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Rho-GTPase–dependent filamentous actin dynamics coordinate vesicle targeting and exocytosis during tip growth

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The dynamic activity of tip-localized filamentous actin (F-actin) in pollen tubes is controlled by counteracting RIC4 and RIC3 pathways downstream of the ROP1 guanosine triphosphatase promoting actin assembly and disassembly, respectively. We show here that ROP1 activation is required for both the polar accumulation and the exocytosis of vesicles at the plasma membrane apex. The apical accumulation of exocytic vesicles oscillated in phase with, but slightly behind, apical actin assembly and was enhanced by overexpression of RIC4. However, RIC4 overexpression inhibited exocytosis, and this inhibition could be suppressed by latrunculin B treatment or RIC3 overexpression. We conclude that RIC4-dependent actin assembly is required for polar vesicle accumulation, whereas RIC3-mediated actin disassembly is required for exocytosis. Thus ROP1-dependent F-actin dynamics control tip growth through spatiotemporal coordination of vesicle targeting and exocytosis.

Introduction

The dynamics of F-actin are achieved through filament treadmilling, i.e., polymerization (growth) at the plus end and depolymerization (shrinkage) at the minus end. Actin dynamics controls many important cellular processes such as animal cell migration, neurite formation, axon guidance, chemotaxis, yeast endocytosis, regulation of gene transcription in developing vertebrate embryos and programmed cell death in the self-incompatibility responses, and the polarization of growth in pollen tubes (Leventhal and Feldman, 1996; Dent and Gertler, 2003; Gu et al., 2003; Haller et al., 2004; Gupton et al., 2005; Mouneimne et al., 2006; Thomas et al., 2006; Toshima et al., 2006). However, the mechanism by which actin dynamics modulate these fundamental processes is largely unclear.

Pollen tube growth is essential for plant sexual reproduction and provides an attractive model system for the study of polarized cell growth. When a pollen grain lands on the stigma, it produces a long pollen tube to deliver sperm to the ovule (Hepler et al., 2001; Johnson and Preuss, 2002). The pollen tube extends by tip growth through highly targeted exocytosis to the tube apex. Growth of the pollen tube is oscillatory and can attain an astonishing rate, up to 1 cm/h, which makes it one of the most rapidly growing cells. All these features are expressed when pollen tubes are cultured in vitro. Therefore, the pollen tube has been widely used as a model system for cell biological, genetic, and molecular analyses of tip growth.

Transmission electron microscopy revealed massive accumulation of vesicles into an inverted cone pattern in the extreme apex of the pollen tube (Lancelle and Hepler, 1992). These vesicles fuse specifically with the apical region of the plasma membrane (PM) to allow rapid polarized tip growth. The molecular and cellular mechanisms underlying this localized exocytosis are poorly understood. The vesicle accumulation pattern suggests that a cytoskeleton-based force is required for the targeting of these vesicles to the tip. Cortical microtubules (MTs) run along the length of pollen tubes but do not extend to the clear zone, and disrupting MTs does not significantly affect tip growth (Åström et al., 1995), which suggests that MTs are unlikely to participate in the vesicle accumulation. Pollen tubes contain extensive axial actin cables and more dynamic F-actin filaments at the tip (Lový-Wheeler et al., 2005; Samaj et al., 2006). The former are excluded from the tip of growing pollen tubes and are proposed to mediate cytoplasmic streaming (Vidali et al., 2001; Cardenas et al., 2005). Low levels of latrunculin B (LatB) do
not affect streaming and actin cables but inhibit growth (Gibbon et al., 1999; Vidali et al., 2001), which is consistent with the notion that the tip-localized dynamic F-actin is important for vesicle targeting. Evidence suggests that the dynamics of the apical F-actin, not just its presence, are critical for polarized pollen tube growth (Gu et al., 2005).

Polarized exocytosis likely requires a signaling network localized to the tip to regulate the cytoskeleton and the targeting, docking, and fusion of vesicles (Zhang et al., 2001; Pellegrin and Mellor, 2005; Brennwald and Rossi, 2007). Recent studies have revealed a signaling network controlling pollen tube growth (Moutinho et al., 2001; Prado et al., 2004; Rato et al., 2004; Gu et al., 2005; Monteiro et al., 2005; Yoon et al., 2006). A key component in this network is the ROP1 GTPase, a member of the plant ROP subfamily of conserved Rho GTPases (Lin et al., 1996; Kost et al., 1999), which function as key molecular switches controlling a variety of cytoskeleton-dependent cellular processes in diverse eukaryotic organisms (Gu et al., 2003; Burridge and Wennerberg, 2004; Sorokina and Chernoff, 2005; Ridley, 2006). ROP1 is preferentially localized to the apical region of the pollen tube PM and is essential for pollen tube growth (Arthur et al., 2003; Chen et al., 2003). Active ROP1 is distributed as an apical cap in the PM apex with a tip-high gradient, and this distribution determines the PM region where growth takes place (Hwang et al., 2005). Thus, the active ROP1 cap could allow for formation of the apical dome of pollen tubes. ROP1 controls pollen tube tip growth through its regulation of tip F-actin dynamics (Fu et al., 2001) and controls actin dynamics by activating at least two pathways regulated by RIC3 and RIC4, respectively. RIC4 promotes F-actin assembly at the tip, whereas RIC3 promotes the formation of tip-focused cytosolic Ca$^{2+}$ gradients, which is required for the disassembly of F-actin (Gu et al., 2005). However, the mechanism by which the ROP1-dependent actin dynamics regulate tip growth is unknown.

Cortical F-actin is thought to be an important regulator of exocytosis. Several findings suggest that cortical F-actin is associated with vesicle targeting but also acts a barrier of vesicle tethering/docking to the target PM (Muallem et al., 1995; Manneville et al., 2003), which implies a need for cortical F-actin to be depolymerized for vesicle tethering/docking. In this paper, we present evidence that ROP1 GTPase activity has an active role in the promotion of polarized exocytosis through its regulation of F-actin dynamics. By visualizing the dynamics of exocytic vesicles and vesicle fusion to the PM, we demonstrate the mode of action for F-actin dynamics in polarized exocytosis: RIC4-mediated F-actin assembly is associated with vesicle accumulation at the tip, whereas RIC3-mediated F-actin depolymerization induces exocytosis to the tip. These observations provide important new insights into the cellular mechanism underlying tip growth, as well as roles of actin dynamics in polarized growth.

