Oscillatory ROP GTPase Activation Leads the Oscillatory Polarized Growth of Pollen Tubes

Jae-Ung Hwang,*† Ying Gu,† Yong-Jik Lee,† and Zhenbiao Yang †

*College of Life Sciences, China Agricultural University, Beijing 100094, China; and †Department of Botany and Plant Sciences and Center for Plant Cell Biology, Institute for Integrative Genome Biology, University of California–Riverside, Riverside, CA 92521

Oscillation regulates a wide variety of processes ranging from chemotaxis in Dictyostelium through segmentation in vertebrate development to circadian rhythms. Most studies on the molecular mechanisms underlying oscillation have focused on processes requiring a rhythmic change in gene expression, which usually exhibit a periodicity of >10 min. Mechanisms that control oscillation with shorter periods (<10 min), presumably independent of gene expression changes, are poorly understood. Oscillatory pollen tube tip growth provides an excellent model to investigate such mechanisms. It is well established that ROP1, a Rho-like GTPase from plants, plays an essential role in polarized tip growth in pollen tubes. In this article, we demonstrate that tip-localized ROP1 GTPase activity oscillates in the same frequency with growth oscillation, and leads growth both spatially and temporally. Tip growth requires the coordinate action of two ROP1 downstream pathways that promote the accumulation of tip-localized Ca²⁺ and actin microfilaments (F-actin), respectively. We show that the ROP1 activity oscillates in a similar phase with the apical F-actin but apparently ahead of tip-localized Ca²⁺. Furthermore, our observations support the hypothesis that the oscillation of tip-localized ROP activity and ROP-dependent tip growth in pollen tubes is modulated by the two temporally coordinated downstream pathways, an early F-actin assembly pathway and a delayed Ca²⁺ gradient-forming pathway. To our knowledge, our report is the first to demonstrate the oscillation of Rho GTPase signaling, which may be a common mechanism underlying the oscillation of actin-dependent processes such as polar growth, cell movement, and chemotaxis.

INTRODUCTION

Oscillating phenomenon is common in biological systems for propagating signals and spatially and temporally coordinating biological processes and is under tight regulation by “the oscillator” at cellular and molecular levels (Bessho and Kageyama, 2003; Patnaik, 2003; Merrow and Roenneberg, 2004). The circadian clock is the most studied oscillation. It regulates diverse metabolic, physiological, and behavioral events in a wide range of organisms on a 24-h basis from the hypocotyl elongation in plants to the sleep/wake cycle of human (Schultz and Kay, 2003; Gachon et al., 2004; Millar, 2004). The mechanism of circadian clock involves a gene expression circuit with both feed-forward and negative feedback controls, resulting rhythmic expressions of key transcriptional regulators (Badieu, 2003; Bessho and Kageyama, 2003; Millar, 2004). In biological systems, there exist many types of noncircadian cellular oscillations with much shorter periods, e.g., seconds or minutes, such as Ca²⁺ waves in plant and animal cells (Allen et al., 1999; Lee et al., 2002), oscillations of metabolites in continuous culture of the budding yeast (Patnaik, 2003), periodic lamellipodial contractions in spreading and migrating animal cells (Giannone et al., 2004), cAMP-mediated waves of cell aggregation in Dictyostelium (Maeda et al., 2004), and oscillatory tip growth of pollen tubes (Hepler et al., 2001). Unlike the circadian clock, many of these rapid oscillations with a period of minutes or even seconds, are unlikely to involve periodic changes in gene expression. The molecular nature of these oscillators remains poorly understood.

Pollens tube tip growth is one good example of the integration of biological rhythms with spatial control of cellular behaviors. For sexual reproduction, pollen tubes extend directionally within female tissues toward the ovule, guided by cell-cell interaction, gravity, and chemoattractant from the ovary (Franklin-Tong, 1999; Lord, 2003; Palanivelu et al., 2003). In this highly competitive journey, pollen tubes show a dramatic rapid elongation of cell length without further cell division and differentiation. The rapid pollen tube growth appears to be facilitated by growth oscillation typically with periods of tens of seconds (Feijo et al., 2001; Holdaway-Clarke et al., 2003; Parton et al., 2003). Recent studies suggest a positive correlation between oscillation and the rate of tip growth (Holdaway-Clarke et al., 2003; Messerli and Robinson, 2003; Parton et al., 2003). Oscillatory growing pollen tubes grow faster than the ones without clear oscillation (Messerli and Robinson, 1997). In addition, the faster growing pollen tubes generally exhibit the shorter periods of oscillation (Holdaway-Clarke et al., 2003; Parton et al., 2003).

The tip growth oscillation appears to be spontaneous, as the oscillation occurs in minimal pollen tube growth medium. Many cellular events associated with tip growth oscillate with the same periodicity of growth rate, including

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Address correspondence to: Zhenbiao Yang (yang@ucr.edu).
tip-localized actin microfilaments (F-actin), membrane trafficking, and intracellular ion gradients and ion fluxes at the tip (Holdaway-Clarke et al., 1997; Messerli et al., 1997; Messerli et al., 1999, 2000; Feijo et al., 2001; Fu et al., 2001; Parton et al., 2001; Zonia et al., 2002). In particular, tip-focused gradients of cytosolic calcium ([Ca$^{2+}$]$_{cyt}$) and tip-localized influxes of extracellular Ca$^{2+}$ ([Ca$^{2+}$]$_{exo}$) have been shown to tightly associate with growth oscillation, lagging behind growth by ~40° and ~120-150°, respectively (Messerli et al., 2000). However, we have no knowledge of molecular mechanisms underlying the oscillations in growing pollen tubes.

Members of the Rho family of small GTPases play a central role in several important cellular responses such as cytokinesis, directional cell movement and growth, and gene expression via regulation of the actin cytoskeleton (Etienne-Manneville and Hall, 2002; Fukuta et al., 2003). Arabidopsis possesses a unique subfamily of Rho GTPases, named ROP (Rho-related GTPase from plants), which are known to regulate tip growth and polar cell expansion in different cell types (Kost et al., 1999; Li et al., 1999; Molenik et al., 2001; Fu et al., 2002; Jones et al., 2002). In particular pollen-specific ROP1, probably together with two other closely related and functionally redundant ROPs (ROP3 and ROP5), has been shown to be an essential regulator of pollen tube tip growth (Kost et al., 1999; Li et al., 1999). ROP1 is preferentially localized to the apical region of the pollen tube plasma membrane (PM) and activates at least two downstream pathways that respectively regulate the generation of the tip-focused [Ca$^{2+}$]$_{cyt}$ gradients and the assembly of dynamic tip-localized actin microfilaments (Kost et al., 1999; Li et al., 1999; Fu et al., 2001; Gu et al., 2003, 2005). ROP1 activates two direct downstream targets, RIC3 and RIC4, which are CRIB motif-containing ROP-interacting proteins (Wu et al., 2001; Gu et al., 2005). RIC3 modulates the formation of the tip-focused [Ca$^{2+}$]$_{cyt}$ gradient probably through the regulation of extracellular Ca$^{2+}$ influxes, whereas RIC4 promotes the assembly of the apical F-actin (Gu et al., 2005). RIC3-dependent calcium promotes the disassembly of the apical F-actin. It was further shown that the two ROP1 downstream pathways counteract to control the dynamics of tip-localized actin microfilaments (Gu et al., 2005).

