii) how do pollen tubes maintain their wall integrity while allowing the apical expansion of the cell wall inflated by the turgor pressure?

The cell wall of pollen tubes in higher plants consists of pectins, callose, cellulose, and hemicelluloses (Dardelle *et al.*, 2010; Chebli *et al.*, 2012). The shape of pollen tubes is, however, determined by the mechanical property of the cell wall in the tip region, where it is devoid of callose (Geitmann *et al.*, 1995; Ferguson *et al.*, 1998; Chebli *et al.*, 2012). While the presence of cellulose and hemicellulose in the apical cell wall of pollen tubes varies among different plant species (Ferguson *et al.*, 1998; Chebli *et al.*, 2012), pectins have been consistently identified as the major component of the cell wall in the tip of pollen tubes across plant species (Li *et al.*, 1994; Geitmann *et al.*, 1995; Jauh and Lord, 1996; Bosch *et al.*, 2005; Parre and Geitmann, 2005). Homogalacturonan (HG), rhamnogalacturonan-I (RG-I), and RG-II are the three major types of pectin found in pollen tubes, contributing to the bearing of internal turgor pressure (Dardelle *et al.*, 2010). Genetic evidence has demonstrated that both cellulose and RG-II are essential for the growth of pollen tubes by maintaining the cell wall integrity (Delmas *et al.*, 2008; Kobayashi *et al.*, 2011; Liu *et al.*, 2011; Wang *et al.*, 2011; Dumont *et al.*, 2014). On the other hand, the structure and mechanics of HGs (hereinafter referred to as the 'pectin') are highly regulated spatiotemporally, and thus have been considered to be the major wall factor that determines the shape and growth rate of pollen tubes (Geitmann *et al.*, 1995; Bosch *et al.*, 2005; Parre and Geitmann, 2005; Kroeger *et al.*, 2009; Fayant *et al.*, 2010; Chebli *et al.*, 2012). The pectin is synthesized in the Golgi apparatus in a highly methylesterified form and is demethylesterified by pectin methylesterase (PME), whose activity can be inhibited by the endogenous PME inhibitor (PMEI). Demethylesterified pectin molecules cross-link via Ca^{2+} bridges (known as the egg-box model) and are much less deformable under turgor pressure, whereas highly methylesterified pectin, lacking Ca^{2+} bridges, is inflatable by turgor pressure. Thus, the mechanical property of the pectin is determined by the level of methylesterification that is controlled by the relative activities of PME and PMEI. Methylesterified pectin, PME, and PMEI are all secreted to the apex of the pollen tube through polar exocytosis (Li *et al.*, 2002; Chebli *et al.*, 2013; Wang *et al.*, 2013), yet the highly methylesterified pectin and the demethylesterified pectin are preferentially distributed to the growing apical region and the shank of pollen tubes, respectively (Bosch *et al.*, 2005; Fayant *et al.*, 2010; Chebli *et al.*, 2012; Wang *et al.*, 2013; Luo *et al.*, 2017). Both PME and PMEI were found to be preferentially distributed to the apical wall as well (Bosch *et al.*, 2005; Rockel *et al.*, 2008; Wang *et al.*, 2013). It was proposed that in tobacco pollen tubes, differential endocytosis in the shoulder region may contribute to the maintenance of the apical distribution of PMEI (Rockel *et al.*, 2008), but how PME and PMEI maintain the tip-high distribution and how the highly methylesterified and demethylesterified pectin are differentially distributed to the apex and the shank are not fully understood, but are conceivably linked to the intracellular regulatory mechanisms underlying apical cell expansion in pollen tubes.

A great deal of work has focused on intracellular regulatory mechanisms underlying pollen tube tip growth, such as those involving the plasma membrane (PM)-localized receptor kinases and cytoplasmic kinases, Ca^{2+}, phospholipids, ROP-type Rho GTPases, fluctuation of ions and pH, cytoskeletal organization and dynamics, and vesicular trafficking. The readers are referred to several excellent recent reviews discussing the roles of various signaling events in the regulation of pollen tube growth, including PM receptors (Higashiyama, 2018; Muschietti and Wengier, 2018; Ge *et al.*, 2019; Johnson *et al.*, 2019), intracellular Ca^{2+} (Steinhorst and Kudla, 2013; Zheng *et al.*, 2019), phospholipids (Sekeres *et al.*, 2015; Heilmann and Ischebeck, 2016), and the fluctuation of the ions and pH (Tavares *et al.*, 2011; Michard *et al.*, 2017; Mangano *et al.*, 2018). This review is intended to focus on how cell wall construction and remodeling are interlinked with the intracellular regulatory mechanisms with an emphasis on ROP signaling in the feedback regulation and its integration with vesicular trafficking that interfaces with both ROP signaling and cell wall construction and remodeling. The evolutionarily conserved Rho GTPases, namely ROPs (Rho-like GTPase from plants), are key regulators of tip growth in pollen tubes (Lin *et al.*, 1996; Kost *et al.*, 1999; Li *et al.*, 1999; Yang, 2002, 2008; Chen *et al.*, 2003; Klahre *et al.*, 2006; Cheung and Wu, 2008; Feiguelman *et al.*, 2018). ROP activation is essential for pollen tube elongation, whereas its deactivation is critical for the maintenance of growth polarity (Hwang *et al.*, 2005, 2010; H. Li *et al.*, 2018). Moreover, the growth domain of the pollen tube is defined by the apical cap of ROP activity (Fig. 1A), which is spatiotemporally regulated by two opposing feedback mechanisms (Yan *et al.*, 2009; Hwang *et al.*, 2010; Luo *et al.*, 2017). RopGEF (Rop guanine nucleotide exchange factor)-dependent positive feedback mediates lateral propagation of ROP activity (Gu *et al.*, 2006; Zhang and McCormick, 2007; Chang *et al.*, 2013; E. Li *et al.*, 2018), whereas negative feedback limits this propagation by RopGAP (Rop GTPase activating protein)-dependent ROP deactivation and possibly

