Vesicle trafficking dynamics and visualization of zones of exocytosis and endocytosis in tobacco pollen tubes

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Vesicle trafficking dynamics and visualization of zones of exocytosis and endocytosis in tobacco pollen tubes

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Abstract

Pollen tubes are one of the fastest growing eukaryotic cells. Rapid anisotropic growth is supported by highly active exocytosis and endocytosis at the plasma membrane, but the subcellular localization of these sites is unknown. To understand molecular processes involved in pollen tube growth, it is crucial to identify the sites of vesicle localization and trafficking. This report presents novel strategies to identify exocytic and endocytic vesicles and to visualize vesicle trafficking dynamics, using pulse-chase labelling with styryl FM dyes and refraction-free high-resolution time-lapse differential interference contrast microscopy. These experiments reveal that the apex is the site of endocytosis and membrane retrieval, while exocytosis occurs in the zone adjacent to the apical dome. Larger vesicles are internalized along the distal pollen tube. Discretely sized vesicles that differentially incorporate FM dyes accumulate in the apical, subapical, and distal regions. Previous work established that pollen tube growth is strongly correlated with hydrodynamic flux and cell volume status. In this report, it is shown that hydrodynamic flux can selectively increase exocytosis or endocytosis. Hypotonic treatment and cell swelling stimulated exocytosis and attenuated endocytosis, while hypertonic treatment and cell shrinking stimulated endocytosis and inhibited exocytosis. Manipulation of pollen tube apical volume and membrane remodelling enabled fine-mapping of plasma membrane dynamics and defined the boundary of the growth zone, which results from the orchestrated action of endocytosis at the apex and along the distal tube and exocytosis in the subapical region. This report provides crucial spatial and temporal details of vesicle trafficking and anisotropic growth.

Key words: Endocytosis; exocytosis, hydrodynamics, lipophilic FM dyes, pollen tube growth, vesicle trafficking.

Introduction

Cell growth patterns are determined by spatial and temporal localization of factors regulating exocytosis and endocytosis, coupled with transeellular and interstitial hydrodynamic flow regulating cell expansion (D’Souza-Schorey and Chavrier, 2006; Kaksonen et al., 2006; Lai and Jan, 2006; Polo and Di Fiore, 2006; Rutkowski and Swartz, 2006; Zonia et al., 2006; Zonia and Munnik, 2007). Cell polarization and asymmetry are special cases of cell growth that are crucial for development in eukaryotes, and occur in yeast, embryos, fungal hyphae, neurons, root hair cells, and pollen tubes (Chant, 1999; Mlodzik, 2002; Lecuit, 2003; Harris and Momany, 2004; Xu and Scheres, 2005). There has been considerable work in recent years on factors that regulate polar growth in plant cells (Holdaway-Clarke and Hepler, 2003; Bosch and Hepler, 2005; Nibau et al., 2006; Campanoni and Blatt, 2007). However, the complexity of vesicle trafficking networks in plant cells complicates investigations to localize specific membrane trafficking pathways, to identify individual exocytic and endocytic vesicles at the plasma membrane, and to understand how vesicle insertion and retrieval are co-ordinated to maintain optimum tension in the plasma membrane during anisotropic growth.

The pollen tube grows by elongation of the vegetative cell through the transmitting tract of the style, and its polarized growth is crucial for fertilization in higher plants (Hepler et al., 2001; Holdaway-Clarke and Hepler, 2003; Campanoni and Blatt, 2007). Previous work established that pollen tube growth is linked with apical cell volume
and is exclusively sensitive to the extracellular osmotic potential (Zonia et al., 2002; Zonia and Munnik, 2004; Zonia et al., 2006). Experimentally triggered changes in apical volume induce specific phosphoinositide and phospholipid signals that regulate growth, including PtdInsP, PtdInsP2, and PtdOH (Zonia and Munnik, 2004, 2006). Ins(3,4,5,6)P4 is a key phosphoinositide signal that regulates anion efflux and affects pollen tube apical volume and growth (Zonia et al., 2002). Cell volume in the apical region oscillates with the same frequency as growth rate oscillations but the cycles are phase-shifted by 180° (Zonia et al., 2006). Hyperosmosis induces decreases in apical volume and growth rate, while hypo-osmosis induces increases in apical volume and growth rate (Zonia et al., 2006). These results indicate that cell elongation and polarized growth result, in part, from vectorial transcellular hydrodynamic flux through the pollen tube apical region (Zonia et al., 2006; Zonia and Munnik, 2007).