The dynamics of the apical F-actin is not only required for polarized growth but also seems to be important for growth oscillation in pollen tubes (Fu et al., 2001). In tobacco pollen tubes, RIC4 overexpression caused stabilization of actin microfilaments at the tip, leading to depolarized growth accompanied by loss of growth oscillation (Gu et al., 2005). Recovering actin dynamics by treating RIC4-overexpressing tubes with latrunculin B (LatB), which promotes actin depolymerization, rescues polar growth and oscillation. Actin dynamics is exhibited as periodic fluctuations of F-actin at the tip of pollen tubes (Fu et al., 2001). Because the activation of the RIC4-dependent actin pathway relies on RIC4 interaction with active ROP1 at the apical domain of the plasma membrane (Gu et al., 2005), it is reasonable to speculate that ROP1 activity at the tip is dynamic in order for it to control actin dynamics. In another word, periodic up- and down-regulation of ROP1 activity might be required for the modulation of growth polarity and oscillation. The idea is supported by the observation that cycling between GTP-bound active and GDP-bound inactive status of ROP1 is critical for normal tip growth of pollen tubes (Li et al., 1999; Gu et al., 2003). Expression of constitutively active forms of ROP1 caused depolarization of growth, generating bulb-shaped pollen tubes, whereas expression of dominant negative forms inhibited tip growth (Kost et al., 1999; Li et al., 1999).

In this report, we have developed a GFP-based assay allowing visualization and quantification of the interaction between active ROP1 and RIC4 in the apical domain of the plasma membrane in growing pollen tubes. Using this assay, we provide evidence that ROP1 activation at the tip oscillates in the same frequency with the oscillation of tip growth. We show that active ROP1-RIC4 interaction leads growth both spatially and temporally, i.e., the focal point of this interaction predicts the future direction of pollen tube growth and this interaction rises ~90° ahead of tip growth rate. Significantly the oscillation of this interaction is regulated by ROP1 downstream signaling events such as calcium and actin microfilaments. These observations support the hypothesis that spatiotemporal regulation of ROP signaling at the tip of pollen tubes, including feedback regulation of ROP activation, is a key mechanism underlying the oscillation of polarized pollen tube growth.

### MATERIALS AND METHODS

#### Transient Expression in Tobacco Pollen Tubes

Mature pollen grains collected from tobacco flowers (Nicotiana tabacum) were used for transient expression of ROP’s, RICs, RopGAP1 (Rop GTPase-activating protein 1), or GFP-mTalin by using a projectile-mediated transformation as described by Fu et al. (2001). For all plasmid constructs used in our experiments, protein coding ORFs were under the control of the pollen-specific promoter LAT52 (Jewell et al., 1991). For analyzing the cellular localization of RIC4 and the morphology of pollen tubes, 0.1–0.2 µg of GFP-RIC4 or GFP-RIC4AC construct was used. To investigate the effect of ROP1, RopGAP1, and DN-rop1 (a dominant negative mutant of ROP1) on the localization of GFP-RIC4AC, 0.3 µg of plasmid DNA expressing ROP1, RopGAP1, or DN-rop1 was used for cotransformation. To study the effect of RIC4 or RIC4AC on the organization of actin microfilaments, 0.4 µg of plasmid DNA encoding GFP-mTalin was cotransformed with 0.4 µg of plasmid DNA encoding RIC4 or RIC4AC. Within 3–6 h of bombardment, pollen tubes were observed by using confocal microscopy. For fluorescence resonance energy transfer (FRET) analysis, 0.7 µg of GFP-RIC4AC and 0.5 µg of other DNA constructs were bombarded and FRET response was measured within 4–7 h. For time-lapse analysis of RIC4 localization or tip-localized F-actin dynamics, 50 ng of GFP-RIC4 or GFP-RIC4AC construct, or 0.2 µg of GFP-mTalin construct were used, respectively. Within 2.5 h after bombardment, pollen tubes were mounted on a thin layer of 0.5% agarose to study the oscillation of tip-localized GFP-RIC4, chemicals such as LatB and LaCl$_3$ were applied to germination medium 2 h after bombardment. To induce accumulation by laser marker, germination medium with working concentration of LatB was applied to one side of cover glass to diffuse into the mounted pollen tube.

#### Confocal Microscopy

For confocal laser scanning microscopy, laser was focused on the median plane, where the apical PM-localized GFP-RIC4 or GFP-RIC4AC signal was the most clear. For single scans, a Bio-Rad MRC 600 (Bio-Rad Laboratories, Hercules, CA) or Leica SP2 (Leica, Wetzlar, Germany) confocal microscope was used. To raise signal-to-noise ratios, fluorescence and laser power for time lapse and 20–25% for single-section scans.

#### Analysis of RIC4 Localization, F-actin Dynamics, and Growth Rate

Time-lapse and Z-series section data were processed and analyzed using a MetaMorph software version 4.5 (Universal Imaging, West Chester, PA). To quantify the relative amount of GFP-RIC4 or GFP-RIC4AC localized at the apical region of the pollen tube PM, the average GFP intensity of the apical PM region (1-avg-pm, Figure 3B), which had a distinct GFP fluorescence from the cytosol, was measured from each median scan of time-lapse recording. The average intensity of cytosolic part (1-avg-cyt) and PM region (1-avg-pm, Figure 3B), which had a distinct GFP fluorescence from the cytosol, was measured from each median scan of time-lapse recording.
of 15-μm circumference in the middle of tube, 3 μm away the end of tube tip (Figure 3B). The level of actin filaments in the tip was calculated by the average intensities of GFP-mTalin (I-avg) within 3 μm from the end of tube tip (Figure 4A). Tip elongation was calculated from the net pixel extension of the tip between two consecutive images and compared with the mean values of I-avg-PM of GFP-RIC4ΔC or I-avg of GFP-mTalin from those two images. Any apparent growth direction change was adjusted by using the image rotation function of MetaMorph (Universal Imaging). Z-stack images of the actin cytoskeleton in the pollen tube (Figure 1C) were constructed from Z-series scans, obtained as described above, and processed using the nearest neighbors deconvolution.

**FRET Assay**

To test in vivo interaction between ROP1 and RIC4ΔC in tobacco pollen tubes, CFP-RIC4ΔC was expressed together with YFP-ROP1 with/without ROP, GAP1 or YFP-DN-rop1. In 4–5 h of germination, sequential scan images were collected using a Leica SP2 confocal microscope: emissions in CFP channel (470–500 nm) and in FRET channel (520–550 nm) with 25% 442-nm laser excitation and 10% YFP emission at 520–550 nm with 50% 514-nm laser excitation. The sequentially scanned images were processed with a MetaMorph software (version 4.5, Universal Imaging). FRET signal was corrected by subtraction of YFP emission due to non-FRET (CFP bleed-through to FRET channel and YFP acceptor excitation by 442-nm laser). Correction factors for non-FRET signals were calculated from CFP donor alone and YFP acceptor alone controls. Corrected FRET signal was normalized with the acceptor amount (FRET efficiency, % of YFP emission with 10% 514-nm laser excitation) for comparison. FRET efficiency was measured at two or three different PM regions where both donor and acceptor were enriched in individual tubes tested.

**In Vitro Pulldown Assay**

In vitro pulldown of MBP-tagged RIC4 or -RIC4ΔC by GST-tagged CA-rop1 (a constitutively active mutant of ROP1) or -DN-rop1 was performed following the procedure described in Wu et al. (2001). Ten micrograms of agrose conjugated GST-ROPIs was loaded with GTP for CA-rop1 and GDP for DN-rop1, respectively, and incubated with 10 μg of MBP-RIC4 or MBP-RIC4ΔC for 2 h at 4°C. MBP-RIC4 and MBP-RIC4ΔC bound to ROP1 were centrifuged down and analyzed by using SDS-PAGE and Western blotting with anti-MBP antibody (New England Biolab, Ipswich, MA).