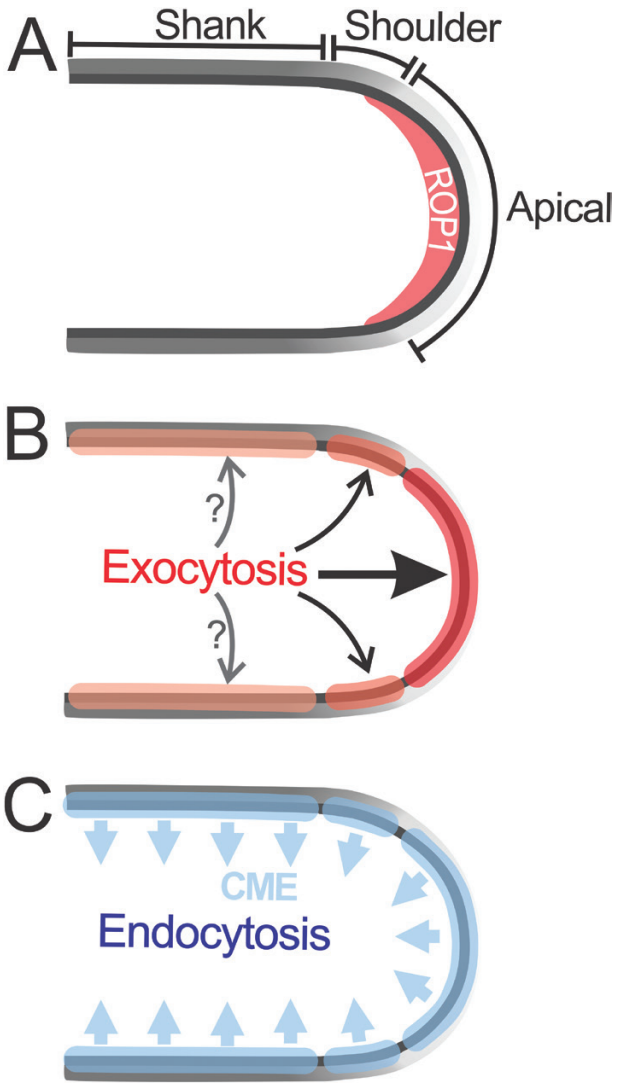

Fig. 1. Proposed sites of exocytosis and endocytosis on the PM of pollen tubes. (A) PM of pollen tubes can be divided into three regions, the apical, shoulder, and shank region, based on the distribution of distinct molecules along the cell surface. An apical cap of active ROP1 defines the growth domain of pollen tubes. (B) The bulk of exocytosis occurs in the apical region, while certain subpopulations of exocytic vesicles may also be secreted to the shoulder region. Evidence also hints at the likely existence of an exocytic site in the shank region. Further identification of the cargos delivered to the shoulder and the shank regions is required to confirm the exocytosis events in the above two sites. (C) Clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) have been proposed to occur in both the apical region and the shank region of pollen tubes; however, only vesicle internalization by the CME has been experimentally observed to occur in the apical, shoulder, and shank region.

by RhoGDI (Rho GTPase guanine nucleotide dissociation inhibitor)-dependent ROP extraction from the PM (Fu *et al.*, 2001; Klahre and Kost, 2006; Klahre *et al.*, 2006; Hwang *et al.*, 2008; Feng *et al.*, 2016). The active ROP interacts with a battery of downstream effectors to organize and coordinate polar vesicle targeting secretion. ROPs activate RIC (ROP-interactive CRIB motif-containing) proteins, which modulate the dynamics of tip-localized actin filaments required for vesicle targeting and docking to the tip (Wu *et al.*, 2000; Gu *et al.*, 2005; Lee *et al.*, 2008). The ROPs may also activate the RIP1/ICR1 (ROP-interactive partner 1/interactor of constitutively active ROPs 1) protein to directly recruit the exocytic machinery (Lavy *et al.*, 2007; Li *et al.*, 2008).

Although the ROP signaling and the cell wall deposition are instrumental for the polar growth of pollen tubes, it is not well understood how these two processes are spatiotemporally coordinated during rapid growth. In particular, little is known about how the signaling network controlled by the ROP GTPase instructs the cell wall deposition in response to various external signals. Recent studies have suggested that exocytosis and endocytosis may coordinate the ROP signaling and the deposition of the cell wall by secreting and gatekeeping of the signal molecules and the cell wall modification enzymes (Luo *et al.*, 2017; H. Li *et al.*, 2018). In this review, we summarize our current understanding of how exocytosis and endocytosis shape the spatial distribution of the active ROP domain and the pattern of the cell wall deposition, and how the ROP signaling directs the deposition of the cell wall through steering the polar exocytosis machinery.