Pollen tubes have extremely rapid growth rates that are supported by an active secretory system, whereby exocytosis at the growth locus delivers new synthetic materials required for cell elongation. Early reports claimed that the pool of very small vesicles in the apical dome of pollen tubes and root hairs were secretory (exocytic) vesicles, and that exocytosis occurs at the apex (Van der Woud et al., 1971; Picton and Steer, 1983). Electron micrographs of freeze-substituted lily pollen tubes confirmed that the apex contains a pool of very small vesicles, with larger vesicles located distally to the apical dome (Lancellle and Hepler, 1992). However, observations of vesicles fused with the apical plasma membrane in these micrographs were rare and gave no information about whether the vesicle was undergoing exocytosis or clathrin-independent endocytosis. The movement of individual vesicles in live pollen tubes has been tracked using computer-assisted video microscopy (de Win et al., 1997, 1998, 1999). Trafficking trajectories were either anterograde or retrograde starting from ~7–10 μm distal to the tip, but within the apical dome many trajectories displayed more random movements (de Win et al., 1997, 1998, 1999). In tobacco pollen tubes, vesicles undergoing anterograde trafficking advanced to within 2–4 μm of the apex during the phase of maximally increasing growth rate, but they were not trafficked all the way to the apex (Zonia et al., 2002). A recent study used total internal reflection microscopy to visualize FM 4-64 labelled vesicles in subapical regions of the pollen tube cylinder and near the apical dome of spruce (Picea meyeri) pollen, and reported observations of exocytosis (Wang et al., 2006). Although it has not previously been possible to directly observe exocytic vesicle fusion with the plasma membrane at the apex in live pollen tubes, the concept of the apex as the site of exocytosis and the locus of growth continues to be the currently accepted model for tip growth (Holdaway-Clarke and Hepler, 2003; Šamaj et al., 2006; Wang et al., 2006; Campanoni and Blatt, 2007).

Endocytic retrieval of excess plasma membrane must occur in tip-growing cells, as the amount of membrane inserted during exocytosis would exceed the quantity required to support new growth (Picton and Steer, 1983). These internalized vesicles could also contain membrane proteins that are recycled by trafficking to the endosomal compartment, as well as bulk fluid and soluble molecules (pinocytosis). Endocytic vesicles can invaginate into coated pits and pinch-off as clathrin-coated vesicles (Maldonado-Baez and Wendland, 2006). Electron microscopy on cryo-fixed serial sections of tobacco pollen tubes showed that the density of clathrin-coated pits was highest in a region from 6–18 μm distal to the tip (Derksen et al., 1995). These data were confirmed recently with immunogold labelling of formaldehyde-fixed Nicotiana sylvestris pollen tubes (Lisboa et al., 2007). Endocytic vesicles can also pinch-off as smooth vesicles in a clathrin-independent mechanism in mammalian cells (Hansen et al., 1991; Iversen et al., 2001; Nichols and Lippincott-Schwartz, 2001; Nichols, 2002; Qualmann and Mellor, 2003). Smooth vesicle endocytosis predominates in some internalizations, and smooth vesicles have been shown to accumulate as a discrete class of endosome that mediates traffic to the Golgi complex (Iversen et al., 2001; Nichols and Lippincott-Schwartz, 2001; Nichols, 2002). Endosomes have been identified in root hairs of Medicago and Arabidopsis (Voigt et al., 2005). It is notable that in diverse organisms from yeast to fruit fly, Rho GTPases have a demonstrated role in regulating clathrin-independent internalization of smooth endocytic vesicles (Qualmann and Mellor, 2003).

Several reports on pollen tubes and root hairs have presented data that are inconsistent with the model of the apex as the growth locus. A study of cell surface expansion properties during root hair growth revealed that the region with the highest rate of expansion was a zone 2–3.5 μm distal to the apex (Shaw et al., 2000). Vesicle trafficking pathways in plant cells have been studied with lipophilic FM dyes that bind to the plasma membrane, are internalized via endocytosis, and subsequently label intracellular vesicles and membranes involved in secretion or degradation (Parton et al., 2001; Bolte et al., 2004). Trafficking of FM dyes from endocytic to exocytic pathways has been observed within 15–30 min (Belanger and Quatrano, 2000a, b; Fischer-Parton et al., 2000; Read and Hickey, 2001). In fungal hyphae, FM 4-64 recycles back to the plasma membrane (Read and Hickey, 2001; Bolte et al., 2004). Time-lapse tracking of dye uptake and distribution pathways in intracellular compartments can reveal information about whether vesicles are involved in early stages including endocytosis or later stages including exocytosis. In lily pollen tubes, FM 4-64 labelled the pool
of small vesicles in the apical dome that have been considered to be exocytic vesicles; however, these were found to undergo retrograde trafficking away from the apex (Parton et al., 2001). Surprisingly, growth rate oscillations in these lily pollen tubes correlated with the cyclic accumulation of FM-labelled vesicles in a region 5–10 μm distal to the apex (Parton et al., 2001).

The goal of the present study was to characterize vesicle pools and vesicle trafficking pathways in pollen tubes, and to determine how membrane dynamics are co-ordinated during growth. Novel microscopic techniques were developed that provided a fresh view on this intensively studied subject. The results enabled identification of sites of endocytosis and exocytosis, and localization of three different classes of vesicles in live pollen tubes. To test a recent prediction that hydrodynamic flow and apical volume increase–decrease could drive pollen tube growth and regulate exocytosis and endocytosis (Zonia and Munnik, 2007), a previously established hydrodynamics approach was implemented and the results enabled detailed spatial mapping of plasma membrane dynamics in the growth zone. This work provides insight into the biomechanics that integrate plasma membrane dynamics and cell growth.