**RESULTS**

A GFP-tagged RIC4 Deletion Mutant (GFP-RIC4ΔC) Reports Active ROP-dependent RIC4 Localization to the Tip

*Arabidopsis* ROP1 GTPase activates two downstream pathways that respectively control the formation of tip-focused [Ca²⁺]cyt gradient and the assembly of tip-localized dynamic actin microfilaments (Li et al., 1999; Fu et al., 2001; Gu et al., 2005). These two ROP signaling targets are known to oscillate in different phases (Messerli et al., 2000; Fu et al., 2001). To understand how ROP GTPase spatiotemporally coordinates these two pathways for tip growth, we need to know whether ROP activity shows spatiotemporal dynamics. To gain insights into the spatiotemporal regulation of ROP activity, we investigated RIC4 localization in growing pollen tubes, because RIC4 was previously shown to interact preferentially with an active form of ROP1 and because its localization to the apex depends on the presence and activation of ROPs (Gu et al., 2005).

In vitro cultured *Arabidopsis* pollen tubes exhibit slow and highly variable growth rates and thus are not suitable for temporal analysis of RIC4 localization (Taylor and Hepler, 1997). We used tobacco pollen for this study, because it elongates fast with relatively homogenous growth rate and it has been a chosen system for transient expression-based gene functional analysis (Read et al., 1993; Kost et al., 1999; Fu et al., 2001; Chen et al., 2003). Extensive studies on ROP signaling and ROP-ROP interaction have been conducted in this system (Kost et al., 1999; Wu et al., 2000; Fu et al., 2001; Wu et al., 2001; Gu et al., 2005). However transient overexpression of RIC4 in tobacco pollen tubes induced depolarized tip growth and eliminated the oscillatory growth by increasing actin microfilament assembly in the tip region (Wu et al., 2001; Gu et al., 2005). To investigate the localization of RIC4 in oscillatory growing pollen tubes, we attempted to identify a RIC4 deletion mutant that is removed of its effector function but retains the capacity to bind active ROP1 as normally as wild-type RIC4 does. Because RIC4 contains a CRIB motif localized in the central region and required for RICs to interact with ROPs, and novel amino acid sequences localized to the N- and C-terminal regions (Wu et al., 2001; Gu et al., 2005), we reasoned that the N- and C-terminal regions likely contain a RIC4 effector domain involved in the regulation of F-actin assembly and that deletion of this domain would eliminate RIC4 activity but not affect its interaction with active ROP1.

We transiently expressed various RIC4 deletion mutants tagged with GFP in tobacco pollen tubes. A short deletion from the N-terminal region enhanced RIC4-induced depolarization of growth, whereas more extensive deletion either altered the PM localization pattern or eliminated the PM localization (Hwang and Yang, unpublished data). However, a C-terminal deletion mutant, RIC4ΔC (133–153), in which the last 21 amino acid residues are removed (Figure 1A), did not induce depolarization of growth but retained the typical apical PM localization (Figure 1B). In the majority of tubes coexpressing RIC4ΔC and GFP-mTalin, actin microfilaments appeared normal like control tubes expressing GFP-mTalin alone (Figure 1C). Therefore, the C-terminal region is required for the effector function of RIC4 in the promotion of tip-localized F-actin assembly.

We next assessed whether RIC4ΔC faithfully reported RIC4 localization and interaction with active ROP1 using several in vitro and in vivo experiments. When transiently expressed in tobacco pollen tubes, GFP-RIC4ΔC was localized to the apical domain of the PM as a tip-high gradient (Figure 1B), similar to GFP-RIC4 localization described (Wu et al., 2001; Gu et al., 2005). We term this localization pattern as the apical cap. Noticeably GFP-RIC4ΔC localization to the apical cap was dependent on ROP1 activation, as is GFP-RIC4 localization (Figure 2A; Gu et al., 2005). ROP1 overexpression increased the distribution and accumulation of

![Figure 1](image-url)
Figure 2. RIC4ΔC binds to tip-localized active ROP1 but does not alter the pollen tube growth. (A) The apical PM localization of GFP-RIC4ΔC is dependent on ROP1 activation. Typical fluorescent images of tobacco pollen tubes expressing GFP or GFP-RIC4ΔC alone or together with Rop1, RopGAP1, DN-rop1, ROP10, or DN-rop10. (B) In vitro pulldown assay of MBP-fused RIC4ΔC with GST-CA-rop1 or GST-DN-rop1 in comparison to MBP-RIC4. Specific binding to active ROP1 is identical to wild-type RIC4. The top panel is Western blot of MBP-RIC4ΔC or MBP-RIC4 with anti-MBP antibody and the lower one is the loading control for GST-CA-rop1 or GST-DN-rop1 used in the assay. CA, CA-rop1; DN, DN-rop1; NC, negative control. (C) FRET assay between CFP-RIC4ΔC and YFP-ROP1 (1st row), YFP-DN-rop1 (2nd row), or YFP-ROP1 in the presence of RopGAP1 (3rd row). Emissions of CFP donor in CFP channel and FRET in YFP channel with excitation at 442 nm and of YFP acceptor in YFP channel with excitation at 514 nm were taken sequentially and processed as described in Materials and Methods. FRET efficiency (% of YFP acceptor emission) was displayed in IMD mode with eight-color hues from red to blue (0–30%) and with intensity of each color indicating the intensity of acceptor. Bar, 10 μm. (D) Dosage effect of GFP-RIC4ΔC expression on pollen tube growth. Compared with GFP-RIC4 of similar amount (similar average GFP intensity in the tip), low and moderate expression (average intensity <150) of GFP-RIC4ΔC did not induce the depolarized growth. Depolarization of tip growth was quantified by measuring the ratio of tube width versus tube length. All the fluorescent images of pollen tubes were taken under the same confocal microscope setting. Average intensity value was displayed in an arbitrary unit (a.u.) from 0 to 255. Sixty tubes for GFP and GFP-RIC4 expressing tubes and 118 tubes for GFP-RIC4ΔC-expressing tubes were pooled based on the average intensity (0–50, 51–100, 101–150, >150) and mean values of width/length ratio were obtained. Small fluctuation in width/length ratio of control tubes (GFP) was not significant (p > 0.1). Significant different width/length ratios from those of control tubes were asterisked (p < 0.05).
increased the accumulation of GFP-RIC4. Similarly, overexpression of dominant-negative rop1 (DN-rop1), which blocks the activation of ROP1 presumably by trapping its endogenous activators, diminished GFP-RIC4 localization to the apical cap. These results show that GFP-RIC4 interacts with ROPI activation-dependent localization to the apical cap, similar to that of GFP-RIC4.

The above observations, together with our previous results showing that RIC4 directly interacts with active GTP-ROP1 but not inactive GDP-ROP1 in vivo (Gu et al., 2005), strongly suggest that the apical cap localization of GFP-RIC4 is the direct result of GFP-RIC4 binding to the activated form of ROP1 that is present in the apical cap. We further tested this hypothesis using in vitro pulldown and in vivo FRET assays (Figure 2, B and C). Pulldown assays showed that MBP-tagged wild-type (WT) RIC4 interacted strongly with a constitutively active GTP-bound form of ROP1 (CA-rop1, CA) but weakly with the dominant negative GDP-bound ROP1 (DN-rop1, DN). Compared with WT RIC4, RIC4 had a slightly increased binding to CA-rop1 and reduced interaction with DN-rop1 (Figure 2B), indicating that removal of the C-terminal domain did not alter the capacity of RIC4 to preferentially interact with active GTP-ROP1. We also tested whether the C-terminal deletion caused promiscuous interaction with other ROPs. In addition to the three functionally overlapping ROPs (ROP1, 3, 5), a group I ROP (ROP8) and three group II ROPs (ROP9, 10, 11) are also expressed in Arabidopsis pollen (Winge et al., 1997; Kost et al., 1999; Li et al., 1999; Cheung et al., 2003; Gu et al., 2003). However, evidence suggests that none of these ROPs functionally interact with RIC4 (Gu et al., 2005). Similarly, none of them interacted with RIC4 in pollen tubes as ROP1 did, because overexpression of ROP8, ROP9, ROP10, or ROP11 had no or little effect on GFP-RIC4 localization to the apical cap (see Supplementary Figure S1).