### Results

#### RabA4d accumulation to the tip is highly dynamic in growing pollen tubes

To understand how vesicle accumulation to the tip is regulated in growing pollen tubes, we used YFP-RabA4d as an exocytic vesicle marker. Arabidopsis thaliana RabA4d is the pollen-specific homologue of RabA4b, which localizes to the plant TGN compartments and/or recycling endosomal vesicles in root hair tips (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1; Preuss et al., 2004). NtRab11b, a possible tobacco orthologue of RabA4d, has also been shown to localize to the transport vesicle–rich apical clear zone in tobacco pollen tubes (de Graaf et al., 2005). When YFP-RabA4d was expressed stably expressed in A. thaliana pollen, it preferentially accumulated at the apical clear zone of pollen tubes in an inverted cone shape (Fig. S1 B). A similar localization pattern was also observed in tobacco pollen tubes transiently expressing this construct (Fig. 1 A). A. thaliana pollen tubes exhibited wiggly growth in vitro cultures and variable growth rates, making it difficult for time-lapse imaging analysis. Therefore, for subsequent analyses, we chose to use tobacco pollen tubes, which exhibit a more uniform shape and constant growth rates. Because the apical clear zone is exclusively occupied by exocytic vesicles, the accumulation of YFP-RabA4d in this area suggests the exocytic vesicle localization of YFP-RabA4d. Moreover, the accumulation of YFP-RabA4d in the apical region was rapidly dissipated by brefeldin A (BFA) treatment (Fig. 1 C). BFA inhibits secretory vesicle formation in plant and animal cells (Kessels et al., 2006; Langhans and Robinson, 2007). Thus, these results suggest that YFP-RabA4d localizes to exocytic vesicles.

A time series of YFP-RabA4d localization showed a highly dynamic process of vesicle accumulation to the tip (Fig. 1 D and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1). The apical clear zone stained by YFP-RabA4d cycled from spanning against the tube apex with a long trail then retracting to the extreme apex region. Thus, accumulation of YFP-RabA4d fluctuates in an oscillatory fashion during pollen tube growth.

#### Oscillatory vesicle accumulation to the tip is temporally associated with tip growth oscillation

Having determined that YFP-RabA4d localized to tip-localized vesicles, we next investigated a possible relation between pollen tube elongation and vesicle accumulation to the tip. A time series of YFP-RabA4d accumulation to the tip was analyzed on confocal microscopy and compared with pollen tube elongation rates. The relative amount of accumulated vesicle at the apex was quantified by measuring the mean intensity of YFP within 5 μm from the tip (Fig. 1 E). Quantitative analysis of fluorescence intensity revealed that the signal intensity of YFP-RabA4d changed in a regular oscillatory fashion during pollen tube growth. The mean growth rate and oscillation period was 29.8 nm/s and 67.8 ± 9.2 s (n = 9), respectively. Fig. 1 G shows that the highest correlation occurs when oscillation of the YFP signal leads the growth oscillation by 10 s. The 10-s lead of YFP signal oscillation corresponds to a 53.1 ± 7.3° phase shift ahead of growth rate oscillation (Fig. 1 F). The mean growth rate and oscillation period was 29.8 nm/s and 67.8 ± 9.2 s (n = 9), respectively. Therefore, tip accumulation of YFP-RabA4d is temporally associated with tip growth and leads to growth bursts.
Figure 1. **Subcellular localization of YFP-RabA4d in growing pollen tubes.** (A and B) Representative confocal images of tobacco pollen tubes transiently expressing YFP-RabA4d [A] or GFP as a control [B]. Shown are single laser sections focused on the midplane of pollen tubes. (C) A representative confocal image of a YFP-RabA4d-expressing tube treated with BFA. 3 h after bombardment, transformed tubes were treated with 1 μg/ml BFA for 1 h before imaging. Shown are single laser sections focused on the midplane of pollen tubes. (D) A time series of a tube showing the dynamics and oscillation of the tip localization of YFP-RabA4d. Confocal images were selected from the entire time series (5-s intervals) of a growing tube expressing YFP-RabA4d. The numbers beneath each image indicate time elapsed from the beginning of the time series. Shown are single laser sections focused on the midplane. Images for this time series are from Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1). (E) A representative median focal plane image of tobacco pollen tube expressing YFP-RabA4d. The mean intensity of the YFP signal was measured in the area within 5 μm from the tip (circle). Bars, 10 μm. (F) Quantitative analysis of the YFP-RabA4d accumulation to the tip and growth rate oscillation. 4 h after bombardment, time-lapse confocal images of pollen tips were collected at 5-s intervals. YFP signal intensity within 5 μm from the tip shown in E of YFP signal (left) was compared with tip elongation rate (right). Similar results were obtained from five individual experiments. Note the mean oscillation period of tip accumulation of YFP-RabA4d was ~68 s. Break is inserted for focus adjustment and reposition of the pollen tube. (G) Cross correlation analysis of F. To quantify the phase relationship between vesicle accumulation and tip growth, the growth curve was shifted with respect to the YFP-RabA4d curve, and the values of the correlation coefficient corresponding to a phase shift were measured. The highest correlation occurs when growth oscillation lags the oscillation of YFP signal by 10 s, which indicates that the growth burst follows YFP-RabA4d accumulation at the tip.
ROP1 activation is required and sufficient for vesicle accumulation to the extreme apex

ROP1 is required for pollen tube growth, and ROP1 activity oscillates ahead of tip growth (Hwang et al., 2005), which suggests that ROP1 signaling may regulate vesicle targeting to the tip. To test this possibility, we coexpressed YFP-RabA4d with wild-type ROP1 in tobacco pollen tubes. Compared with control tubes, pollen tubes overexpressing a low level of wild-type ROP1 showed more rapid tip elongation and oscillation of the apical YFP-RabA4d but no remarkable difference in distribution of YFP-RabA4d (Fig. 2 A). The period of the apical YFP-RabA4d oscillation was 37.1 ± 7.6 s (n = 6), which was faster than that in control pollen tubes (Fig. 2 B).