Materials and methods

Plant material and culture

Pollen from Nicotiana tabacum was isolated and stored as described previously (Zonia et al., 2002, 2006; Zonia and Munnik, 2004). For FM pulse-chase labelling and confocal microscopy, culture chambers were assembled on microscope slides using silicone isolators (Invitrogen-Molecular Probes Europe, Leiden, The Netherlands) and pollen was cultured as described previously (Zonia et al., 2006). For high-resolution differential interference contrast (DIC) time-lapse microscopy, pollen was germinated in glass Petri dishes at 23 °C on a platform shaker at 50 rpm with a culture density of 0.1 mg ml⁻¹ in germination medium (Zonia et al., 2002, 2006; Zonia and Munnik, 2004). Pollen was used for imaging from 3 h to 5 h after the start of germination.

FM labelling and confocal microscopy

The lipophilic styryl fluorophores FM 1-43 and FM 4-64 (Invitrogen-Molecular Probes Europe) have distinct excitation and emission maxima that enable separation of the wavelengths on a confocal microscope. Emission wavelengths for FM 1-43 and FM 4-64 are 626 and 734 nm, respectively. Pollen was germinated and cultured for 2 h before loading cells with FM 1-43. Pollen tubes were allowed to incorporate FM 1-43 for 1.5–2 h before addition of FM 4-64. Each fluorophore was added to the culture medium at a concentration of 2 μM. Similar results were obtained irrespective of which dye was added first, although FM 1-43 uptake was slower than FM 4-64. Endocytic internalization and trafficking of the fluorophores were observed using a Zeiss LSM510 confocal microscope. Images were captured starting at 1 min after addition of the second fluorophore FM 4-64. For experiments manipulating osmotic potential and cell volume, methods and results were used that were established in previous reports (Zonia et al., 2002, 2006; Zonia and Munnik, 2004, 2007). For the current studies, pollen was germinated and labelled with 2 μM FM 1-43 as above, then either 100 mM NaCl or one-half volume of H₂O (final culture:H₂O=2:1) was added to the cultures with gentle swirling, and after 1 min 2 μM FM 4-64 was added. Images were captured as above, starting at 1 min after addition of FM 4-64 and 2 min after the start of osmotic treatment.

High-resolution refraction-free time-lapse DIC microscopy

DIC microscopy was performed with an Olympus AX70 Provis upright microscope equipped with high-definition differential interference contrast. A ×60 U-PlanApo NA 1.20 water-immersion objective was used. Spherical aberration was reduced to zero by positioning the water-immersion objective directly above the live pollen tubes growing in glass Petri dishes, without any refractive interface between the sample and the objective to degrade the image quality. Images were captured with a Sony Power HAD 3CCD colour video camera and Lucia Image Analysis version 4.5 (Laboratory Imaging, Prague, Czech Republic). Images were grabbed during 5–10 min growth sequences under data-stream conditions, which translate to 0.21 s image⁻¹. The sequential time-lapse growth sequence images were contrast-enhanced and transformed into AVI video format with Lucia Image Analysis (Laboratory Imaging). For Figs 2F, 3D, and 5A, individual images were selected from the time-lapse sequences and imported into Adobe Photoshop v. 6.0 for minimal processing and assembly of the composite panels.

Measurement of vesicle diameters

High-definition refraction-free time-lapse DIC image sequences from five different pollen tubes captured at a rate of 0.21 s image⁻¹ for 5–10 min were employed for these studies. Images were sampled at selected intervals ranging from 10 s to 20 s as required. Each digital image was enlarged, smoothed with a median filter to enhance the signal-to-noise ratio, and then a contrast algorithm was applied to sharply define the vesicle perimeters. Vesicles in three different regions were measured, and, for each image, 3–20 vesicles in each region were measured (depending on the vesicle density). A total of 140 vesicles was measured for each region in the image sequences for each pollen tube, then the data were pooled to give a total of 700 vesicles in each of the three regions for five pollen tubes. Thus, a total of 2100 vesicles was measured for the entire analysis. The series of measurements was performed twice for verification. Vesicle diameters were measured using the calibrated length-measuring function of Lucia Image Analysis (Laboratory Imaging). The regions measured are defined as follows. The apical dome region extended from the tip to the location where the semi-hemispherical dome joined the cylindrical body of the tube, which averaged 3 μm distal to the apex. The adjacent region spanned the area from 3 μm to 10 μm distal to the tip. The distal region spanned the area from 10 μm to 40 μm distal to the tip. The data for each region for all five pollen tubes were pooled, exported to SigmaPlot 7.1 for analysis, and plotted as histograms. A total of 97.2% of all vesicles measured were ≥200 nm. There were 59 vesicles in the size range of 150–200 nm that were clearly visualized in the apical dome, or 2.8% of the total number of vesicles measured. The vesicle diameters reported in this study do not represent absolute vesicle dimensions, which would have to be measured from electron micrographs, but they do reflect the relative size distribution of vesicles in unperturbed living cells that are undergoing dynamic growth. Furthermore, these measured values are consistent with values obtained from electron micrographs (Picton and Steer, 1983; Kroh and Knuiman, 1985; Lancelle and Hepler, 1992).