The lack of RIC4 interaction with other ROPs (9, 10, and 11) is further supported by the observation that a dominant negative mutant of ROP10, which would be expected to block the activation of group II ROPs when overexpressed, did not affect the localization of GFP-RIC4 (Figure 2A). These observations strongly support the conclusion that the removal of the C-terminal domain of RIC4 does not cause a promiscuous RIC4 interaction with other ROPs that normally do not bind RIC4 (Gu et al., 2005).

We next investigated the in vivo interaction of RIC4 with active ROP1 using FRET analysis. FRET signal was obtained from YFP emission with an excitation at 442 nm for CFP and corrected for non-FRET signals (i.e., the CFP donor bleed-through into YFP channel and YFP emission from acceptor excited at 442 nm, see Materials and Methods for details). For comparison between treatments, FRET signals were normalized with the amount of acceptor and were presented as FRET efficiency (% of YFP emission resulting from 442-nm vs. 514-nm excitation; Figure 2C and Supplementary Figure S2). Nonspecific FRET between soluble CFP and YFP-ROP1 in the cytosol was negligible (an efficiency 0.1 ± 2.8%; n = 19; Supplementary Figure S2). FRET between CFP-RIC4 and YFP-ROP1 was prominent in the tip of pollen tubes (Figure 2C, first row). The distribution and intensity of PM-localized FRET signals were correlated with the localization of CFP-RIC4 to the apical PM region. The mean FRET efficiency in the tip of pollen tubes expressing CFP-RIC4 and YFP-ROP1 was 14.0 ± 6.1% at the PM and 5.9 ± 4.3% in the cytosol, respectively (58 independent PM areas and 15 independent cytosolic parts from 20 cells). This suggests that the interaction of RIC4 with ROP1 primarily occurred at the apical region of the PM. To test whether the FRET signals were the result of the interaction of CFP-RIC4 with GTP-bound or GDP-bound form of ROP1, we examined FRET between CFP-RIC4 and YFP-DN-rop1. FRET between CFP-RIC4 and YFP-DN-rop1 were dramatically lower than those with YFP-ROP1 (Figure 2C, second row, and Supplementary Figure S2), having a mean FRET efficiency of 7.4 ± 2.4% at the PM (p < 0.05, 32 regions from 15 cells, Supplementary Figure S2). This value was not significantly different from either mean cytosolic FRET efficiency for the same pair (7.2 ± 2.5%, n = 32) or cytosolic FRET efficiency for CFP-RIC4/YFP-ROP1. These results suggest that RIC4 interacts preferentially with the active form of ROP1 localized at the apical region of the PM in pollen tubes.

To further test whether the in vivo interaction between CFP-RIC4 and YFP-ROP1 was dependent on ROP activity, we analyzed their FRET in tubes coexpressing RopGAP1, which promotes the conversion of GTP-bound to GDP-bound form of ROPs (Wu et al., 2000). As shown in Figure 2C, RopGAP1 significantly suppressed the FRET efficiency between CFP-RIC4 and YFP-ROP1 at the PM to 10.3 ± 8.0% (p < 0.05, 22 regions from 11 cells). The relatively high variation in these tubes was probably due to variable RopGAP1 expression levels in different tubes. RopGAP1 expression suppressed ROP1 overexpression-induced depolarized growth. In those cases where depolarized growth was completely suppressed, the PM FRET efficiency was similar to that in the cytosol (Figure 2C, third row). In those tubes whose depolarized growth was less suppressed (presumably due to weak RopGAP1 expression), relatively high FRET signals were found at the apical cap (Supplementary Figure S2). Cytosolic FRET signals were not significantly different between cells expressing YFP-ROP1 with and without RopGAP1. These results suggest that RIC4 localization to the apical PM region is the direct result of its binding to the endogenous GTP-bound active form of ROP1 and/or closely related ROPs (collectively ROP1 hereafter for convenience) and thus this reflects the relative activity of ROP1 localized to the apical region of the PM in growing pollen tubes.

We were interested in using transiently expressed GFP-RIC4 for assessing relative ROP1 activity at the tip of normal growing pollen tubes. For this purpose, we determined the maximum level of transient expression for this construct that did not alter pollen tube elongation and tube morphology, because we expected that excessive expression of GFP-RIC4 would compete with endogenous RIC4 and thus affect polar growth in pollen tubes. As shown in Figure 1B, GFP-RIC4 displayed the typical localization pattern of RIC4, but its expression at moderate levels did not induce depolarized tip growth (Figure 2D). Induction of depolarized growth, which was measured by increased ratios of tube width over tube length (Fu et al., 2001; Gu et al., 2005), was analyzed from a population of tubes expressing various levels of GFP-RIC4 (indicated by the average GFP intensity of the tip). Low and moderate levels of GFP-RIC4 expression (relative intensity <150 in Figure 2D), had no effects on the polarity of pollen tube growth like the expression of soluble GFP (Figure 2D). At high levels of expression (intensity >150), RIC4 slightly induced depolarized growth, which might have resulted from residual effector activity of RIC4 or blockage of endogenous ROP signaling by overwhelming RIC4 in all experiments described.
below, we used a small amount of GFP-RIC4ΔC construct (50 ng) for transient expression, which produced a GFP-RIC4ΔC level that had no significant effects on pollen tube morphology.

**RIC4 Localization to the Apical Cap Oscillates Ahead of Tip Growth**

Having determined that GFP-RIC4ΔC localization to the tip can be used to report the distribution and accumulation of active ROP1 at the apical region of the pollen tube PM, we next investigated possible spatiotemporal dynamics of GFP-RIC4ΔC localization. A time series of GFP-RIC4ΔC localization was analyzed using confocal microscopy and compared with pollen tube elongation rates (Figure 3, B and C). The relative amount of GFP-RIC4ΔC accumulated at the apical cap was quantified from the median section of longitudinal confocal scans. Assuming that a pollen tube is a cylinder with a hemisphere at the tip and that GFP-RIC4ΔC localization to the tip forms a symmetric gradient, emanating from the very summit of the hemisphere (Figure 3B), the total amount of GFP-RIC4ΔC localized to the apical cap is linearly proportional to the GFP-RIC4ΔC amount on a single longitudinal section of the apical cap. Thus, the average intensity of GFP-RIC4ΔC at the apical cap (I-avg-pm) in the median scans reflects the total amount of RIC4ΔC at the apical cap (Figure 3B and see Materials and Methods for details). If we define pollen tube growth as net surface area increase, the growth is proportional to the height increase of the cylinder that corresponds to the linear tip elongation of a pollen tube. Thus, the comparison of I-avg-pm and the linear tip elongation rate well represents the temporal relationship between the relative GFP-RIC4ΔC amount at the apical cap and pollen tube growth. Hereafter, growth rate means linear tip elongation of pollen tubes per unit time.