To further assess the involvement of ROP1 activity in vesicle targeting, we examined the effect of modulated ROP1 activity on vesicle accumulation in tobacco pollen tubes. Dominant-negative rop1 (DN-rop1) overexpression induced distinct changes in YFP-RabA4d distribution (Fig. 2 C, b). Compared with control (157.3 ± 8.8 s, n = 15), tip accumulation of YFP-RabA4d in a DN-rop1–expressing pollen tube was significantly reduced (102.9 ± 5.8 s, P < 0.05, n = 15; Fig. 2 D).

Conversely, constitutively active rop1 (CA-rop1) expression produced tip-focused localization of YFP-RabA4d (Fig. 2 C, c). Strong YFP signal was detected in the extreme apical region, and distribution of YFP-RabA4d was less extended to the sub-apical area (193.25 ± 12.91, P < 0.05, n = 15; Fig. 2 D). Clearly, decreased ROP1 activity inhibited the accumulation of YFP-RabA4d–stained vesicles to the tip, whereas active ROP1 promoted vesicle accumulation to the extreme apex of the clear zone.

Tip accumulation of YFP-RabA4d is dependent on cortical F-actin structure

We next tested whether ROP1 mediates vesicle accumulation to the tip through ROP1-dependent F-actin dynamics. First, we examined whether F-actin is required for RabA4d localization to the tip of pollen tubes, as shown for RabA4b localization to the tip of root hairs (Preuss et al., 2004). We treated YFP-RabA4d–expressing pollen tubes with 5 nM LatB, which has been shown to disrupt tip F-actin but not actin cables (Vidali et al., 2001). LatB treatment completely disrupted the apical localization of YFP-RabA4d (Fig. 3 A, b). We then assessed whether the ROP1-dependent F-actin is required for YFP-RabA4d targeting. ROP1 downstream targets (RIC3 and RIC4) counteract to control F-actin dynamics (Gu et al., 2005). RIC3 promotes the disassembly of the apical F-actin through a tip-focused Ca²⁺ gradient (Fig. S2 B; available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1; Gu et al., 2005). In RIC3-overexpressing tubes, YFP-RabA4d staining no longer exhibited the distinct inverted cone shape but was evenly dispersed in the cytoplasm (Fig. 3 A, c), similar to that induced by LatB. Quantitative analysis revealed no tip enrichment of vesicles in RIC3-overexpressing pollen (52.99 ± 7.4, P < 0.05, n = 15; Fig. 3 C). RIC3 overexpression-induced disruption of vesicle accumulation was suppressed by LaCl₃, which blocks tip calcium influxes and stabilizes the apical F-actin (Fig. 3 A, d). These observations indicate that apical F-actin is necessary for vesicle delivery and accumulation to the tip.

We next examined the effect of RIC4 overexpression on YFP-RabA4d localization. RIC4 overexpression stabilizes the apical cortical F-actin and reduces growth polarity in pollen tubes (Fig. S2 C; Gu et al., 2005). RIC4 overexpression altered the distribution of YFP-RabA4d from the inverted cone shape to a near cup shape (Fig. 3 B, a). YFP-RabA4d signal right behind the cortex of the apical region was significantly increased, (202.39 ± 10.68, P < 0.05, n = 13; Fig. 3 C). Furthermore, this subcortical accumulation of YFP-RabA4d became less polarized toward the extreme apex, i.e., it was extended beyond the extreme apex. Similar changes in YFP-RabA4d distribution were induced by treatment with LaCl₃ (Fig. 3 B, b) or jasplakinolide (Fig. 3 B, c). Together with the requirement of apical F-actin for targeting vesicles to the tip, these results suggest that the RIC4-dependent cortical F-actin in the tip allows the accumulation of vesicles to the cortical and subcortical region of the extreme apex.

Because the balanced counteraction between the RIC3 and RIC4 pathways results in apical F-actin dynamics, which are important for polarized tip growth (Gu et al., 2005), we tested whether these F-actin dynamics are required for the normal pattern of vesicle accumulation in the tip. As shown in Fig. 3 B (d), LatB treatment recovered the normal vesicle accumulation in RIC4-overexpressing tubes. Similarly, coexpression of RIC3 and RIC4 also resulted in the recovery of normal vesicle accumulation at the tip (Fig. 3 B, e). Taken together, these results suggest that RIC4-dependent F-actin is required for the accumulation of vesicles to the cortical and subcortical region of the extreme apex, whereas the ROP1-dependent F-actin dynamics (resulting from the checks and balances between the RIC3 and RIC4 pathways) are critical for the dynamic accumulation of vesicles in the tip of pollen tubes.