In terms of classical optics, the resolution that can be achieved (rₑₑ) is dependent on the wavelength and the aperture and properties of the objective. This is called the Rayleigh criterion and
is given by \( r_{\text{airy}} = 1.22 \lambda_d / 2 \) NA. For the microscope system used in this work, operated in normal bright field mode, the limit of resolution that can be achieved according to the Rayleigh criterion is 203 nm. However, during operation of the microscope configured for high-resolution DIC, the resolution that can be achieved must be computed in the spatial frequency domain and is dependent on the lateral displacement between the axial and orthogonal wavefronts passing through the sample and the contrast transfer function (Inoué, 1995; Pawley, 1995). Under optimum conditions, the theoretical limit of resolution in the spatial frequency domain can approach 0.5 \( r_{\text{airy}} \) (Inoué, 1995; Pawley, 1995), or 102 nm for this microscope system. In practice, this theoretical limit generally is not achieved, but the resolution that is achieved is always greater than the limit of resolution according to the Rayleigh criterion. Additional parameters of the image data that must be considered are the signal-to-noise ratio (visibility) and the Nyquist criterion (Pawley, 1995). Both of these parameters were optimized for this report. Finally, and most importantly, imaging was further optimized by configuring the system to eliminate spherical aberration (Keller, 1995). This was achieved by the use of a water-immersion objective that was positioned directly above the cells in aqueous medium, without interference of a cover slip refractive interface to degrade the image quality. It was confirmed that resolution of the dynamical features presented in this report was obscured by spherical aberration when cells were viewed through cover slips (using the same objective) or through oil immersion (using an oil-immersion objective with equivalent NA) (data not shown). The confidence level set for the spatial frequency limit of resolution in this report is 150 nm. Other studies using highly optimized DIC microscopy report spatial frequency limits of resolution of 35–50 nm (Grzywa et al., 2006; Li et al., 2007).

Results

**Pulse-chase labelling with FM 1-43 and FM 4-64 identifies endocytic vesicles**

A pulse-chase labelling method was developed to identify discrete vesicle pools in growing tobacco pollen tubes. Two lipophilic FM dyes that have different emission wavelengths were used for these studies (Fig. 1A, B). In the pulse-chase experiments, cells were first labelled with FM 1-43 (green) for 1.5–2 h to saturate intracellular vesicle pools, and then labelled with FM 4-64 (red) to distinctly visualize sites of endocytic uptake and to track endosomal trafficking pathways. This strategy enabled identification of discrete endocytic and endosomal trafficking pathways as FM 4-64 was internalized and mixed with pre-labelled FM 1-43 pools. The first site where the two dyes mix (yellow) defines the site of endocytosis and retrieval of excess membrane inserted during exocytosis and growth of the plasma membrane. This site was labelled within 1–2 min and was located at the apex and along the apical dome (Fig. 1C). FM 4-64 rapidly labelled the pool of small vesicles located in the apical dome within 3–5 min (Fig. 1D). These vesicles undergo retrograde trafficking, and form the characteristic inverse-cone shaped pool of small vesicles extending from the apex (Fig. 1D, E). This entire pool of vesicles incorporates FM 4-64 within 8–10 min (Fig. 1E). These results indicate...
that small vesicles located in the apical dome are endocytic and result from membrane retrieval. These vesicles undergo retrograde trafficking and appear to enter an endosomal compartment.

A second mechanism for FM 4-64 internalization occurs along the shank of the pollen tube, starting at ~6 μm distal to the apex. These vesicles are significantly larger than those observed at the apex and their fluorescence emission is red-shifted, indicating that there is little or no mixing with pre-labelled vesicle compartments (Fig. 1E). Although a few vesicles of this endocytic pathway appear at the earliest time points (Fig. 1C), significant accumulation occurs after ~8–10 min (Fig. 1E). These membrane properties and kinetics of internalization indicate that endocytosis in the distal pollen tube is different from that at the apex.

Control experiments that labelled pollen tubes first with FM 4-64 for 2 h followed by FM 1-43 gave similar results, although the uptake of FM 1-43 was significantly slower than that of FM 4-64 (Fig. 1F).

Pulse-chase labelling with FM 1-43 and FM 4-64 identifies exocytic vesicles

Distinct intracellular pathways were sufficiently labelled by 20 min after addition of FM 4-64 and 2 h after addition of FM 1-43 to enable identification of three different pools of vesicles: endocytic/endosomal (yellow), exocytic (green), and coated endocytic vesicles (red) (Fig. 2). Degradative pathways including vacuolar membrane were not significantly labelled at these time points (Fig. 2). Close inspection of time-lapse images of growing pollen tubes revealed further information about vesicle trafficking dynamics and subcellular localization. Endocytosis at the apical plasma membrane is constitutive (Fig. 2A). Exocytic vesicles and organelles were not trafficked to the apex, but approached to within 2–5 μm distal to the apex (Fig. 2A). Exocytic vesicles were observed to fuse with the plasma membrane in a zone spanning 3–10 μm distal to the apex (Fig. 2B-E). FM 1-43-labelled membrane (green) is observed in the plasma membrane in the subapical region (Fig. 2B-E). Vesicle fusion with the plasma membrane in this region was also observed using refraction-free high-resolution time-lapse DIC microscopy (Fig. 2F, see also Movie 1 in Supplementary data available at JXB online). Endocytosis of coated vesicles along the shank of the pollen tube was constitutive, but less active than membrane retrieval at the apex (Fig. 2A).