Membrane trafficking during pollen tube tip growth

Pollen tube growth requires active membrane trafficking for the cell surface increase and the spatiotemporal coordination of regulatory molecules. Exocytosis delivers the cell membrane, cell wall materials, and signaling molecules to the apical expanding region (Li *et al.*, 2002; Lee *et al.*, 2008; Wang *et al.*, 2013; Bloch *et al.*, 2016; Luo *et al.*, 2017), while endocytosis retrieves excess membrane (Picton and Steer, 1983; Derksen *et al.*, 1995; Bove *et al.*, 2008; Ketelaar *et al.*, 2008) and signaling molecules to maintain the boundary of their active PM domains (H. Li *et al.*, 2018). An alternative mode of exocytosis, kiss-and-run exocytosis, where secretory vesicles release their content through partial fusion between the membrane of the exocytic vesicle and the PM and leave the PM after the closure of the vesicles, is also proposed to mediate the fast polar secretion without delivering excess membrane materials (Bove *et al.*, 2008). Although this alternative exocytosis represented a major type of exocytosis in tobacco BY-2 protoplasts and maize coleoptile protoplasts (Weise *et al.*, 2000; Bandmann *et al.*, 2011), it has not been directly observed in pollen tubes and is unlikely to regulate the abundance or spatial distribution of PM-residing signaling proteins. Identification of the PM regions where exocytosis and endocytosis occur is critical for understanding how these processes regulate tip growth in pollen tubes. Based on the distribution of various intracellular and extracellular molecules along the cell surface, the pollen tube PM can be divided into three regions (Fig. 1A): the apical region containing active ROPs; the shoulder region with underneath distribution of longitudinally arranged actin bundles known as the actin fringe; and the shank region with various components of the clathrin-mediated endocytosis machinery (Hwang *et al.*, 2005; Lovy-Wheeler *et al.*, 2005; Gadeyne *et al.*, 2014; Rounds *et al.*, 2014; H. Li *et al.*, 2018; Muro *et al.*, 2018; Kaneda *et al.*, 2019).

The site of exocytosis in pollen tubes has been studied using various methods, and two contrasting models have been proposed. Two studies proposed the PM at the shoulder region as the site of exocytosis in pollen tubes by microscopic analysis of vesicles labeled with lipophilic styryl FM dyes (Bove *et al.*, 2008; Zonia and Munnik, 2008). However, without directly visualizing the cargos delivered through exocytosis, these two studies studied the trafficking events in the tip of pollen tubes by observing the dynamics of FM dye-labeled membrane compartments via advanced microscopy techniques, such as FRAP (fluorescence recovery after photobleaching) and STICS (spatiotemporal image correlation spectroscopy). Considering the highly dynamic trafficking events at the apex of pollen tubes, these indirect observations might not be efficient in distinguishing actual exocytosis events from other membrane trafficking events, such as endocytosis and membrane dilution (Luo *et al.*, 2016). In addition, the evidence supporting exocytosis at the subapical PM by differential interfence contrast (DIC) imaging of vesicle fusion events is misleading (Zonia and Munnik, 2008), because at the tip region it is unable to resolve the fusion events of small vesicles (Derksen *et al.*, 1995), which are under the detection limit of the optical microscope used in this study.

Electron microscopic observation has indicated the accumulation of exocytic vesicles at the extreme tip region (Derksen *et al.*, 1995). This is further supported by observing the secretion site of the cargos transported by polar exocytosis, such as pectin (Bloch *et al.*, 2016; Luo *et al.*, 2017), PME (Wang *et al.*, 2013), and a membrane-localized receptor-like kinase (PRK1) (Lee *et al.*, 2008; Luo *et al.*, 2016). By correcting the effect of endocytosis and membrane dilution, Luo *et al.* (2016) accurately measured the relative rate of exocytosis at the apical region of Arabidopsis pollen tubes and found that the apex of the PM has higher exocytosis activity than the shoulder region of the PM. These results together demonstrate that the bulk of exocytosis occurs in the apical region but does not exclude the existence of exocytosis in the shoulder region for some subpopulations of exocytic vesicles (Fig. 1B). Interestingly, while subunits of the exocyst complex in plants such as SEC3 (Bloch *et al.*, 2016), SEC6, SEC8, and Exo70A1 (Hala *et al.*, 2008) showed polar distribution in an apex-rich pattern, SEC3 can redistribute to the shoulder and shank region of the PM in non-growing pollen tubes (Bloch *et al.*, 2016). A subcellular localization study of the members in the exocyst subunit EXO70 family in tobacco pollen tubes also revealed distinct PM domains of potential exocyst targeting sites (Sekeres *et al.*, 2017). These observations suggest a likely existence of multiple distinct sites of exocytosis on the PM of pollen tubes, but identification of cargos delivered to the shoulder region is needed to confirm the exocytosis site at the shoulder of pollen tubes. It should be noted that the exact locations of exocytosis may differ in pollen tubes from different species, given the large variations in diameters and growth speed of pollen tubes among different species.

The mapping of endocytosis sites in pollen tubes was carried out by tracing the membrane trafficking events with FM dyes and charged nanogold particles (Moscatelli *et al.*, 2007; Bove *et al.*, 2008; Zonia and Munnik, 2008). It was suggested that clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) occurred in both the apical and shank regions (Moscatelli *et al.*, 2007). Indeed, the clathrin light chain (CLC) and heavy chain (CHC) proteins have been localized to the apical, shoulder, and the shank regions of the pollen tube PM (Blackbourn and Jackson, 1996; Zhao *et al.*, 2010; H. Li *et al.*, 2018; Muro *et al.*, 2018; Kaneda *et al.*, 2019), and the internalization of CLC has been observed in all of these regions by live imaging in two recent studies (H. Li *et al.*, 2018; Kaneda *et al.*, 2019) (Fig. 1C). Internalization of active ROP1 by REN4-activated CME at the apical PM of slow-growing pollen tubes and the PM at the shoulder region of fast-growing pollen tubes further demonstrates the importance of CME for dynamic regulation of the active ROP1 domain during pollen tube growth (H. Li *et al.*, 2018) (see below). Hence, selective internalization of cargos by endocytosis at multiple PM regions may allow robust dynamic demarcation of functionally distinctive PM domains.