Development of a FRAP-based method for monitoring exocytosis in pollen tubes

To monitor exocytic activity at the tip of pollen tubes, we developed a novel strategy involving FRAP of receptor-like kinase (RLK)-GFP, whose targeting to the PM completely depends on exocytosis. GFP was fused to the C terminus of the A. thaliana pollen-specific RLK and used as a membrane marker protein (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1). RLK (At5g35390) shares high sequence similarity to the tobacco pollen–expressed receptor kinase that was used as a PM marker (Cheung et al., 2002). When transiently expressed in tobacco pollen tubes, RLK-GFP preferentially localized to the apical region of the pollen tube PM, although a weaker signal was also observed at the apical region of the cytoplasm as an inverted cone shape (Fig. 4 A). This localization pattern of RLK-GFP was similar to that of other proteins that are targeted to the pollen tube PM through exocytosis (de Graaf et al., 2005). Furthermore, PM localization of RLK-GFP was completely disrupted by BFA treatment (Fig. 4 B). These results provide strong evidence that RLK-GFP was inserted into the PM through exocytosis.
Figure 2. **Modulation of ROP1 activity affects vesicle accumulation to the tip.** (A) A time series of YFP-RabA4d staining in tobacco pollen tube overexpressing ROP1. Confocal images obtained were focused in the median plane. The numbers beneath each image indicate the time elapsed from the first image. A lower amount of ROP1 plasmid (0.1 μg) was used to weakly express ROP1. A low level of wild-type ROP1 expression induced rapid elongation of pollen tubes. Shown are single laser sections focused on the midplane. (B) Quantitative analysis of A. The mean intensity of YFP-RabA4d accumulated at the tip region and growth rate were determined from each time-lapse confocal image. YFP signal intensity and growth rate were measured every 10 s and plotted as a function of the time. Note that the period of oscillation was reduced to 37.1 ± 7.6 s (n = 6). (C) Representative images of YFP-RabA4d alone (a) or together with DN-rop1 (b) or CA-rop1 (c). DN-rop1 inhibited accumulation of YFP-RabA4d to the tip, whereas CA-rop1 induced tip-focused YFP-RabA4d accumulation. Shown are single laser sections focused on the midplane. Bars, 10 μm. (D) Quantification of YFP-RabA4d accumulation in representative wild-type (control), DN-rop1 OX, and CA-rop1 OX pollen tubes. Pixel intensity values along a central transect (indicated by the box) through each image was measured [left]. Similar results were obtained from ~20 individual pollen tubes.
was reduced to 10% of its original level (Fig. 4 C, top middle). GFP signals started to reappear in the apical dome within 30 s and reached >90% of their original intensity 75 s after bleaching (Fig. 4 E and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1). The half-time of fluorescence recovery in the apical membrane area was \( \frac{1}{35} \) s (\( n = 5 \)). The recovery first occurred in the center of the apical dome and then gradually moved laterally (Fig. 4 C). Furthermore, different regions of the PM apex exhibited a gradient of FRAP rates, with the fastest recovery being in the center of the apical dome.

To monitor vesicle fusion to the PM in growing pollen tubes, tobacco pollen tubes expressing RLK-GFP underwent FRAP. We photobleached a section of the apical region and monitored the recovery of RLK-GFP fluorescence in the bleached region of the PM every 5 s. We reasoned that the recovery was caused by exocytic activity at the tip of pollen tubes. An example of the FRAP analysis of an RLK-GFP-expressing tube is shown in Fig. 4 C. Prior to photobleaching, RLK-GFP appeared as a bright line associated with the PM (Fig. 4 C, top left). After photobleaching, the fluorescence signal in the apical PM area was reduced to 10% of its original level (Fig. 4 C, top middle). GFP signals started to reappear in the apical dome within 30 s and reached >90% of their original intensity 75 s after bleaching (Fig. 4 E and Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1). The half-time of fluorescence recovery in the apical membrane area was \( \frac{1}{35} \) s (\( n = 5 \)). The recovery first occurred in the center of the apical dome and then gradually moved laterally (Fig. 4 C). Furthermore, different regions of the PM apex exhibited a gradient of FRAP rates, with the fastest recovery being in the center of the apical dome.

Figure 3. The accumulation of YFP-RabA4d vesicles to the tip is associated with the apical F-actin dynamics. (A) F-actin is associated with YFP-RabA4d accumulation to the tip. YFP-RabA4d was transiently expressed alone (a) or coexpressed with RIC3 (b) in tobacco pollen tubes. For chemical treatment, control pollen tubes (YFP-RabA4d alone) or RIC3-overexpressing pollen tubes were treated with 5 nM LatB (c) or 100 \( \mu \)M LaCl\(_3\) (d), respectively, for 1 h. Tip accumulation of YFP-RabA4d was completely disrupted by RIC3 overexpression (b) or LatB treatment (c). LaCl\(_3\) treatment nearly restored normal accumulation of YFP-RabA4d in RIC3-overexpressing tubes (d). All images show a midplane section. (B) Accumulation of tip F-actin causes depolarized localization of YFP-RabA4d to the tip. YFP-RabA4d was transiently expressed alone (b and c) or coexpressed with the indicated RICs (RIC4 [a] and RIC3 + RIC4 [e]). 3 h after bombardment, control pollen tubes (YFP-RabA4d) were treated with 50 \( \mu \)M LaCl\(_3\) for 1 h (b) or 100 nM jasplakinolide for 10 min (c) before imaging. RIC4 OX (a), LaCl\(_3\) (b), or jasplakinolide treatment (c) induced increased accumulation of YFP-RabA4d near the cortex over an expanded region of the apex. Treatment with LatB (d) or coexpression with RIC3 (e) restored pollen tube growth and normal vesicle accumulation to the tip in RIC4-overexpressing tubes. Arrows indicate the depolarized localization of YFP-RabA4d. All images were taken from the midplane. Bars, 10 \( \mu \)m. (C) Quantitative comparison of YFP-RabA4d accumulation. Pixel values were measured along a central transect through the fluorescence image in wild-type, RIC3 OX, and RIC4 OX pollen tubes. Similar results were obtained from \( \sim \)20 individual pollen tubes.
expected exocytic activity in the corresponding region of a growing pollen tube. The lack of recovery in the subapical region suggests that RLK-FRAP could not have resulted from either

(Fig. 4 F). When a subapical region was bleached, FRAP was negligible (Fig. 4, D and E; and Video 3). The rate of RLK-GFP FRAP in different regions of the PM was consistent with the