Relative size distribution of vesicles in growing pollen tubes

Vesicle trafficking in pollen tubes is very dynamic and a spectrum of vesicle sizes can be observed in different regions of the growing pollen tube (see Movie 1 in the Supplementary data at JXB online). As discussed above, pulse-chase labelling indicated the existence of three types of vesicles involved in pollen tube growth (Fig. 2). The relative size distribution of vesicles in different regions of growing pollen tubes could provide important functional information about the dynamics of vesicle trafficking pathways, in addition to yielding first clues about the potential volume of cargo loads in different populations of vesicles. Therefore, the diameters of 700 vesicles located in each of three regions were measured from refraction-free high-resolution DIC time-lapse images of growing pollen tubes (n=5) (Fig. 3). For these studies, the microscope was configured to eradicate refractive blur by using a water-immersion objective placed directly over live cells growing in glass dishes (see Materials and methods). This enabled non-invasive measurements in growing pollen tubes and preserved spatial and temporal information about vesicle dynamics.

The regions examined were the apical dome, the adjacent region spanning from 3 μm to 10 μm distal to the apex, and the region spanning from 10 μm to 40 μm distal to the apex (Fig. 3D). The results were plotted in histograms (Fig. 3A–C), and indicated that distinctly sized vesicles accumulate in each of the three regions. Vesicles in the region of the apical dome had a mean diameter of 405±7 nm, and the distribution was heavily skewed toward very small vesicles (Fig. 3A). The mean diameter for vesicles in the adjacent zone was 573±6 nm, and the distribution indicates the presence of two populations (Fig. 3B). The mean diameter in the distal region was 726±6 nm, and the histogram corresponds to a normal distribution (Fig. 3C). It is notable that each region contains statistically distinct vesicle populations, in addition to a low frequency of vesicles from each of the other two regions. This suggests that different vesicles are sequestered in different zones of the pollen tube.

Hydrodynamic flux selectively induces endocytosis versus exocytosis

There is a strong correlation between membrane tension and vesicle insertion or retrieval in many cell types including mammalian and plant (guard cells). Exocytosis is stimulated by increased membrane tension and endocytosis is stimulated by decreased membrane tension (Kubitscheck et al., 2000; Apodaca et al., 2002; Shope et al., 2003; Meckel et al., 2005). Hydrodynamic flow and the resultant apical volume increase–decrease could correlate with changes in membrane tension and thereby induce increases in exocytosis or endocytosis (Zonia and Munnik, 2007). Here this is tested in pollen tubes using pulse-chase labelling with FM dyes and osmotic manipulation of pollen tube growth (see Materials and methods).

Treatment with 100 mM NaCl inhibits pollen tube growth and exocytosis, and stimulates endocytic retrieval.
of plasma membrane at the apex (Fig. 4A). During prolonged hypertonic treatment, a distinct zone was revealed that had significantly decreased labelling on the plasma membrane (Fig. 4B). This is the result of prolonged activation of endocytic membrane retrieval at the apical plasma membrane coupled with inhibition of exocytosis in the adjacent region. Hyperosmosis and cell volume decrease blocks secretion of new vesicles (green fluorescence) in the growth zone, and endocytosis at the apex clears FM dyes out of the plasma membrane. Thus, this strategy enables visualization of the active growth zone in pollen tubes, which emerges as the apical region lacking FM dyes in the plasma membrane (Fig. 4B).

Conversely, hypoosmotic treatment with addition of H$_2$O to a final volume of 1:2 (H$_2$O:culture) stimulated pollen tube growth and exocytosis, and attenuated endocytic membrane retrieval at the apex (Fig. 4C). During longer
hypotonic treatment, exocytosis was significantly increased in the growth zone to support increased growth rate and cell expansion (Fig. 4D). Endocytosis of coated vesicles in the shank of the pollen tube was only slightly attenuated in response to hypo-osmotic cell swelling (Fig. 4D).

Morphology of anisotropic growth

Morphological changes at the cell surface in the growth zone reflected the spatial dynamics of exocytic membrane insertion and endocytic membrane retrieval (Fig. 5; see also Movie 2 in the Supplementary data at JXB online). The pollen tube shown in Fig. 5A and Movie 2 was undergoing a very strong growth pulse, with a clearly defined region of new growth. The region of rapidly deposited new growth had a decreased tube diameter compared with the older region. Cell elongation during growth was tracked and was equivalent to the length of cell expansion in the growth zone (Fig. 5A, arrows). Exocytosis of new membrane and cell wall materials adjacent to the apex (Fig. 5A, 222.2 s, arrows) created a flexible region that elongated under the force of hydrodynamic flux and continued exocytosis (Fig. 5A, 266.0 s and 298.5 s, arrows). Excess plasma membrane was retrieved by endocytosis at the apex, and the cell surface of the apical dome underwent only minor changes during the growth pulse (Fig. 5A).