Roles of endocytosis and exocytosis in directing the cell wall deposition/modification and maintaining cell wall integrity

Exocytosis and endocytosis regulate the mechanical property of the cell wall in pollen tubes by forming two pectin zones with distinct levels of pectin methylesterification, a soft expandable zone with highly methylesterified pectin at the expanding tip and a stiff zone in the shank region with demethylesterified pectin that is cross-linked by Ca^{2+} bridges (Chebli *et al.*, 2013). Dynamic adjustment of the soft pectin zone determines the rate and direction of the pollen tube elongation and is essential for navigating the pollen tube to female reproductive cells (Fig. 2) (Luo *et al.*, 2017). Polar exocytosis delivers highly methylesterified pectin, PME, and PMEI to the expanding tip. Based on the distribution of AtPPME1 and AtPMEI2 in tobacco pollen tubes, it was proposed that the PME activity is inhibited by PMEI in the apical region to maintain the methylesterified status of pectin, and that PMEI is internalized by endocytosis in the shoulder region to establish a border between the two pectin zones by releasing the activity of PME, which converts pectin to the demethylesterified form (Rockel *et al.*, 2008). Furthermore, defects in pollen tube guidance or reduced pollen tube growth were frequently observed to be associated with an abnormal pattern of pectin accumulation and pectin modification when proteins involved in polar exocytosis, such as RABA4d (Szumlanski and Nielsen, 2009) and Exo70C2 (Synek *et al.*, 2017), were compromised. Similarly, mutations on genes encoding Arabidopsis AP180 N-terminal homolog (ANTH) proteins, AtAP180 and AtECA2, which are CME adaptor proteins, also generate abnormal pectin zones with mislocalized demethylesterified pectin and produce pollen tubes with morphological aberration (Kaneda *et al.*, 2019). It is therefore almost certain that exocytosis and endocytosis are indispensable for the pectin dynamics in the cell wall, but it is still unanswered how exocytosis and endocytosis mediate the establishment of the specific pattern of pectin zones and how

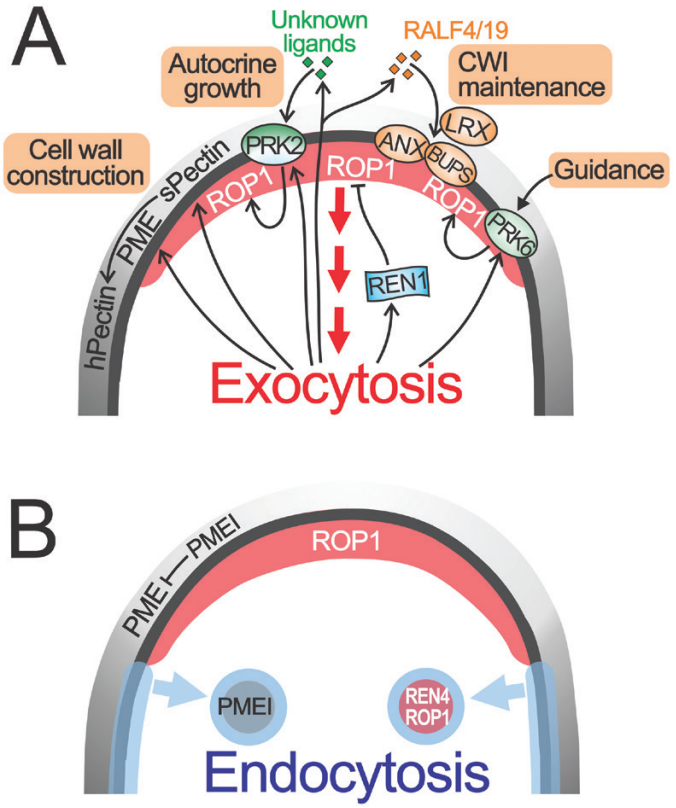

Fig. 2. Role of exocytosis and endocytosis in the coordination and fine-tuning of the active ROP1 domain and the cell wall dynamics. (A) Reciprocal regulation of polar exocytosis and ROP signaling coordinated the active ROP1 domain and cell wall deposition. ROP GTPase organizes and coordinates polar secretion by regulating the dynamics of the actin filaments and recruiting the exocytosis machinery through a battery of downstream effectors. Exocytosis regulates the pollen tube growth and guidance through multiple pathways. (i) Exocytosis delivers building blocks of the cell wall, including pectin, PME, and PMEI to the apical growth domain, where the PME activity is inhibited by the PMEI to maintain the soft methylesterified status of pectin (sPectin). In the shoulder region, where the activity of PME is released due to internalization of the PMEI, pectin is demethylesterified by PME and cross-linked via Ca^{2+} bridges to form a hard network of stiff pectin polymers (hPectin). (ii) Exocytosis mediates positive feedback regulation on the ROP signaling by polar secretion of ligands and their receptors (PRK2) to the growth domain in an autocrine manner. (iii) Exocytosis delivers REN1 RopGAP to the tip of pollen tubes for global inhibition of active ROP1. (iv) Exocytosis participates in maintenance of the cell wall integrity (CWI) by secreting signal peptides (RALF4/19) and CWI sensor LRX family proteins; the secreted RALF4/19 peptides are also sensed by the PM-localized ANX1/2 and BUPS1/2 protein complexes. (v) Preferential delivery of the receptor-like kinase PRK6, the receptor for the guidance peptides AtLUREs, to the side of the PM that facing the AtLUREs is likely to be mediated by polar exocytosis. (B) Endocytosis shapes growth domains in pollen tubes using at least two mechanisms: it releases the activity of AtPPME1 by selective internalization of its inhibitor AtPMEI2 in the shoulder region and establishes a border between the two pectin zones; and it internalizes active ROP1 by REN4-mediated endocytosis in the apical and the shoulder region on the PM to maintain an optimal level of active ROP1 in the extreme apex and control the robustness of growth directionality in pollen tubes.