The mean growth rates and oscillation periods for tubes expressing GFP-RIC4ΔC were comparable to those for untransformed tubes or the ones expressing GFP alone: mean growth rate and mean period length of untransformed control tubes are 25.1 ± 10.6 nm/s and 81 ± 24 s (n = 7), whereas those of GFP-RIC4ΔC expressed tubes are 30.5 ± 9.1 nm/s and 72 ± 29 s (n = 10). These were also similar to what was previously reported for untransformed tobacco pollen tubes (Zonia et al., 2002). In growing pollen tubes, I-avg-pm of GFP-RIC4ΔC oscillated with the same periodicity as growth rate (n = 22, Figure 3, A and C). The oscillation of I-avg-pm (GFP-RIC4ΔC) was not due to changes in the total GFP-RIC4ΔC amount in the apical region of the cytoplasm, because the average cytosolic GFP intensity in this region only showed slight changes lagging the growth rate oscillation (Figure 3C). The slight fluctuations in the cytosolic GFP-RIC4ΔC amount may be mainly resulted from the volume change of the tip region, because GFP-expressing tubes showed similar fluctuations (unpublished data). In addition, the periodic insertion of nascent PM materials could not have accounted for the GFP-RIC4ΔC oscillation for the following two reasons. First, in some pollen tubes that ceased growth upon treatment with LatB or LatC, several cycles of dampening GFP-RIC4ΔC I-avg-pm oscillation was observed without measurable tip elongation (unpublished data). Furthermore, GFP-RIC4ΔC oscillation was not in the opposite phase of periodic tip extraction (surface area increase). For fast growing tubes (> ~20 nm/s), the oscillation period of tip growth and I-avg-pm of GFP-RIC4ΔC was 50–160 s. Based on comparison of period length of oscillations, I-avg-pm oscillation was ahead of growth rate oscillation by 18 s in a typical 70-s period oscillation (asterisks and arrows in Figure 3C). In another word, I-avg-pm oscillation had a ~90° phase shift ahead of growth rate oscillation. Similar periodic fluctuations were observed for GFP-RIC4 localization to the tip (Supplementary Figures S4 and S5). These results indicate that RIC4 localization to the apical cap, i.e., the apparent tip-localized ROP1 activity, is oscillatory and leads growth bursts in growing pollen tubes. These results extend previous observations showing that ROP1 is critical and sufficient to drive pollen tube tip growth (Li et al., 1999; Gu et al., 2003).

**Tip-localized Actin Microfilaments Oscillate in a Similar Phase with RIC4 Localization to the Tip**

ROP1 regulates the assembly of tip-localized F-actin and the formation of tip-focused [Ca²⁺]c gradients, respectively, and the balance between these two pathways is critical for tip growth (Gu et al., 2005). It would be very interesting to understand how ROP temporally coordinates these two pathways, which seem to oscillate in different phase with the tip growth (Messerli et al., 2000; Fu and Yang, 2001). In a previous article, we found that tip-localized F-actin seemed to oscillate roughly in the opposite phase of but ahead of growth (Fu et al., 2001). However, this time-lapse analysis involved imaging with low resolution (one or two pixel extension each growth peaks) and thus had difficulty in determining a precise temporal relationship between tip elongation and the assembly of actin microfilaments because of high noise to signal ratio. Here, we investigated the oscillation of tip-localized actin microfilaments using a high-resolution time-lapse analysis (Figure 4 and see Materials and Methods). The level of F-actin in the apex was measured by averaging intensity of GFP-mTalin within 3 μm from the very tip at 5- or 10-s intervals (Figure 4A).

The pollen tubes we investigated displayed slow oscillatory tip growth of longer period compared with control tubes (Figure 4B). It is likely to be caused by slight inhibitory effect of GFP-mTalin on the oscillatory tip growth by altering F-actin dynamics (Cheung and Wu, 2004). We chose tubes with weak GFP fluorescence to minimize the possible inhibitory effect (Figure 4A; Fu et al., 2001). Correlation of period lengths was analyzed between each growth pulse and associated leading or lagging pulse of individual GFP-mTalin oscillations (n = 7). We found that the level of tip-localized GFP-mTalin oscillated ahead of growth rate oscillation (15–30 s shift ahead of a 70–160-s period): the phase shift of tip-localized F-actin assembly to the associated growth pulse is slightly <90°. This result suggest that tip-localized actin microfilaments oscillate in a similar phase with or slightly behind GFP-RIC4ΔC (Figures 3C and 4B), consistent with a role for RIC4 in promoting the assembly of actin microfilaments (Gu et al., 2005). Similar results were obtained using another GFP-based F-actin marker, GFP-fABD2 (second actin binding domain from AtFimbrin1; Sheahan et al., 2004; unpublished data). Simultaneous imaging of actin microfilaments and RIC4ΔC should more precisely define the temporal relationship between ROP1 activity and actin microfilaments. However this is not possible with the current reporter systems of GFP derivatives: YFP is easily bleached, whereas GFP and mRFP have low quantum yields and are of relatively slow expression. The observed oscillation of F-actin level (Figure 4A) was not due to changes in the amount of free GFP-mTalin in the tip region, because the average GFP intensity fluctuation in the tip region of pollen tube expressing soluble GFP was much smaller and clearly lagged the growth oscillation (unpublished data). Our results support the hypothesis that active ROP controls two signaling targets that oscillate in different phases: an early RIC4-dependent assembly of tip-localized...
Figure 3. The relative amount of GFP-RIC4ΔC localized at the apical plasma membrane oscillates with the growth rate in growing pollen tubes. (A) Representative fluorescent (bottom row) and corresponding transmission (top row) images of a tobacco pollen tube showing one round of GFP-RIC4ΔC oscillation. Fluorescent images were obtained focused at the median plane and numbers on top left in each transmission images are time (seconds) elapsed from the first one (T = 0). Bar, 10 μm. (B) The average intensity of GFP-RIC4ΔC localized to the apical PM (I-avg-pm) was measured by drawing a line (Left, dotted dark line at top of hemisphere) on the PM area of GFP-RIC4ΔC localization (Left, thick dark gray line of hemisphere). The average intensity of cytosolic GFP-RIC4ΔC (I-avg-cyto) was obtained from the area 3 μm away the tip end (circled area). I-avg-pm and I-avg-cyto were compared with tip elongation rate that represents the net surface increase in pollen tubes (Right). (C) Oscillations of the growth rate, I-avg-pm and I-avg-cyto of GFP-RIC4ΔC. I-avg-pm oscillations are 10–20 s ahead of growth rate oscillation (asterisks and arrows, ~90° ahead of growth). We analyzed eight representative tubes and obtained the same phase relation between oscillatory GFP-RIC4ΔC localization to the tip and the tip growth (average 87 ± 17 degrees, n = 8). Breaks in the curves are because of repositioning the tube that had grown out of the frame.
actin filaments that leads the way for subsequent growth surge and a delayed formation of tip-focused $[Ca^{2+}]_{cyt}$ gradients that slightly lags the growth peak (Holdaway-Clarke et al., 1997; Messerli and Robinson, 1997; Messerli et al., 2000; Feijo et al., 2001).