These dynamics are illustrated in the model of cell growth (Fig. 5B). Exocytosis occurs in a zone adjacent to the apex. The newly deposited cell wall is more visco-plastic than the mature cell wall (Dumais et al., 2006) and expands under the force of vectorial hydrodynamic flux driving the apical dome outward (Zonia et al., 2006; Zonia and Munnik, 2007). Endocytic membrane retrieval occurs at the apical plasma membrane, and these small vesicles enter the endosomal retrograde trafficking stream. Vectorial hydrodynamic flux coupled with endocytosis at the apex organizes the orientation of growth polarity, in that excess membrane incorporated during exocytosis flows toward the apex during cell elongation, where it is internalized. Pollen tube growth is predominantly unidirectional, but there can also be a very low level of cell expansion in the opposing direction. This is due to the continual deposition of new materials in the growth zone, followed by cell expansion in this region (Fig. 5B). Endocytosis of coated vesicles occurs in the distal region of the growth zone and along the shank of the pollen tube. Thus, the active growth zone is delimited by the orchestrated action of exocytosis adjacent to the apical
dome, coupled with endocytosis at the apex and at the shank of the pollen tube (Fig. 5B).

Discussion

This report presents a novel pulse-chase labelling strategy using FM 1-43 and FM 4-64 that enables visualization of sites of endocytosis and exocytosis. The results reveal detailed spatial and temporal information about vesicle localization and vesicle trafficking pathways in growing tobacco pollen tubes. The emergent model resolves several long-standing questions about anisotropic growth in these cells and sheds new light on functions of proteins and signals regulating growth. Transcellular hydrodynamic flux appears to integrate exocytosis, endocytosis, and cell elongation during pollen tube growth.
vesicles have been shown to mediate trafficking to known to be important in mammalian cells and smooth synthesis and growth. Smooth vesicle endocytosis is bulky cell wall materials and proteins required for cell to be recycled, but would not be required to carry vesicles; they contain membrane and proteins destined to be recycled, but would not be required to carry bulky cell wall materials and proteins required for cell synthesis and growth. Smooth vesicle endocytosis is known to be important in mammalian cells and smooth vesicles have been shown to mediate trafficking to endosomal compartments (Hansen et al., 1991; Iversen et al., 2001; Nichols and Lippincott-Schwartz, 2001; Nichols, 2002; Qualmann and Mellor, 2003). Endocytic membrane retrieval at the apex is crucial for the mechanics of pollen tube growth, in that it recycles proteins to the endosomal compartment and also functions in the maintenance of polar growth in the growth zone (discussed in the following sections).

The second endocytic pathway occurred along the shank region of the pollen tube, starting ~6–10 μm distal to the apex (Fig. 2). These vesicles are considerably larger than endocytic vesicles at the apex, suggesting that they may be clathrin-coated. This result is in agreement with previous work that identified clathrin-coated vesicles in this region in tobacco pollen tubes. Electron microscopy on cryo-fixed serial sections of tobacco pollen tubes showed that the density of clathrin-coated pits was highest in a region from 6 μm to 18 μm distal to the tip (Derksen et al., 1995). Thus, endocytosis in the distal region of the pollen tube observed in Fig. 1 could result from coated pit assembly and internalization. Two distinct endocytosis pathways, including one mediated by clathrin, were recently reported in a study following uptake of charged nanogold particles into tobacco pollen tubes (Moscatelli et al., 2007).

The distal endocytic vesicles incorporate primarily FM 4-64, indicating that membrane inserted during exocytosis (labelled with FM 1-43) undergoes only a low level of retrograde flow from the site of insertion (Figs 2, 4). During cell elongation, newly inserted exocytic membrane mainly undergoes anterograde flow toward the apex, where excess membrane (labelled with both FM 1-43 and FM 4-64) is internalized via smooth vesicle endocytosis (Figs 1, 2, 4). Anterograde flow of newly inserted membrane is clearly revealed during hyperosmotic manipulation of cell volume, which attenuates exocytosis and drives increases in smooth endocytosis at the apex (Fig. 4B). In these cells, both dyes are rapidly cleared out of the plasma membrane in the growth zone, while distal plasma membrane retains FM 4-64 (Fig. 4B).

The sites of endocytosis

Two different sites of endocytosis were identified: along the apical plasma membrane and in the distal region adjacent to the apical dome (Fig. 2). Endocytosis at the apical plasma membrane clearly involved membrane recycling as vesicles incorporated both FM 1-43 and FM 4-64 (Figs 1, 2, 4). These vesicles undergo retrograde transport and appear to be trafficked to endosomal compartments (Figs 1, 2; Movies 1 and 2 in the Supplementary data at JXB online). Endocytic vesicles internalized along the apical plasma membrane have the smallest diameters of all vesicles in the pollen tube (Fig. 3), in agreement with previous reports (Picton and Steer, 1983; Kroh and Knuiman, 1985; Lancelle and Hepler, 1992). This probably reflects the fact that these vesicles are required to carry smaller cargo loads than exocytic vesicles; they contain membrane and proteins destined to be recycled, but would not be required to carry bulky cell wall materials and proteins required for cell synthesis and growth. Smooth vesicle endocytosis is known to be important in mammalian cells and smooth vesicles have been shown to mediate trafficking to