they integrate inputs from both the internal signaling network and the external positional cues, such as guidance signals.

Recent studies suggest the involvement of exocytosis and endocytosis in regulating cell wall integrity maintenance during pollen tube growth. Pollen tube elongation is driven by a massive turgor pressure of ~0.2 MPa (Benkert *et al.*, 1997), therefore the cell wall of pollen tubes is under enormous mechanical stress. Cell wall integrity (CWI)-sensing modules, including extracellular RAPID ALKALINIZATION FACTOR 4/19 (RALF4/19) ligands and their receptors LEUCINE-RICH REPEAT EXTENSIN (LRX) family proteins and several membrane-localized CrRLK1L receptor kinases [Buddha's Paper Seal 1/2 (BUPS1/2) and ANXUR1/2], are required to maintain the CWI (Ge *et al.*, 2017; Mecchia *et al.*, 2017). Obviously, the secretion of the extracellular RALF4/19 and LRX proteins relies on functional exocytosis. Furthermore, the recycling of ANXUR1/2 to the tip of the pollen tube was reported to require loading by two ANTH domain-containing proteins, PICALM5a and PICALM5b, which are both involved in the endocytic pathway (Muro *et al.*, 2018). Damping the CWI-sensing signal by reducing the expression level of RALF4/19 enlarges the soft zone of methylesterified pectin and results in pollen tube burst (Mecchia *et al.*, 2017). Therefore, CWI-sensing feedback regulates the distribution of the pectin zones. Further elucidation of the downstream CWI signaling pathways will be necessary for understanding how the CWI-sensing feedback regulates exocytosis- and endocytosis-mediated cell wall delivery and modification.

Missing links between ROP signaling and organization of actin filaments

Polar exocytosis requires the apical accumulation of exocytic vesicles in pollen tubes, which is thought to be independent of microtubules but relies on actin filaments (Cardenas *et al.*, 2008; Bou Daher and Geitmann, 2011; Rounds *et al.*, 2014; Fu, 2015; Qu *et al.*, 2017). Early studies using immunostaining or fluorescent protein labeling methods have revealed distinct structural organization of actin filaments in different regions of pollen tubes, including an array of actin bundles (cables) in the shank region and a cortical fringe (collar) of short parallel actin cables around the shoulder region (Lovy-Wheeler *et al.*, 2005; Cheung *et al.*, 2008). It is believed that the array of actin bundles in the shank region supports organelle movement and cytoplasmic streaming through the actomyosin system, while the more dynamic actin fringe mediates targeted exocytosis of signaling components and cell wall materials to the growing tip (for reviews, see Cheung and Wu, 2008; Yang, 2008; Chebli *et al.*, 2013; Cai *et al.*, 2015; Hepler and Winship, 2015). However, with the development of better labeling methods and more sensitive microscopic instruments, two populations of actin filaments have been visualized to be polymerized from the apical PM: one population extends in the shank-ward direction which forms the actin fringe, and the other novel population of actin filaments extends to the inside of the pollen tube (Fu *et al.*, 2001; Gu *et al.*, 2005; Liu *et al.*, 2015; Zhang *et al.*, 2016; Qu *et al.*, 2017; Lan *et al.*, 2018). This novel population of internal actin filaments has been observed to prevent the entry of large organelles into the clear cone and act together with the actin fringe to facilitate the formation of the 'V-shaped' cone region (Fu *et al.*, 2001; Gu

et al., 2005; Qu *et al.*, 2017). The studies involving ROP1 effector proteins RIC3 and RIC4 suggest that the dynamics of the apical actin filaments are required for polar exocytosis to the tip of pollen tubes (Gu *et al.*, 2005; Lee *et al.*, 2008). It was proposed that RIC4-promoted assembly or stabilization of the apical actin filaments allows the accumulation of vesicles in the extreme apex, while RIC3-mediated disassembly of the same actin filaments is required for vesicle docking or fusion (Lee *et al.*, 2008). This hypothesis is consistent with the results from the studies of several actin-binding proteins (Liu *et al.*, 2015; Jiang *et al.*, 2017; Li *et al.*, 2017; Qu *et al.*, 2017; Lan *et al.*, 2018).