RIC4 Localization to the PM Predicts the Direction of Tip Growth

Because the localization of RIC4 to the apical cap, which presumably corresponds to the apical PM region where secretory vesicles are targeted for tip growth precedes growth burst (Figure 3), we hypothesized that ROP1 activation is spatially regulated to define the region of the PM for growth. This idea is also consistent with an increase in the size of the GFP-RIC4 containing apical cap is correlated with an increase in radial tube expansion or tip swelling induced by RIC4 or ROP1 overexpression (Li et al., 1999; Wu et al., 2001; Gu et al., 2005). This hypothesis predicts that GFP-RIC4 relocates toward the future growth site in a pollen tube to undergo growth reorientation. To test this, we analyzed temporal changes in the distribution of GFP-RIC4 in tubes undergoing slow growth redirection at a dramatic angle. It was reported that in response to stimuli that were applied uniformly, for example, heat shock or exposure to hyperosmotic medium, pollen tubes displayed sequential cytological events: cessation of tip growth, tip swelling, and reestablishment of polarized growth in a new direction (Malho et al., 1995). Tobacco pollen tubes seemed to respond in the same way; application of LatB, LaCl$_3$, or mannitol during the culture caused a bulge in the tube with both flanking sides forming an angle (Figure 5A). To analyze the detailed temporal relation between redirection of tip growth and spatial ROP activity, we applied 0.5 nM LatB to individual growing tubes. On LatB treatment, pollen tubes initially responded by tip growth arrest, and then oscillatory growth was recovered in 20–40 min in a new direction (Figure 5). Concurrent with growth cessation, the tip-high GFP-RIC4 gradient disappeared (unpublished data) and was subsequently replaced with even distribution of GFP-RIC4 to the apical area of the plasma membrane extending to the shank region, indicating loss of polarity (a and b in Figure 5B). In 10 min tip swelling occurred, probably due to nonpolarized growth. This was followed by recoupling of GFP-RIC4 to a locus on the swollen tip (b–e in Figure 5B) and subsequent reestablishment of a GFP-RIC4 gradient and resumption of polarized tip growth (d and e in Figure 5B). In rapidly growing tubes, GFP-RIC4 or GFP-RIC4/C relocation was generally not apparent because of small directional changes and of relatively high prepulse growth rate. But during a few marked growth redirections accompanying slow prepulse growth rate, GFP-RIC4/C relocation was detected before growth surge toward a new direction (Supplementary Figure S5). These observations further support the importance of spatial regulation of ROP1 activity in defining the polarity of tip growth in pollen tubes.

The Amount of RIC4 Localized to the Apical Cap Is not Correlated with the Rate of Growth

To gain further insights into the roles of ROP GTPase in tip growth oscillation, a series of correlation analyses was performed by taking advantages of varying oscillation periods and amplitudes during the growth of individual pollen tubes ($n = 7$). As described above, high levels of RIC4 localization to the apical cap were temporally ahead of
growth burst, i.e., period lengths of growth rate pulses had higher correlation with those of the leading I-avg-pm pulses than those of lagging I-avg-pm pulses: correlation coefficients were \(0.77 \pm 0.17\) and \(0.27 \pm 0.35\), respectively. Interestingly, the amplitudes of I-avg-pm oscillations and the associated growth surges did not show meaningful correlation (Figure 6A). In another word, differently from the ion fluxes, which temporally lag tip growth (Messerli et al., 1999), a large increase in RIC4 localization to the apical PM region does not lead to a relatively large growth rate increase. This indicates that although ROP is essential to pollen tube tip growth, the level of RIC4 localization to the apical cap does not necessarily determine the amplitude of growth rate.

Given that RIC4 localization to apical PM regions precedes growth burst and predicts new growth direction, how do we explain the lack of a close correlation between the level of tip-localized RIC4 and growth rate? Our previous studies show that efficient tip growth requires not just the presence of but the dynamics of tip-localized actin microfilaments (Fu et al., 2001; Gu et al., 2005). The process of exocytosis is known to require rapid actin reorganization (Eitzen, 2003). In the pollen tube tip, RIC4-mediated F-actin assembly is probably favorable for targeting of vesicles to the tip but may actually block the fusion of vesicles to the PM. Indeed, we found the mean level of tip-localized RIC4 was negatively correlated with the peak growth rate in a growing pollen tube (Figure 6B). A similar negative correlation was found between the level of tip-localized F-actin and the growth rate: the sustained increase of F-actin level was coupled with relatively lower growth rate (Figure 4B).

**F-actin Dynamics Is Important for ROP Activity and Growth Oscillations**

Because stabilization of the apical F-actin by RIC4 overexpression results in growth depolarization as well as loss of growth oscillation (Gu et al., 2005), we speculated that the dynamics of the apical F-actin (i.e., a coordination of F-actin assembly with disassembly) is an important mechanism underlying the oscillations of tip growth and tip-localized ROP activity. To test this hypothesis, we investigated whether treatments with LatB, which promotes the dynamics of actin filaments by sequestering monomer actin and thereby increasing F-actin depolymerization, restore oscillatory tip growth in tubes expressing GFP-RIC4. Because GFP-RIC4 overexpression induced depolarized tip growth (Figure 2D),...
only pollen tubes with weak GFP fluorescence were investigated. In the absence of LatB, 10 of 13 tubes expressing GFP-RIC4 did not show any measurable growth over noise level or detectable oscillation of GFP-RIC4 localized to the apical cap for 15–20-min recording. The other three tubes grew slowly at a lower frequency of oscillation (Figure 7A; Supplementary Figure S4): mean growth rate and oscillation period were 9 ± 2.8 nm/s and 227 ± 117 s (n = 3), respectively. In the presence of 0.5 nM LatB, the average tube length was much longer even with higher GFP-RIC4 expression levels, and 4 of 8 cells tested showed measurable growth of shorter period (Figure 7A; Supplementary Figure S5). The mean growth rate and period length were 23.6 ± 16.9 nm/s and 98 ± 44 s (n = 4), significantly different from control tubes without LatB treatment (p < 0.1). These results suggest that actin dynamics in the tube tip is crucial for the oscillation of ROP signaling and tip growth.

If actin dynamics is indeed important for growth oscillation, we would predict that a complete removal of apical actin microfilaments from the tip would eliminate growth oscillation. However, this prediction cannot be tested because complete removal of tip actin microfilaments causes growth arrest. To allow further testing of the role of actin microfilaments in growth oscillation, we resorted to using a subinhibitory dosage of LatB (0.2–0.5 nM) to reduce actin microfilaments from pollen tubes expressing GFP-RIC4ΔC, which does not affect actin assembly (Figure 1C). These low concentrations of LatB have no effects on the organization of axial actin cables in the base of the tube, but appear to affect tip-localized actin microfilaments (Fu et al., 2001; Vidali et al., 2001). As shown in Figure 7A, LatB treatment neither significantly changed mean growth rate, nor did it alter mean period length. Compared with a mean growth rate of 30.5 ± 9.1 nm/s and a mean period of 72 ± 29 s in control (n = 10), LatB-treated cells had a mean growth rate of 28.4 ± 6.1 nm/s and a mean period of 69 ± 20 s (n = 6). However, the amplitude of tip growth oscillations was decreased (Figure 7B) without altering mean growth rate: a decrease in peak values and a slight increase in basal (interpulse) values (mean ratio of peak over basal growth rate is 1.7 ± 0.33 for LatB-treated tubes and 2.2 ± 1.01 for untreated control tubes, p < 0.01). Similarly, the amplitude of tip-localized ROP activity also decreased without significant change in the mean values of growth rates and oscillation periods (Figure 7C): mean ratio of peak over basal I-avg-pm is 1.2 ± 0.11 for LatB-treated tubes and 1.3 ± 0.19 for untreated control tubes (p < 0.01). These observations suggest that F-actin assembly in the tip is involved in a sustained RIC4 accumulation (due to the sustained activation of ROPs). This hypothesis can explain why actin dynamics is critical for tip growth oscillation.

**Calcium Is Important for ROP Activity and Tip Growth Oscillation**

Previous reports have shown that a tip-focused gradient of cytosolic Ca2+ ([Ca2+]cyt) is regulated by active ROP1 and is required for F-actin dynamics (Li et al., 1999; Gu et al., 2005). To test whether the [Ca2+]cyt gradient is involved in controlling the oscillations, we investigated the effect of LaCl3, which is known to block Ca2+ influxes essential for establishing the [Ca2+]cyt gradient (Malho et al., 1995). To alter the tip-focused [Ca2+]cyt gradient without its complete dissipation, low concentrations of LaCl3 (1–10 μM) were applied to the germination medium. For the majority of treated tubes, no measurable growth over noise level was observed, and GFP-RIC4ΔC remained localized to the apical cap but did not show oscillation (unpublished data). A small number of tubes displayed measurable oscillatory tip growth with lower growth rates and longer growth periods (Figure 7A, D; n = 6): average growth rate was 19.8 ± 5.5 nm/s and period length was 105 ± 30 s. Noticeably these pollen tubes also displayed significantly decreased RIC4 accumulation at the tip (the amplitude of RIC4 oscillation), compared with
untreated tubes with similar mean growth rate (Figure 7, C and D): mean ratio of peak over basal I-avg-pm is 1.1 ± 0.07 for LaCl₃-treated tubes and 1.3 ± 0.19 for untreated control tubes (p < 0.01).