The site of exocytosis

Exocytosis occurs in a zone that spans from 3 μm to 10 μm distal to the apex (Fig. 2). Exocytic vesicles traffic to within 2–5 μm of the apex, in agreement with earlier reports (Parton et al., 2001; Zonia et al., 2002). Exocytic vesicle fusion with the plasma membrane was observed using both the pulse-chase labelling technique (Fig. 2A–E) and refraction-free high-resolution DIC microscopy (Fig. 2F; Movie 1 in the Supplementary data at JXB online). FM 1-43-labelled membranes derived from exocytic vesicles are clearly observed in the plasma membrane in the growth zone (subapical region) (Fig. 2A–E). Vesicles in this region sort into two size classes, with average
diameters of ~400 nm and ~650 nm (Fig. 3). It is suggested that the population of vesicles in this region could include endosomal vesicles, exocytic vesicles, clathrin-coated vesicles, as well as some small organelles, with exocytic vesicles sorting into the class with smaller diameters (~400 nm) that is labelled with FM 1-43 (green) (Fig. 2). Vesicle sizes and distributions measured here are consistent with values obtained from electron micrographs of tobacco, lily, and Tradescantia pollen tubes (Picton and Steer, 1983; Kroh and Knuiman, 1985; Lancelle and Hepler, 1992). Indeed, one report indicated that exocytic vesicles in tobacco pollen tubes could be of the order of 400–500 nm (Kroh and Knuiman, 1985).

These results resolve long-standing questions inherent in the currently accepted model of pollen tube tip growth. Vesicles traffic along the actin cytoskeleton, which has an axial organization in the cylindrical cell (Miller et al., 1995; Hepler et al., 2001; Holdaway-Clarke and Hepler, 2003; Smith and Oppenheimer, 2005). Most actin filaments terminate ~4–7 μm distal to the apex, although there is evidence that very fine filaments can extend closer to the apex during certain phases of the growth cycle (Fu et al., 2001). There has been speculation about how exocytic vesicles traverse the distance from the point where most actin filaments terminate to the presumed site of exocytosis at the apex. This report shows that exocytic vesicles are trafficked directly to the site of exocytosis, which is adjacent to the apical dome. Thus, delivery of exocytic vesicles to the site of insertion is not a rate-limiting step for growth. Furthermore, it has been unclear why the very smallest vesicles would be selected to transport the bulky cargoes of cell wall components and plasma membrane proteins that are crucial for rapid synthetic growth of the cell. This report shows that exocytic vesicles are the abundant class of vesicles with intermediate diameters, well suited for transport of components required for cell wall synthesis and growth.

Consequences of growth zone dynamics for cell growth and stability

The spatial organization of endocytosis and exocytosis sites functions to delimit the active growth zone to the apical region (Fig. 5). Polarized growth is maintained in part by the anterograde flow of newly inserted membrane, with excess membrane internalized via smooth vesicle endocytosis at the apex (Figs 2, 4). Optimum membrane tension and growth rate could be rapidly adjusted by changing the rate of endocytosis at the apex and/or at the distal site (Fig. 4B, D). The balance between rates of endocytosis and exocytosis confers a pivotal regulatory node that can be easily integrated into cellular networks controlling pollen tube growth.

Pollen tubes must grow through the style to reach the ovule and affect fertilization (Hepler et al., 2001). The pollen tube apex encounters the greatest stress and resistance as the cell penetrates the stigma and grows through the stylar tissue. If the apex was the locus of growth, then the site of new cell wall synthesis would be located at the point of greatest structural vulnerability of the cell, and so functions to amplify structural instability. Greater structural stability would be conferred if the site of new cell synthesis was displaced from the apex. This report confirms this prediction and shows that in fact the locus of exocytosis and growth is adjacent to the apical dome (Figs 2, 5).

Hydrodynamic control of pollen tube growth

Previous reports showed that hydrodynamically driven cell volume changes in the pollen tube apical region could trigger the start of the growth cycle and provide a motive force for cell elongation (Zonia et al., 2002, 2006; Zonia and Munnik, 2004, 2007). The current work shows that transcellular hydrodynamic flux can also affect the rates of exocytosis and endocytosis (Fig. 4). Hypo-osmosis leads to cell swelling, which stimulates exocytosis and attenuates endocytosis (Fig. 4C, D). Apical cell volume increase not only promotes exocytosis but also triggers the start of the growth cycle (Zonia et al., 2006; Zonia and Munnik, 2007). Conversely, hyperosmosis leads to cell shrinking, which increases endocytic membrane retrieval at the apex while attenuating exocytosis (Fig. 4A, B). Apical cell volume decrease also dissipates the motive force driving cell elongation (Zonia et al., 2006; Zonia and Munnik, 2007). Recent work on growth of Chara corallina cells has shown that turgor pressure drives the movement of cell wall materials from the plasma membrane into the cell wall (Proseus and Boyer, 2005, 2006). Increasing pressure could drive larger polysaccharides into the wall, while decreasing pressure caused a decrease in wall deposition (Proseus and Boyer, 2005, 2006). Thus, oscillations in apical hydrostatic pressure during pollen tube growth could affect the balance of endocytosis and exocytosis, wall deposition and assembly, and cell elongation during growth.