Figure 4. FRAP of RLK-GFP-stained apical PM in tobacco pollen tubes. (A) PM localization of RLK-GFP in a tobacco pollen tube transiently expressing a LAT52:RLK-GFP construct. GFP was fused to the C terminus of A. thaliana pollen-specific receptor kinase. Confocal images were taken from the midplane. (B) BFA effect on the localization of RLK-GFP. Transformed pollen tubes expressing RLK-GFP were treated with 1 μg/ml BFA for 1 h before imaging. BFA completely disrupted PM localization of RLK-GFP. The focus is on the midplane. Bars, 10 μm. (C and D) FRAP analysis of RLK-GFP in growing pollen tubes. Photobleaching was performed and recovery was analyzed in the apical area (C) and subapical area (D) of the pollen tube PM. A series of time-lapse images was recorded every 5 s for 3 min. The numbers in each image indicate elapsed time after photobleaching. The bleached area is marked by boxes. Note that photobleaching did not affect pollen tube elongation. The mean growth rate and oscillation period was 30.92 nm/s and 64 ± 6.5 s (n = 5), respectively, after photobleaching. These were similar to what was observed in the GFP-expressing pollen tube. These time series correspond to Videos 2 and 3 (available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1). Bars, 10 μm. (E) Quantitative analysis of FRAP for C and D. Fluorescence recovery was measured by calculating the mean GFP signal intensity on the PM of the region of interest. Fluorescence at the apical PM area recovered completely in 75 s, with a 35 ± 3 s half-time of recovery ( ), whereas little fluorescence recovery occurred in the subapical PM area ( ). Similar results were obtained from five individual experiments. (F) Quantitative FRAP analysis at four different regions of the membrane. The intensity of recovered GFP signal is plotted as a function of the time. Fluorescence recovery was fast at the apical PM. The rate of signal recovery gradually became slower away from the center of the apex. The recovered GFP signal was measured every 5 s for 80 s. The regions [1–4] where FRAP was measured are indicated in C.
DN-rop1 significantly decreased the rate of fluorescence recovery (Fig. 5 A). In most cases, the recovery of GFP signal at the bleached membrane reached only 30% of its original intensity 3 min after photobleaching (n = 5; Fig. 5 B). DN-rop1 inhibition of the FRAP was not the result of reduced cell growth because CA-rop1 expression inhibited cell expansion but not the FRAP. Thus, these results indicate that ROP1 inactivation inhibits exocytosis to the site of cell growth and support the hypothesis that ROP1 is required for pollen tube growth at least in part via its role in regulating exocytosis.

CA-rop1 expression suppressed pollen tube elongation and induced tip swelling 4 h after pollen was bombarded with the CA-rop1 construct. The rate of fluorescence recovery was slightly reduced in these pollen tubes (Fig. 5 C). Quantitative analysis of FRAP showed that 90 s after bleaching, 80% of the original signal was recovered (n = 5; Fig. 5 B). Interestingly, the recovery occurred simultaneously throughout the whole bleached region of the PM, and the recovery rate was identical in different regions of the PM (Fig. 5, C and D). Therefore, ROP1 activity controls exocytosis to the site of growth

Active ROP1 is distributed to the apical region of the PM as an apical cap with a tip-high gradient; this cap determines the growing region of the pollen tube tip and is required for tip growth (Hwang et al., 2005). The apical cap and the gradient of active ROP1 seem to correspond to the apical region showing RLK-GFP FRAP. From these observations, we hypothesized that active ROP1 controls pollen tip growth by modulating the site and the rate of exocytosis. To test this hypothesis, we performed RLK-GFP FRAP experiments on tubes expressing CA-rop1 or DN-rop1. DN-rop1 significantly decreased the rate of fluorescence recovery (Fig. 5 A). In most cases, the recovery of GFP signal at the bleached membrane reached only 30% of its original intensity 3 min after photobleaching (n = 5; Fig. 5 B). DN-rop1 inhibition of the FRAP was not the result of reduced cell growth because CA-rop1 expression inhibited cell expansion but not the FRAP. Thus, these results indicate that ROP1 inactivation inhibits exocytosis to the site of cell growth and support the hypothesis that ROP1 is required for pollen tube growth at least in part via its role in regulating exocytosis.

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these results indicate that ROP1 activity determines the site of vesicle exocytosis.

**RIC4- and RIC3-mediated actin dynamics are required for exocytosis**

To understand how ROP1 regulates exocytosis, we investigated the effect of RIC3 and RIC4 on RLK-GFP FRAP. As expected, RIC3 overexpression, which eliminated tip-localized F-actin, inhibited the RLK-GFP FRAP due to its disruption of vesicle accumulation to the tip (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1). Surprisingly, RIC4 overexpression also greatly inhibited the FRAP (n = 5; Fig. 6 A). Even 3 min after photobleaching, only 10% of the original fluorescence was recovered in the tip membrane. Similar results were observed in LaCl3- or jasplakinolide-treated pollen tubes (Fig. S5). Because both RIC4 and jasplakinolide cause the accumulation of stable F-actin, these results suggest that the accumulation of the apical F-actin inhibits exocytosis.

To test whether the inhibitory effect of RIC4 overexpression on exocytosis was the result of F-actin stabilization, FRAP was performed on RIC4-overexpressing tubes treated with 5 nM LatB or coexpressed with RIC3. RIC3-mediated Ca2+ signaling was shown to promote the disassembly of RIC4-dependent F-actin accumulation. Additionally, LatB treatment suppresses the RIC4-overexpression phenotype and recovers the normal F-actin structure (Gu et al., 2005). As shown in Fig. 6 (B and C), LatB or RIC3 rapidly recovered the FRAP. GFP signals in F-actin structure (Gu et al., 2005). As shown in Fig. 6 (B and C), LatB or RIC3 rapidly recovered the FRAP. GFP signals in F-actin, these results suggest that the accumulation of stable F-actin, these results suggest that the accumulation of the apical F-actin inhibits exocytosis.

**Discussion**

In this paper, we have established a cellular mechanism by which Rho GTPase-dependent F-actin dynamics modulate tip growth in pollen tubes. We show that RIC4 activation of F-actin assembly is necessary for vesicle accumulation to the tip, whereas the RIC3-dependent Ca2+ signal reduces vesicle accumulation at the tip, though its role in activating F-actin disassembly is required for vesicle exocytosis to the growth site. These results together with our previous studies (Fu et al., 2001; Gu et al., 2005) provide strong evidence that ROP1-mediated F-actin dynamics play an important role in spatiotemporal coordination of vesicle targeting and exocytosis, leading to polarized cell growth. Actin dynamics have also been shown to be critical for exocytosis in animal cells, and Rho GTPases are well known to control polarized growth in fungi and neuronal cells through their regulation of actin dynamics. The Rho GTPase–actin dynamics–exocytosis pathway we established might also provide a mechanism for the control of exocytosis and polarized cell growth in these systems.