The organization and turnover of actin filaments in pollen tubes is regulated by a battery of actin-binding proteins that function in nucleating (by formins), bundling (by fimbrins, villins, and LIM proteins), and depolymerization (by actin-depolymerizing factor/cofilins) of actin filaments (for reviews, see Higaki *et al.*, 2007; Ren and Xiang, 2007; Chen *et al.*, 2009; Qu *et al.*, 2014; Fu, 2015). Interestingly, the activities of many actin-binding proteins are regulated *in vitro* by Ca^{2+} (Xiang *et al.*, 2007; Wang *et al.*, 2008; Zhang *et al.*, 2010; Zhou *et al.*, 2015), which is an essential ionic signal molecule for pollen tubes (Hepler *et al.*, 2012; Steinhorst and Kudla, 2013; Michard *et al.*, 2017; Zheng *et al.*, 2019). Yet, it is still not clear how the *in vivo* activity of these actin-binding proteins is regulated by the Ca^{2+} ion in pollen tubes. On the other hand, ROP GTPase has been found to regulate polar exocytosis by modulating the organization of the actin filaments at the tip of Arabidopsis pollen tubes through its effector proteins RIC3 and RIC4 (Gu *et al.*, 2005; Lee *et al.*, 2008). In addition, NtRAC1, a ROP GTPase in tobacco pollen tubes, was proposed to modulate the activity of an actin-depolymerizing factor, NtADF1, through phosphorylation (Chen *et al.*, 2003). However, the biochemical mechanism underlying the ROP signaling-mediated organization of actin filaments is still unclear. Since the Arp2/3 actin-nucleating complex is not required for pollen tube growth (Li *et al.*, 2003), ROPs are unlikely to target this complex at the tip of pollen tubes. Given the critical role of formins in activating the nucleation of the dynamic apical actin filaments, RIC4 might link ROPs to formins, but how ROPs or RIC4 may regulate formin activity remains to be studied. Two compounds have been identified to affect both the ROP activity and the organization of actin filaments; further study of their binding targets might help us to understand the missing link between ROP signaling and actin organization (Laggoun *et al.*, 2019).

Pollen tube growth reorientation such as turning towards attractants also provides an opportunity to study the link between ROP signaling and actin organization in the regulation of polar exocytosis. Two studies have both shown redistribution of the apical actin structure to the future turning direction before the actual morphological change in the tip of pollen tubes (Bou Daher and Geitmann, 2011; Qu *et al.*, 2017). It has been proposed that the asymmetric opening of Ca^{2+} channels may be one of the driving forces (Bou Daher and Geitmann, 2011). Indeed, a recent study has revealed the preferential enrichment of a Ca^{2+} channel, CNGC18, in the PM facing an unknown pollen tube attractant through selective exocytosis involving MLO5/9 (Meng *et al.*, 2020). Consistently, disrupting actin filaments by the actin-depolymerizing drug latrunculin B decreased the cytosolic Ca^{2+} gradient in the tip of pollen tubes (Cardenas *et al.*, 2008), probably due to inhibition of the polar exocytosis of Ca^{2+} channels to the PM of pollen tubes. The finding that active ROP preferentially accumulates in the PM region facing the pollen tube attractant AtLURE before the turning of pollen tube (Luo *et al.*, 2017) also provides a future opportunity for understanding the link between ROP signaling and the dynamics of Ca^{2+} and actin organization by observing the spatiotemporal pattern and relationship between ROP activity, Ca^{2+} level, and actin organization during the turning.

Exocytosis-coordinated generation of the apical polarity domain of ROP activity defines tip growth and controls growth direction

The ROP GTPase regulates the polarity of the pollen tube, which is defined by an apical domain of active ROP1 on the PM known as the apical cap (Lin *et al.*, 1996; Li *et al.*, 1999; Hwang *et al.*, 2005). It has been shown that the active ROP spatiotemporally regulates polar exocytosis by reorganizing the cytoskeleton and recruiting the exocyst through its effectors (Gu *et al.*, 2005; Lavy *et al.*, 2007; Lee *et al.*, 2008; Li *et al.*, 2008), while polar exocytosis also plays a fundamental role in establishing the domain of active ROP by active delivery of the molecular components regulating ROP signaling (Hwang *et al.*, 2008; Lee *et al.*, 2008; Luo *et al.*, 2017). The components involved in the positive feedback regulation of the ROP signaling, including the potential autocrine signal peptides Lat52 (Tang *et al.*, 2002), LTP5 (Chae *et al.*, 2009), and the candidate ligand receptor PRK2 (Tang *et al.*, 2002; Lee *et al.*, 2008; Chang *et al.*, 2013), are suggested to be targeted to the tip through polar exocytosis (Luo *et al.*, 2017). PRK2 in turn directly activates ROP1 via RopGEFs (Zhang and McCormick, 2007; Chang *et al.*, 2013; Zhao *et al.*, 2013). Therefore, ROP1-dependent exocytosis appears to be a key to the positive feedback loop of the autocrine signaling to generate the apical cap of the ROP1 activity in pollen tubes.

Apart from the critical feedforward role for exocytosis in the initial formation of the apical ROP1 activity cap, exocytosis is also required for maintaining the apical cap by targeting a key negative regulator of ROP1 signaling, REN1 (Hwang *et al.*, 2008; Luo *et al.*, 2017). REN1 is a RopGAP targeted to the apical PM, where ROP1 is activated. Loss of function of the REN1 RopGAP leads to the lateral expansion of the apical ROP1 activity and thus severe growth depolarization and breakdown of tip growth, a phenotype analogous to that caused by constitutive activation of ROP1 (Li *et al.*, 1999; Klahre *et al.*, 2006; Hwang *et al.*, 2010). Interestingly, REN1 targeting to the apical PM is mediated by exocytosis, thus exocytosis plays a crucial role for REN1-mediated down-regulation of the apical ROP1 activity and the maintenance of the tip growth polarity. Hence, exocytosis maintains a balance between the positive and negative feedback regulation of the ROP1 activity, and is required for the generation and maintenance of the apical ROP1 cap (Luo *et al.*, 2017). Computational modeling and

experimental validation show that a moderate reduction in exocytosis to a threshold level leads to lateral spreading of the apical ROP1 activity and thus growth depolarization, whereas severe defects in exocytosis passing the threshold level eliminate the apical ROP1 activity (Luo *et al.*, 2017). From these analyses, it was proposed that the exocytosis-coordinated positive and negative feedback system of Rho GTPase signaling provides a design principle for rapid tip growth in pollen tubes and probably other similar cell systems such as fungal hyphae (Fig. 2) (Luo *et al.*, 2017).