Conclusions

This report has provided a detailed spatial and temporal map of vesicle trafficking pathways in growing tobacco pollen tubes and has identified sites of exocytosis and endocytosis. Mapping was enabled by a novel pulse-chase labelling technique with two lipophilic FM dyes that have different emission wavelengths (Fig. 1). This technique allowed visualization of three distinct vesicle pools involved in anisotropic growth (Fig. 2). High-resolution refraction-free time-lapse DIC microscopy enabled measurement of vesicle sizes and showed that discretely sized vesicles accumulate in each of these three regions (Fig. 3; Movies 1 and 2 in the Supplementary data at JXB online).
Constitutive endocytic membrane retrieval from the apical plasma membrane generates the pool of small vesicles in the apical dome (Figs 1–4). These vesicles undergo retrograde trafficking and appear to enter endosomal compartments (Figs 1–4; Movies 1 and 2 in the Supplementary data at JXB online). Endocytosis of coated vesicles occurs along the shank of the pollen tube, starting at ~6 μm distal to the apex (Figs 2, 4). Exocytosis of new plasma membrane and cell wall materials occurs in a zone ~3–10 μm distal to the apex (Fig. 2).

These results lead to a new model of pollen tube growth (Fig. 5). Optimum growth is regulated by the orchestrated action of endocytosis at the apex and along the distal tube and exocytosis adjacent to the apical dome. During cell elongation, newly inserted membrane flows out from the site of exocytosis, primarily toward the apex. Hydrodynamic regulation of exocytosis and endocytosis rates converges with hydrodynamic regulation of cell elongation. Apical cell volume oscillations may also correlate with activation of cellular signals such as Ca2+, phospholipids and phosphoinositides, and Rho GTPases (Zonia and Munnik, 2007, and references therein). Thus, hydrodynamic flux appears to be a key integrator of anisotropic pollen tube growth.

**Supplementary data**

**Movie 1.** High-resolution refraction-free time-lapse DIC microscopy of pollen tube oscillatory growth. Images were captured at a rate of 0.21 s image−1, contrast-enhanced, and compressed into video format. The video shows 600 s real-time live cell imaging compressed to 289 s video time.

**Movie 2.** High-resolution refraction-free time-lapse DIC microscopy of pollen tube oscillatory growth. Images were captured at a rate of 0.21 s image−1, contrast-enhanced, and compressed into video format. The video shows 600 s real-time live cell imaging compressed to 290 s video time. During strong growth pulses, the force of transcellular hydrodynamic flux causes rapid shifts in pollen tube positions, resulting in sudden out-of-focus jumps in the live movie.

**Fig. S1 (Fig. 1 corrected for red-green color blindness).** Pulse-chase labelling with FM 1-43 and FM 4-64 reveals sites of endocytosis in growing pollen tubes. These images were corrected by changing green to blue. Thus, FM 1-43 (green) is represented as blue, FM 4-64 (red) is perceived as grey for color-blind readers, and mixture of FM 1-43 + FM 4-64 remains yellow. Pollen tubes were labelled with FM 1-43 (green) for 2 h, osmotically stressed for 1 min, and then labelled with FM 4-64 (red) for 1 min before the start of imaging. Times in min indicate elapsed time after the start of FM 4-64 labelling. (A-B) Hyperosmotic treatment with 100 mM NaCl increases endocytosis. (C-D) Hypoosmotic treatment with the addition of H2O to a final volume of 1:2 (H2O:medium) increases exocytosis. Bar=10 μm.

**Fig. S2 (Fig. 2 corrected for red-green color blindness).** Vesicle dynamics in growing pollen tubes. These images were corrected by changing green to blue. Thus, FM 1-43 (green) is represented as blue, FM 4-64 (red) is perceived as grey for color-blind readers, and mixture of FM 1-43 + FM 4-64 remains yellow. The pollen tube shown in (A-E) was labelled with FM 1-43 (green) for 2 h and FM 4-64 (red) for 20 min before the start of imaging. (A) Overlay images show an elapsed time-course of 96 s of growth, with the start of imaging labelled as t=0 s. (B) The sequence 36–60 s shows exocytosis of vesicles into the plasma membrane and resultant expansion of the cell surface (arrow). Top row, overlay; center, green channel; bottom row, red channel. (C) Close-ups of the overlay images marked with arrows in B. FM 1-43 labelled membrane vesicles (green) are in the plasma membrane. (D) The sequence 168–192 s shows exocytosis of a single vesicle into at the plasma membrane (arrow). Top row, overlay; center, green channel; bottom row, red channel. (E) Close-ups of the overlay images marked with arrows in D. (F) Exocytosis of a vesicle into the plasma membrane (arrow) observed with high-resolution refraction-free time-lapse DIC microscopy. Elapsed time shown is 1.4 s. Bars=10 μm.

**Fig. S3 (Fig. 4 corrected for red-green color blindness).** Osmotic manipulation of transcellular hydrodynamic flux and pollen tube cell volume selectively increases endocytosis versus exocytosis. These images were corrected by changing green to blue. Thus, FM 1-43 (green) is represented as blue, FM 4-64 (red) is perceived as grey for color-blind readers, and mixture of FM 1-43 + FM 4-64 remains yellow. Pollen tubes were labelled with FM 1-43 (green) for 2 h, osmotically stressed for 1 min, and then labelled with FM 4-64 (red) for 1 min before the start of imaging. Times in min indicate elapsed time after the start of FM 4-64 labelling. (A-B) Hyperosmotic treatment with 100 mM NaCl increases endocytosis. (C-D) Hypoosmotic treatment with the addition of H2O to a final volume of 1:2 (H2O:medium) increases exocytosis. Bar=10 μm.

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