**Oscillatory apical vesicle accumulation is temporally associated with pollen tip growth**

Our result showed a dynamic localization pattern of YFP-RabA4d in the apical clear zone of pollen tubes. The apical clear zone is exclusively occupied by exocytic vesicles that are accumulated in an inverted cone pattern (Parton et al., 2001; de Graaf et al., 2005), which is identical to the YFP-RabA4d localization we showed in this study. The effect of BFA on the YFP-RabA4d localization pattern strongly suggests a Golgi-derived secretory vesicle localization of this fusion protein (Fig. 1). However, these observations do not exclude the possibility that YFP-RabA4d was also present in recycling endocytic vesicles. FM4-64–stained endosomal vesicles that are recycled back to the PM by exocytosis also accumulate in an inverted cone shape (Parton et al., 2001; Camacho and Malho, 2003). YFP-RabA4d could be localized to both TGN-derived secretory vesicles and recycling endosomal vesicles that are targeted to and fused with the apical PM for tip growth in pollen tubes. Regardless of the secretory or endosomal nature of the RabA4d vesicles, their localization pattern suggests that they represent exocytic vesicles accumulated to the apical clear zone. The exocytic nature of these vesicles is also supported by the dynamics of their accumulation to the tip. On visualizing vesicles using YFP-RabA4d, we demonstrated that the RabA4d vesicle accumulation to the tip oscillates in the same periodicity as the tip growth oscillation. Interestingly, the peak of the RabA4d vesicle accumulation precedes the growth burst by ~10 s in a 68-s period of oscillation, which is consistent with the notion that their exocytosis results in pollen tube growth.

**Tip-localized ROP1 controls tip growth by modulating tip-targeted exocytosis**

Tip growth requires spatiotemporal coordination of exocytic vesicle targeting, tethering, and fusion to the apical PM region, but the mechanism underlying this coordination is not well understood. Many data indicate that ROP1 and its orthologues control pollen tube tip growth (Li et al., 1999; Arthur et al., 2003; Klahre et al., 2006). Our evidence suggests that ROP GTPase does so by regulating both tip-targeted vesicle accumulation and exocytosis. First, the apical accumulation of RabA4d vesicles oscillate with the same period as both the oscillation of the apical ROP1 activity and pollen tube elongation rates but in a phase that is between the two (this paper; Hwang et al., 2005). Second, DN-rop1 impaired vesicle accumulation to the tip, whereas CA-rop1 expression promoted the RabA4d vesicle accumulation to the apical region, causing the movement of more RabA4d vesicles closer to the apical PM region (Fig. 2). Thus, the tip-localized active ROP1 promotes the targeting and/or capturing of exocytic vesicles to the cortical region of the pollen tube tip.

ROP1 activity also plays an important role in the spatiotemporal control of vesicle exocytosis. Using FRAP analysis, we showed that vesicle exocytosis is highly active in the apical cap of the PM, where active ROP is localized. The rate of exocytosis forms a tip-high gradient, which corresponds to the gradient of active ROP1 (Hwang et al., 2005). Interestingly, CA-rop1
expression induced depolarization of exocytosis, which is consistent with its induction of depolarized distribution of active ROP to the PM and growth depolarization (Li et al., 1999; Hwang et al., 2005). Our observations are in the line with the fact that ROP1 activity spatially predicts the site and direction of tip growth (Hwang et al., 2005). Taken together, we propose that the tip-localized ROP1 activity spatiotemporally coordinates the targeting and exocytosis of vesicles to the apical PM region, resulting in tip growth.

Rho GTPase coordination of polar accumulation and exocytosis of vesicles to tip-growing sites may be a common mechanism for the control of tip growth in various systems. ROP GTPase has also been shown to control polarized tip growth in root hairs, a process also known to require ROP
The tip expansion, and the compensatory exocytosis should immediately follow the tip expansion, which could explain why Ca\(^{2+}\) slightly lags behind the

dock and fuse to the PM until the Ca\(^{2+}\) level is high to disassemble cortical F-actin. The exact timing of vesicle exocytosis has yet to be determined, but

required for the accumulation of exocytic vesicles to the tip but inhibits vesicle exocytosis to the apical PM (orange). Meanwhile, ROP1 also activates

is activated at the apical region of the PM, and active ROP1 subsequently activates two distinct pathways. The RIC4 pathway promotes F-actin assembly

is expected to be in phase with Ca\(^{2+}\), which slightly lags behind tip expansion (Messerli et al., 2000). If so, the PM at the tip may be stretched during

vates the Arp2/3 complex that nucleates actin to form actin cables that are thought to be important for targeting of exocytic vesicles (Martin et al., 2007). Rho GTPases have also been shown to regulate tip growth in filamentous fungi (Bauer et al., 2002). Our results also suggest that the RIC4-dependent cortical F-actin inhibits the exocytosis of the vesicles targeted by the RIC4-dependent mechanism. RIC4 overexpression almost completely inhibited vesicle fusion to the PM, despite inducing accumulation of exocytic vesicles near the cortex. RIC4-dependent cortical actin meshwork presumably blocks vesicle tethering to the PM. Moreover, our results suggest that the RIC3–Ca\(^{2+}\)-dependent pathway promoting depolymerization of this meshwork is also required for vesicle exocytosis to the apical PM.

Our results suggest that tip-localized ROP1 signaling coordinates the accumulation and exocytosis of vesicles to the tip of pollen tubes through ROP1-dependent actin dynamics. Pollen tube tips contain a cortical actin meshwork localized to the extreme tip and a cortical actin fringe behind this network (Fig. S2; Gibbon et al., 1999; Fu et al., 2001; Gu et al., 2005; Lovy-Wheeler et al., 2005). Our previous work shows that ROP1 controls pollen tube tip growth through its regulation of the dynamics of these F-actin structures and their oscillation downstream signaling events such as tip-focused calcium gradient and tip-localized F-actin (Molendijk et al., 2001; Jones et al., 2002). Rho GTPases such as Cdc42 are also known to regulate exocytosis in yeast and filamentous fungi (Adamo et al., 2001). In yeast, Cdc42 has been shown to regulate exocytosis by activating exocyst, a protein complex involved in the tethering of exocytic vesicles to the PM. Cdc42 also activates the Arp2/3 complex that nucleates actin to form actin patches, but it is unclear whether actin patches are involved in vesicle accumulation to the growing site. However, Cdc42 signaling appears to indirectly influence the formation of actin cables that are thought to be important for targeting of exocytic vesicles (Martin et al., 2007). Rho GTPases have also been shown to regulate tip growth in filamentous fungi (Bauer et al., 2004). Although their mode of action is unclear, it would not be surprising that they also regulate vesicle targeting and exocytosis in these fungi.