A recent study has identified *trans*-Golgi network (TGN)-localized YPT-interacting protein 4a (YIP4a) and YIP4b that contribute to the delivery of ROPs to the root hair initiation sites (Gendre *et al.*, 2019). Given the similarity between the root hair and pollen tube, the delivery of ROP itself to the domain of active ROP by a ROP signaling pathway may also be facilitated by exocytosis in pollen tubes.

The integrated modeling and experimental studies suggest that the exocytosis-coordinated design principle underlying tip growth also provides a core mechanism for growth guidance in pollen tubes (Luo *et al.*, 2017). The integration of the exocytosis-coordinated ROP signaling into the mechanisms for growth guidance is further supported by a study showing that the receptor for the AtLURE1 guidance signal is PRK6 that also directly interacts with RopGEFs (Takeuchi and Higashiyama, 2016; Yu *et al.*, 2018). Interestingly, in the presence of a pollen tube attractant AtLURE1, its receptor PRK6 rapidly re-localizes to the side facing the attractant before the pollen tube reorientation (Takeuchi and Higashiyama, 2016). It is highly likely that the relocalization of PRK6 is mediated by the targeting of PRK6 to the PM region facing the attractant via ROP1-dependent exocytosis, because PRK6 may locally promote exocytosis by activating ROP1 in the same PM region (Takeuchi and Higashiyama, 2016; Luo *et al.*, 2017). The computational modeling predicts that this core mechanism is also required for the shifting of the active ROP domain towards the attractant side (Luo *et al.*, 2017).

Exocytosis mediates the coordination of the active ROP domain with the distribution of pectin zones

It was proposed that a regulatory network regulates pectin dynamics by orchestrating the PME activity in the pollen tube (Bosch and Hepler, 2005). Accumulating evidence suggests that the ROP signaling is central to this regulatory network to direct the cell wall dynamics during the pollen tube growth and guidance (Fig. 2). The active ROP domain on the PM is tightly associated with the distribution of the pectin zones in the growing pollen tubes. The enlargement of the active ROP domain in several mutants, such as *raba4d*, *ren1-1*, and the *tri-gdi* mutants, often accompanies an abnormal distribution of the pectin zones, where the soft pectin zone enriched with highly methylesterified pectin is enlarged (Szumlanski and Nielsen, 2009; Feng *et al.*, 2016; Luo *et al.*, 2017). Both the active ROP domain and the site of pectin deposition relocalize to the same side of the pollen tube attracted by the AtLURE peptide before the pollen tube actually turns to the attractant (Luo *et al.*, 2017). These all strongly suggest an underlying intracellular mechanism that coordinates the distribution of the active ROP domain and the organization of the pectin zones in the pollen tube.

Given the facts that exocytosis delivers the cell wall materials to the expansion site, and that exocytosis and the ROP signaling reciprocally regulate the formation of the active ROP domain, it is highly likely that exocytosis may coordinate the spatial dynamics of the two aforementioned processes. This hypothesis is validated by Luo *et al.* (2017) using mathematical modeling and experimental approaches, wherein the active ROP domain recruits the exocytosis machinery, which in turn guides the distribution of the soft pectin zone. This mechanism further explains how pollen tube guidance is achieved, in which the external guidance cue instructs asymmetric cell wall deposition through redirecting the exocytosis by the locally activated ROP signaling (Luo *et al.*, 2017). However, this model is a simplified framework that only considers a limited number of pathways. A full understanding of the coordination between the active ROP domain and cell wall dynamics still requires further studies on several other factors or pathways, including endocytosis, Ca^{2+}, and phosphoinositide signaling, which all play important roles in regulating pollen tube growth and guidance. A recent study revealed that two AGC kinases in Arabidopsis restrict the active ROP domain to the apical PM by phosphorylating RopGEFs and targeting them to the apical PM (E. Li *et al.*, 2018). It will be interesting to investigate whether the initiation of AGC kinase activity and the polar targeting of RopGEFs depend on the ROP activity, which would add another layer of complexity to the current model.

REN4-mediated endocytosis acts as a rheostat to control the robustness of tip growth polarity by fine-tuning the apical ROP1 activity

Endocytosis is known to regulate signaling transductions and ion homeostasis in plant cells by selective internalization of PM-localized receptors and channels (PaezValencia *et al.*, 2016). Although the ROP signaling has been reported to suppress the endocytosis of PIN1 through regulating the actin filaments in pavement cells (Nagawa *et al.*, 2012), whether endocytosis also involves the regulation of the ROP signaling in pollen tubes was completely unknown until recently. This puzzle has been partly resolved by the identification of the REN4 protein, which binds and internalizes active ROP in the shoulder region of the PM by recruiting the endocytosis machinery (H. Li *et al.*, 2018). Via dual interactions with Endocytosis Adaptor of Pollen Tube (EAP1, an ANTH-related cargo adaptor for clathrin-mediated endocytosis) and GTP-bound active ROP1, REN4 initiates the endocytosis of and removes active ROP1 from the PM at the apical or the shoulder region in slow-growing and fast-growing pollen tubes, respectively (H. Li *et al.*, 2018). By doing so, REN4 ensures that an optimal level of active ROP1 is precisely localized to the extreme apex needed for the rapid and unidirectional growth of pollen tubes

(H. Li *et al.*, 2018). Consequently, loss of REN4 function results in wiggly pollen tubes with reduced elongation rates (H. Li *et al.*, 2018). Therefore, the REN4-mediated endocytosis of active ROP1 acts as a rheostat for fine-tuning the apical ROP1 activity and controls the robustness of growth directionality in pollen tubes by canceling the noise of active ROP1 distribution away from the extreme apex.