Taken together with our previous results (Gu et al., 2005), these observations suggest that tip-localized Ca²⁺ influxes and a steep tip-focused [Ca²⁺]cyt gradient are essential for ROP activity/tip growth oscillations.

**DISCUSSION**

Our results reported here provide evidence that ROP GT-Pase is a key molecular oscillator that controls the periodic tip growth of pollen tubes. This work represents the first example of a Rho GTPase oscillation that allows spatiotemporal coordination of Rho GTPase-mediated cellular responses. Our data show that a localized activation of ROP
signaling in the apical region of the pollen tube plasma membrane spatially predicts the direction of and temporally leads the burst of pollen tube growth. In support of the essential role for ROP in the control of growth oscillation, we demonstrate that both F-actin and Ca\textsuperscript{2+} pathways downstream of active ROPs participate in the oscillation of ROP signaling and oscillatory tip growth, providing a potential feedback mechanism underlying the oscillation of ROP GTase signaling.

**ROP GTase Is a Key Oscillator Leading Periodic Pollen Tube Growth**

Our studies indicate that the periodic regulation of ROP GTase signaling underpins the oscillation of polarized pollen tube growth. By visualizing GFP-RIC4\textsubscript{AC} in live tobacco pollen tubes, we have demonstrated RIC4 is localized to the apical domain of the plasma membrane as the form of an apical dome, termed the apical cap. The RIC4 apical cap shows a spatiotemporal dynamics in growing pollen tubes. Our data collectively suggest that the spatiotemporal dynamics of RIC4 localization, as reported by GFP-RIC4\textsubscript{AC}, represents the dynamics of ROP1 activity in the apical domain of the plasma membrane. First, RIC4 preferentially binds active form of ROP1, and its localization to the apical cap in pollen tubes is dependent on the activation of ROP1 (Gu et al., 2005). Second, the RIC4 deletion mutant RIC4\textsubscript{AC}, which lacks the RIC4 effector function but retains the full capability to bind active ROP1, behaved like RIC4 in terms of its in vivo interaction with ROP1 and its spatiotemporal localization pattern (Figures 2; Supplementary Figures S4 and S5). RIC4\textsubscript{AC} specifically interacted with the active form of ROP1 in vitro. Third, the amount of GFP-RIC4\textsubscript{AC} localized to the apical cap was tightly correlated with ROP1 activity. ROP1 overexpression enhanced GFP-RIC4\textsubscript{AC} localization to the apical cap, whereas inhibition of ROP1 activation by RopGAP1 or DN-rop1 expression abolished this localization pattern (Figure 2). Fourth, FRET analysis shows that the spatial distribution of CFP-RIC4\textsubscript{AC} interaction with YFP-ROP1 was identical to the pattern of CFP-RIC4\textsubscript{AC} localization to the apical cap. Finally, GFP-RIC4\textsubscript{AC} localization to the apical cap oscillated in spite of a constant level of cytosolic GFP-RIC4\textsubscript{AC}, indicating that GFP-RIC4\textsubscript{AC} oscillation in the tip was not due to fluctuation in its local amount but due to its capacity to localize to the apical cap in a GTP-ROP-dependent manner in pollen tubes. However, our results do not reveal whether or not GFP-RIC4 or -RIC4\textsubscript{AC} localization reports any changes in cytosolic active ROPs if such changes occur. Our results show the direct interaction of RIC4 with active ROP1 but do not exclude possible involvement of other molecules in the active ROP1-dependent RIC4 localization to the apical cap. Nonetheless our observations have clearly demonstrated the spatiotemporal dynamics of ROP signaling at the tip of pollen tubes, underscoring the central role for ROP1 in coupling the spatial control with the temporal control of pollen tube growth (Li et al., 1999; Zheng and Yang, 2000).

Our studies strongly support the hypothesis that tip-localized ROP activation leads the tip growth of pollen tubes. Pollen tube growth oscillation has long been documented, and several cellular and ionic activities have been shown to oscillate, including tip-focused cytosolic calcium gradients as well as tip-localized calcium, potassium, and proton influxes, chloride effluxes, and assembly of fine actin filaments (Messerli et al., 1999, 2000; Fu et al., 2001; Zonia et al., 2002). All these factors except for the ROP/RIC4-dependent actin filaments oscillate in phase with or lagging behind the tip growth, indicating that they are associated with growth itself or postgrowth events. In this report, we show that GFP-RIC4\textsubscript{AC} localization to the tip oscillates in the same periodicity with the oscillating tip elongation of pollen tubes. Correlation analysis with growth rate oscillation indicates that an increase in the apical localization of GFP-RIC4\textsubscript{AC} precedes that in pollen tube elongation rates, similar to the oscillation of RIC4-dependent tip-localized actin filaments.

Given that GFP-RIC4\textsubscript{AC} localization reports ROP activation, we propose that ROP activation at the apical PM region is a critical leading factor that controls pollen tube growth oscillation. This hypothesis is supported by observations described in this report or our previous work (Li et al., 1999; Gu et al., 2005). Consistent with the leading role for ROP activation in growth burst, several studies show that the localization of ROPs to the plasma membrane and their activation are required and sufficient for pollen tube growth (Kost et al., 1999; Li et al., 1999). Low levels of ROP1 overexpression enhance the accumulation of ROP1 at the apical region of the plasma membrane and promote pollen tube elongation, whereas DN-rop1 mutant expression inhibits tube growth (Li et al., 1999; Gu et al., 2003). Increased distribution of ROP1 to the apical region of pollen tubes resulting from high levels of ROP1 overexpression is associated with increased radial expansion or depolarized growth of pollen tubes (i.e., increased area at the tip where new growth occurs; Li et al., 1999). Importantly we have shown that the RIC4-containing apical cap (accordingly the active ROP-containing region) predicts the direction of new growth (Figure 5). Therefore, ROP activation appears to lead growth burst both spatially and temporally. This also agrees with our observations that ROP activates two downstream pathways, RIC4- and RIC3-regulated pathways, and the targets of these two pathways respectively oscillate in a similar phase or lagging behind the apparent ROP activation. Finally, the central role of periodic ROP activation in oscillatory pollen tube growth is also supported by our observations that the periodic ROP activity is regulated by the two ROP downstream pathways.

**How Is ROP Activity Oscillation Established?**

In principle, inherent biological rhythms, such as oscillatory pollen tube growth, must be controlled by self-organizing mechanisms involving both feed-forward and feedback regulations. Circadian clocks are known to be composed of an autoregulatory transcription and/or posttranslational-based feedback circuits of clock genes (Badiu, 2003). In cAMP-mediated chemotropic responses of *Dictyostelium*, cellular cAMP level spontaneouly oscillates under control of an oscillatory circuit including adenyl cyclase and phosphodiesterase, and the activities of these enzymes are regulated by cAMP (Maeda et al., 2004).