ROP1-mediated F-actin dynamics spatiotemporally coordinate vesicle targeting and fusion to the tip

Our results suggest that tip-localized ROP1 signaling coordinates the accumulation and exocytosis of vesicles to the tip of pollen tubes through ROP1-dependent actin dynamics. Pollen tube tips contain a cortical actin meshwork localized to the extreme tip and a cortical actin fringe behind this network (Fig. S2; Gibbon et al., 1999; Fu et al., 2001; Gu et al., 2005; Lovy-Wheeler et al., 2005). Our previous work shows that ROP1 controls pollen tube tip growth through its regulation of the dynamics of these F-actin structures and their oscillation (Fu et al., 2001; Gu et al., 2005). ROP1 not only activates RIC4 to promote the assembly of the apical F-actin but also activates the RIC3–Ca\(^{2+}\) pathway to promote the disassembly of the apical F-actin (Gu et al., 2005). By manipulating agents in these two pathways, we show that the RIC4-dependent F-actin is required for the accumulation of exocytic vesicles to the region near the cortex of the pollen tube tip. We propose that RIC4-mediated F-actin generates the force required for vesicles to be targeted to a region near the cortex. Colocalization of RabA4d with the cortical actin meshwork in RIC4-overexpressing tubes suggests that the meshwork is important for vesicle accumulation, but a role for the actin fringe in this process is also possible.

Our results also suggest that the RIC4-dependent cortical F-actin inhibits the exocytosis of the vesicles targeted by the RIC4-dependent mechanism. RIC4 overexpression almost completely inhibited vesicle fusion to the PM, despite inducing accumulation of exocytic vesicles near the cortex. RIC4-dependent cortical actin meshwork presumably blocks vesicle tethering to the PM. Moreover, our results suggest that the RIC3–Ca\(^{2+}\)-dependent pathway promoting depolymerization of this meshwork is also required for vesicle exocytosis to the apical PM.

From the results presented here, we propose a model in which ROP GTPase-dependent F-actin dynamics function as a key regulator of polarized tip growth through two counteracting pathways regulating F-actin assembly and disassembly, which spatiotemporally coordinate targeted vesicle accumulation and exocytosis (Fig. 7). The spatial coordination of these two cellular processes is achieved through the formation of the active ROP1 cap in the tip of pollen tubes, which

Figure 7. **A model for the coordination of vesicle transport and exocytosis by ROP1-dependent actin dynamics during oscillatory pollen tip growth.** (A) Pollen tip growth is controlled by locally distributed ROP1 activity through its effect on actin assembly and disassembly at the tip of pollen tubes. ROP1 is activated at the apical region of the PM, and active ROP1 subsequently activates two distinct pathways. The RIC4 pathway promotes F-actin assembly required for the accumulation of exocytic vesicles to the tip but inhibits vesicle exocytosis to the apical PM (orange). Meanwhile, ROP1 also activates the RIC3–calcium pathway. As RIC3-dependent Ca\(^{2+}\) levels rise, the tip F-actin is depolymerized, and vesicles can then tether on and fuse with the PM. This event is accompanied by reduced vesicle accumulation in the tip (blue). The RIC3 and RIC4 pathways counteract to control F-actin dynamics and consequently coordinate vesicle targeting and exocytosis. (B) A temporal relationship among ROP1, F-actin, and Ca\(^{2+}\) oscillation based on this work and previous studies (Messerli et al., 2000; Hwang et al., 2005). An approximate temporal relationship among ROP1 activity, tip F-actin accumulation, tip expansion (growth) rate, and tip Ca\(^{2+}\) concentration is illustrated using different color-coded curves, whereas the peak points of vesicle accumulation and anticipated exocytosis are indicated by arrows. Tip-localized active ROP1 activates both RIC4 and RIC3, respectively, leading to rapid actin assembly and a delayed Ca\(^{2+}\) gradient formation and actin disassembly. We propose that when F-actin is assembled, vesicles are targeted to the tip, but do not dock and fuse to the PM until the Ca\(^{2+}\) level is high to disassemble cortical F-actin. The exact timing of vesicle exocytosis has yet to be determined, but is expected to be in phase with Ca\(^{2+}\), which slightly lags behind tip expansion (Messerli et al., 2000). If so, the PM at the tip may be stretched during the tip expansion, and the compensatory exocytosis should immediately follow the tip expansion, which could explain why Ca\(^{2+}\) slightly lags behind the tip expansion.

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activates the RIC4-mediated assembly in the apical cortex of pollen tubes as well as the RIC3-mediated formation of a tip-focused Ca\(^{2+}\) gradient that in turn leads to disassembly of the apical F-actin. The temporal coordination of vesicle targeting and exocytosis involves the time delay of the RIC3–Ca\(^{2+}\) pathway so that F-actin–dependent vesicle targeting is temporarily followed by exocytosis requiring the RIC3–Ca\(^{2+}\)-dependent actin depolymerization. RIC4-mediated F-actin assembly oscillates in a phase slightly behind the apical ROP1 activity but well ahead of the growth burst (Hwang et al., 2005). In this paper, we show that the apical accumulation of exocytic vesicles oscillates in a phase also ahead of growth burst. The Ca\(^{2+}\) gradient is known to oscillate in a phase slightly behind the growth burst (Messerli et al., 2000) and thus is clearly behind that of the apical F-actin, though both act downstream of ROP1 (Gu et al., 2005). The time delay for the RIC3–calcium pathway allows for the RIC4-dependent assembly of the apical F-actin, which mediates the apical accumulation of exocytic vesicles. The F-actin blockage of these vesicles from tethering to the apical PM is then released when subsequent activation of the RIC3–calcium pathway leads to the disassembly of the apical F-actin, which induces a burst of exocytosis. The RIC3-mediated Ca\(^{2+}\) may also play a role in activating vesicle fusion, although this has not been demonstrated in pollen tubes. Thus, this spatiotemporal coordination of vesicle targeting and exocytosis also explains the oscillatory nature of pollen tube tip growth.