The regulation of the ROP1 activity by REN4-mediated endocytosis is functionally distinct from the REN1 RopGAP-dependent inactivation of ROP1 (Hwang *et al.*, 2008; Luo *et al.*, 2017; H. Li *et al.*, 2018). As discussed above, the exocytosis-mediated targeting of REN1 is to balance the exocytosis-dependent positive feedback activation of ROP1 and its lateral propagation, and thus is essential for the tip growth system (Fig. 2), as the absence of REN1 leads to an extensively lateral spreading of active ROP1 and the breakdown of the tip growth system (Hwang *et al.*, 2008; Luo *et al.*, 2017). In contrast, the absence of REN4 does not abolish tip growth (H. Li *et al.*, 2018). In addition to being a fine-tuning function, however, the REN4-mediated endocytosis of active ROP1 also has a safeguarding function in the regulation of the ROP1 activity essential for tip growth. It was shown that moderate overexpression of ROP1, which only slightly depolarized pollen tube growth in a wild-type background, dramatically caused lateral spreading of active ROP1 and a loss of tip growth in the *ren4-1* mutant (H. Li *et al.*, 2018). Furthermore, potential additional functions of REN4-mediated endocytosis and its regulation deserve further investigation. For instance, it will be interesting to investigate whether REN4-mediated endocytosis regulates the relocalization of the active ROP domain by preferential internalization of ROP in the PM that is not facing the attractant during the pollen tube guidance, whether and how the ROP signaling may regulate endocytosis, especially the endocytosis at the apical PM where it overlaps with the active ROP domain, and whether the active ROP domain is fine-tuned by a reciprocal regulation between ROP signaling and endocytosis. Lastly, it should be noted that the identification of active ROP1 as cargo for clathrin-mediated endocytosis is probably only the tip of the iceberg of the regulation and function of endocytosis in pollen tube tip growth. We need to fully explore why CME is essential for pollen tube growth and why many CME adaptors such as ANTH and ENTH proteins are expressed in pollen tubes (H. Li *et al.*, 2018; Muro *et al.*, 2018; Kaneda *et al.*, 2019).

Conclusions and perspectives

A sophisticated model for the ROP GTPase-dependent mechanisms underlying tip growth in pollen tubes has emerged. The polarity of the pollen tube is regulated by the ROP signaling pathway, but the cell expansion is physically regulated by the extensibility of the cell wall. The polarity and future growth site of pollen tube are defined by an active ROP domain, which is established and maintained by a pair of opposing positive and negative feedback regulations controlled by exocytosis that is in turn controlled by active ROP1 localized in the apical PM as an apical cap. The endocytosis further restrains and stabilizes the active ROP domain by internalizing the laterally propagated ROP in the apical and shoulder region. The extensibility of the cell wall in the pollen tube is mainly regulated by the cell wall pectin, which formed two distinct pectin zones, with a soft pectin zone at the expanding tip and a stiff pectin zone at the shank region. Both pectin and its modification enzymes are delivered by ROP1-dependent exocytosis, but the formation of the distinct zones requires the internalization of PMEI by endocytosis. Therefore, both ROP signaling and cell wall dynamics are regulated by exocytosis and endocytosis. To achieve the maximum efficiency of growth and guidance in pollen tubes, the active ROP domain and the pectin dynamics must be coordinated tightly via exocytosis-based events. Nonetheless, the current understanding of the exocytosis-coordinated mechanism only provides a simplified framework underlying pollen tube growth and guidance. Further studies will expand and improve this framework by fine-tuning the current pathways and including new regulatory modules. Firstly, exocytosis has multiple target sites on the PM of the pollen tube, but it remains to be determined whether various membrane signaling components are delivered to distinct sites of the PM via different populations of vesicles. Secondly, the role of endocytosis during pollen tube growth and guidance is not yet fully understood, such as whether the tip endocytosis that overlaps with the active ROP domain regulates the ROP signaling, and whether the relocalization of the AtLURE receptor PRK6 also requires the input from endocytosis. Thirdly, Ca^{2+}, as a secondary messenger, may regulate both actin organization and vesicle trafficking, and is also part of the negative feedback regulation of the ROP activity (Gu *et al.*, 2005; Yan *et al.*, 2009), but whether and how the spatial distribution of Ca^{2+} channels are regulated by exocytosis and endocytosis need further investigation. Lastly, several species of phosphoinositides have been found to participate in the regulation of exocytosis and endocytosis (Sousa *et al.*, 2008; Zhao *et al.*, 2010; Pleskot *et al.*, 2012; Bloch *et al.*, 2016; Hempel *et al.*, 2017), but how these phosphoinositides regulate vesicular trafficking and how they are integrated with the ROP signaling network remain poorly understood. With the advanced super-resolution light microscopy technologies and additional live-cell molecular and cellular markers, the field of tip growth using pollen tubes as a model system will continue to be fertile in the coming years.

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