Our results support a unique mechanism by which two ROP signaling downstream pathways participate in the establishment of ROP activity oscillation. The RIC4-dependent actin assembly pathway seems to mediate a feed-forward regulation of ROP activation. The RIC3-dependent calcium-signaling pathway may negatively feedback-regulate ROP activity by counteracting RIC4-dependent actin pathway (Gu et al., 2005; see Figure 8, model). This model is consistent with the temporal relationship between the two downstream pathways (Figure 8). The accumulation of tip-localized actin microfilaments peaks ~90° ahead of growth (Fu et al., 2001; this report), whereas the RIC3-modulated tip-focused [Ca\textsuperscript{2+}]\textsubscript{cyt} gradient oscillates slightly behind growth (Messerli et al., 2000). Thereby an early actin-mediated feed-forward regulation would allow tip-localized ROP activity
to rapidly reach a peak, which corresponds to a delayed build-up of the tip-focused \[\text{Ca}^{2+}\]_{\text{cyt}} gradient. The delay in the increase in \[\text{Ca}^{2+}\]_{\text{cyt}} may be explained by a requirement for a long cascade of RIC3-mediated signaling events leading to \[\text{Ca}^{2+}\]_{\text{cyt}} accumulation. The rise of \[\text{Ca}^{2+}\]_{\text{cyt}} above a threshold level would lead to the fall in the tip-localized ROP activity (Figure 8). As described below, this hypothesis is strongly supported by our data described here and previous work. Nonetheless the identification of other components in these feedback loops will more directly test this hypothesis.

Polarity control in yeast cells and moving animal cells is known to involve feed-forward regulation of Rho GTPases (Li et al., 2003; Wedlich-Soldner et al., 2003; Xu et al., 2003). There is emerging evidence that ROP is also subject to feed-forward regulation in its control of polarized growth of pollen tubes and that a proper control of this feed-forward regulation is important for maintaining polarized growth (Wu, Hwang, and Yang, unpublished results). Overexpression of ROPs or expression of constitutively active rop mutant caused depolarization of tip growth and eliminated oscillatory growth (Li et al., 1999). The depolarization of growth is apparently caused by a feed-forward-mediated lateral amplification of ROP activation (Wu, Hwang, and Yang, unpublished results). Similarly RIC4 overexpression caused depolarized growth due to stabilization of tip actin microfilaments. Promotion of actin depolymerization at the tip, e.g., by LatB treatments, recovered polarized growth both in ROP1-overexpressing and RIC4-overexpressing tubes (Fu et al., 2001; Gu et al., 2005), implicating ROP/RIC4-dependent actin polymerization in ROP feed-forward regulation. We found that LatB treatment significantly reduced the peak of GFP-RIC4\text{C} localization to the tip, which is associated with peak assembly of tip F-actin (Figures 4 and 7D). This suggests that tip-localized assembly of actin filaments contributes to maintaining a high level of RIC4 or ROP activity at this apical cap through the feed-forward regulation.

Actin-mediated feed-forward regulation of Rho GTPase signaling appears to be a common theme in different organisms. In yeast cells, it was proposed that actin-dependent exocytosis is involved in the positive feedback activation of Cdc42 at the site of polarity establishment, probably by replenishing factors required for Cdc42 activation to the site.

![Figure 8](image-url)

Figure 8. Models for the phase difference between oscillations and for plausible feed-forward and feedback regulation of ROP activity. (A) Expected phase difference between tip growth (black dotted line), tip-localized cytosolic \[\text{Ca}^{2+}\] increase (gray dotted line), and tip-localized ROP/RIC4 activity (gray solid line) oscillations. Tip-localized F-actin oscillation is in similar phase with or slightly lagging ROP/RIC4 activity oscillations. We speculate the threshold of \[\text{Ca}^{2+}\]_{\text{cyt}} in the pollen tube tip for down-regulation of tip-localized ROP/RIC4 activity: above the level, down-regulation of ROP activity may be promoted (black arrows), whereas below the level, ROP activation may be favored (gray arrows). (B) A model for ROP activity regulation by two downstream pathways. It is expected that fast RIC4-dependent pathway for F-actin assembly positively regulates the accumulation of active ROP at the tip apical plasma membrane. This tip-localized F-actin assembly may contribute to keep \[\text{Ca}^{2+}\]_{\text{cyt}} below the threshold for ROP down-regulation. Slow-activation of RIC3-dependent increase in the tip-localized \[\text{Ca}^{2+}\]_{\text{cyt}} is expected to promote the down-regulation of tip-localized ROP activity.
of initial Cdc42 activation (Pruyne and Bretscher, 2000; Wedlich-Soldner et al., 2003). In chemotropic responses of mammalian neutrophil cells, actin polymerization has also been implicated to act downstream of Rac/Cdc42 for the establishment of polarity for cell movement (Xu et al., 2003), but the mechanism for the actin action is unknown. In root hair development, exocytosis might be involved in localizing ROPs to the site of root hair development (Molendijk et al., 2001). However, it seems unlikely that actin-mediated exocytosis plays a sole role in tip actin-mediated feed-forward regulation of growth oscillation in pollen tubes, because the localization of RIC4 at the tip rises ahead of that of growth rate and is not positively correlated with growth (Figures 3 and 6). Therefore, the mechanism by which the level/dynamics of tip-localized actin polymers controls ROP activity remains to be studied.

Ca2⁺ is one critical signaling component regulating tip growth of pollen tubes (Pierson et al., 1994; Malho and Treuwavas, 1996). It forms a tip-high gradient and oscillates slightly behind tip growth (Messerli et al., 2000). The formation of the tip-focused [Ca2⁺]gradient is mediated by the RIC4 signaling pathway, another tip-localized ROP downstream pathway (Gu et al., 2005). The slow accumulation of [Ca2⁺]gradient after ROP activation at the tip and the counteraction of Ca2⁺gradient is necessary for the oscillation of tip-localized ROP activity and tip growth (Figure 7).

Our previous work has shown that tip-localized Ca2⁺ and F-actin pathways counteract each other (Gu et al., 2005). Regulation of tip F-actin dynamics may be one mechanism by which Ca2⁺ contributes to the control of tip-localized ROP activation. However, this does not seem to be the primary mechanism, because LatB treatment did not recover La3⁺-suppressed oscillation of tip growth and RIC4 localization to the tip (unpublished data). Recent progress in the study of the regulation of Ras-superfamily GTPases may provide insights into this mechanism (Bivona et al., 2003; Caloca et al., 2003; Holinstat et al., 2003; Price et al., 2003; Walker et al., 2003; Walker et al., 2004). In animal cells, several regulators of Ras superfamily small GTPases, including guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs), are reported to either contain binding motifs for Ca2⁺ or Ca2⁺ sensors or to be regulated by Ca2⁺-dependent modification (Ebinu et al., 1998; Kawasaki et al., 1998; Cullen and Lockyer, 2002; Walker et al., 2004). There are also reports showing Ca2⁺-dependent phosphorylation of RhoGDI alpha and p115RhoGEF (Holinstat et al., 2003; Price et al., 2003). Ca2⁺ regulation of cellular localization or activities of small G proteins is a common mechanism. In pollen tubes, cytosolic calcium oscillation may also contribute to the regulation of localization and activation of ROP. Therefore it seems possible that the regulation of ROP activity in oscillatory pollen tube growth by [Ca2⁺]gradient increase involves both calcium promotion of actin disassembly and a more direct calcium regulation of the cycling between GTP- and GDP-bound forms of ROP. Nonetheless, an important future challenge is to elucidate the mechanism by which calcium regulates the oscillation of ROP activity.

In conclusion, we have shown that the spatially localized ROP activity oscillates and is a key oscillator leading the oscillation of tip growth in pollen tubes. The oscillation of ROP activity appears to be regulated by two downstream pathways, whose counteraction may in part participate in the control of ROP activity and tip growth oscillation. Our results indicate that the oscillatory tip growth of pollen tubes provides an interesting model system for the study of the oscillation of Rho GTPase signaling and its role in coordinating spatial control with temporal dynamics of cell growth.

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