Our findings beg an interesting future question: Is the coordinate regulation of vesicle targeting and exocytosis by the ROP1-dependent actin dynamics coupled to additional ROP1-dependent pathways? One potential pathway is the tethering of exocytic vesicles through exocyst, which is known to tether exocytic vesicles in yeast and animals (Boyd et al., 2004; Beronja et al., 2005). Exocyst components have been genetically shown to be essential for tip growth in plants (Cole et al., 2005; Synek et al., 2006). Interestingly, a recent study has implicated a novel ROP effector, ICR1, interacting with the SEC3 homologue of an exocyst component, which suggests that ROPs could be involved in regulating the tethering of exocytic vesicles through exocyst (Lavy et al., 2007). This potential ROP downstream pathway would be analogous to the yeast Cdc42 interaction with SEC3 to recruit exocyst to the site of polar growth in yeast.

Our findings may also have a broad implication in understanding the regulation of exocytosis in other eukaryotic cells because the dual role of F-actin dynamics in vesicle targeting and exocytosis may provide a paradigm for the cellular control of exocytosis in eukaryotic cells. Evidence from mammalian cells apparently supports this hypothesis. Actin assembly and disassembly have both been implicated in the regulation of exocytosis in mammalian cells, in which actin was found to both mediate movement of and hinder exocytosis of secretory granules in the subplasmalemmal region (Lang et al., 2000). In neuroendocrine cells, treatment with low concentrations of LatB increases secretion (Gasman et al., 2004). Nonetheless, further studies should determine how common the actin dynamics coordination of vesicle targeting and exocytosis is in eukaryotic cells.

### Materials and methods

#### Plant materials and growth conditions
Nicotiana tabacum plants were grown in growth chambers at 22°C under 12 h of dark/12 h of light.

#### DNA manipulation and plasmid construction
All plasmids used for transient expression in pollen were constructed in a pLATS2 vector as described previously (Fu et al., 2001; Wu et al., 2001). cDNA for the *A. thaliana* pollen-expressed RIK was amplified by PCR with gene-specific primers (5'-GCCCTAGAATTCCTCCATGCAGCCGGC-3' and 5'-CGGATCCGTTAAGGCAAAGGTACCAAC-3') and subcloned into a pLATS2:GFP vector.

#### Particle bombardment-mediated transient expression in tobacco pollen
Mature pollen grains collected from tobacco plants were used for transient expression with a particle bombardment procedure as described previously (Fu et al., 2001), 1 μg YFP-RabA4d or 0.5 μg LAT52:RiK-GFP plasmid DNA was used to coat the 0.5-mg gold particles. For investigating the effect of ROP1 signaling, 0.2 μg of CA-rop1, DN-rop1, RIC3, or RIC4 plasmid DNA was used with 1 μg YFP-RabA4d and 0.5 μg RIK-GFP plasmid DNA each. The pollen grains were incubated for 4 h before observation under a confocal microscope.

#### Drug treatments
3 h after bombardment, 5 μM LatB and 100 mM LaCl\(_3\) stock solution were added to the germination medium to a final concentration of 5 nM and 50 μM, respectively. For BFA treatment, 1 μg/ml BFA was included in germination medium in a 1-mg/ml stock solution. To determine the effect of jasplakinolide on exocytosis, a stock solution of 100 μM jasplakinolide (Invitrogen) was added to germinated pollen to a final concentration of 100 nM.

#### Confocal microscopy
For confocal laser scanning microscopy, the laser was focused on the median plane, where the apical PM-localized RIK-GFP or YFP-RabA4d signal was clearest. For single scans, a confocal microscope (SP2; Leica) was used with 488-nm laser excitation and emission at 500–570 nm for GFP, 514-nm laser excitation and emission at 520–550 nm for YFP, and 442-nm laser excitation and emission at 470–500 nm for CFP. Fluorescence images of the pollen tube were collected using a 63× water immersion lens (Leica) and confocal software (ICS; Leica), zoomed 4–8x with a 1,024 x 1,024 frame and 400-Hz scanning speed.

#### Analysis of YFP-RabA4d–stained vesicle accumulation and growth rate
4 h after bombardment, midplane sections of the pollen tubes were scanned in 5-s intervals. Time-lapse data were processed and analyzed using ImageJ (http://rsb.info.nih.gov/ij) software. To quantify the relative amount of YFP-RabA4d and localization at the apical region of the pollen tube, the mean YFP intensity of the apical region was measured from each median scan of a time-lapse image. The level of YFP intensity of the apical region was calculated by averaging the intensity of YFP signal within 5 μm from the end of the tube tip. Tip elongation was calculated from the net pixel extension of the tip between two consecutive images.

#### FRAP analysis
FRAP was performed on a confocal microscope (SP2) with a 63× water immersion lens. Fluorescence within the region of interest (ROI) was photobleached by 10–15 iterations of the 488-nm laser line at 100% emission strength, and then fluorescence recovery was recorded with 25% of laser power at 5-s intervals for 3 min. To quantify the fluorescence recovery, the mean GFP intensity was measured by drawing a line on the membrane of the ROI. Time-lapse data were processed and analyzed with use of ImageJ software.

### Online supplemental material
Fig. S1 shows expression and localization of YFP-RabA4d in *A. thaliana*. Fig. S2 shows effect of RIC3 and RIC4 overexpression on RabA4d accumulation and F-actin structure. Fig. S3 shows FRAP analysis of RIK-GFP after BFA treatment. Fig. S4 shows RIK-GFP FRAP analysis of pollen tubes overexpressing RIC3. Fig. S5 shows FRAP analysis on pollen tubes treated with LaCl\(_3\) and jasplakinolide. Video 1 shows the dynamic accumulation of YFP-RabA4d in a growing pollen tube. Videos 2 and 3 show FRAP analysis of RIK-GFP at the apical tip and subapical region, respectively. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200801086/DC1.
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