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Differential Organelle Movement on the Actin Cytoskeleton in Lily Pollen Tubes

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We have examined the arrangement and movement of three major compartments, the endoplasmic reticulum (ER), mitochondria, and the vacuole during oscillatory, polarized growth in lily pollen tubes. These movements are dependent on the actin cytoskeleton, because they are strongly perturbed by the anti-microfilament drug, latrunculin-B, and unaffected by the anti-microtubule agent, oryzalin. The ER, which has been labeled with mGFP5-HDEL or cytochalasin D tetramethylrhodamine, displays an oscillatory motion in the pollen tube apex. First it moves apically in the cortical region, presumably along the cortical actin fringe, and then periodically folds inward creating a platform that transects the apical domain in a plate-like structure. Finally, the ER reverses its direction and moves basipetally through the central core of the pollen tube. When subjected to cross-correlation analysis, the formation of the platform precedes maximal growth rates by an average of 3 s (35–40°). Mitochondria, labeled with Mitotracker Green, are enriched in the subapical region, and their movement closely resembles that of the ER. The vacuole, labeled with carboxy-dichlorofluorescein diacetate, consists of thin tubules arranged longitudinally in a reticulate network, which undergoes active motion. In contrast to the mitochondria and ER, the vacuole is located back from the apex, and never extends into the apical clear zone. We have not been able to decipher an oscillatory pattern in vacuole motion. Because this motion is dependent on actin and not tubulin, we think this is due to a different myosin from that which drives the ER and mitochondria. Cell Motil. Cytoskeleton 2007.

Key words: endoplasmic reticulum; mitochondria; vacuole; actin; myosin

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Abbreviations used: carboxy-DCFDA, carboxy-dichlorofluorescein diacetate; CLSM, confocal laser scanning microscopy; DIC, differential interference contrast; GFP, green fluorescent protein; TMR, tetramethylrhodamine.

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INTRODUCTION

Polarized tip growth is exhibited by various cell types such as pollen tubes, root hairs, fungal hyphae, moss protonemata, and neurons, and permits these cells to explore their environment [Hepler et al., 2001]. Just as the cell as a whole is polarized, so are its contents. For example, the intracellular components of a pollen tube can be divided into zones in which the growing tip is filled with secretory vesicles that fuse with the membrane to drive elongation [Steer and Steer, 1989; Derksen et al., 1995; Hepler et al., 2001]. This vesicle rich area and the region adjacent to it are mostly devoid of large organelles, such as starch containing amyloplasts and the vacuole, and thus is called the clear zone; however it does contain endoplasmic reticulum (ER), Golgi dictyosomes, and mitochondria. Beyond the clear zone the cytoplasm becomes granular, due primarily to amyloplasts, and this difference between the clear zone and the starch grain rich shank of the pollen tube is clearly visible. Despite the process of cytoplasmic streaming, which constantly moves these components both forward and rearward, the cytoplasmic elements retain their polarized organization. It seems plausible that like the streaming itself, the maintenance of intracellular polarity depends on the activity of the actin cytoskeleton [Steer, 1990; Geitmann and Emons, 2000; Vidali et al., 2001; Vidali and Hepler, 2001; Cárdenas et al., 2005].

Pollen tube actin filaments are organized in long axial bundles distributed throughout the shank of the lily pollen tube [Lancelle and Hepler, 1992; Lovy-Wheeler et al., 2005]. However, in the extreme apex, actin filaments form a cortical fringe of parallel bundles [Lovy-Wheeler et al., 2005], whose position remains close to the apex, suggesting that actin turnover must keep pace with the growth rate of the pollen tube. At the base of the clear zone is the area where the cytoplasmic streaming reverses direction as revealed by the granular amyloplasts [Vidali and Hepler, 2001]. However, close inspection at high resolution differential interference contrast (DIC) reveals that small particles move forward in the cortical cytoplasm of the clear zone and rearward through the core of the tube, indicating that streaming occurs close to the pollen tube apex.

For many years it has been evident that actin MFs and not microtubules, form the tracks along which streaming occurs, based on the sensitivity of the process to cytochalasin or latrunculin and not to colchicine or oryzalin. In recent years acto-myosin based motility in plants has received considerable attention [Liebe and Menzel, 1995; Boevink et al., 1998; Nebenführ et al., 1999; Mano et al., 2002; Van Gestel et al., 2002; Collings et al., 2003; Kim et al., 2005], and it is becoming clear that the motor protein that actually drives particle motion is myosin XI [Shimmen and Yokota, 2004; Wang and Pesacreta, 2004]. It seems pertinent that class XI myosins of plants show considerable (40%) similarity to class V found commonly in yeast and animal cells [Kinkema et al., 1994]; this similarity may explain the cross-reactivity of myosin V antibodies to small organelles in pollen tubes [Miller et al., 1995]. In mammalian systems, there is mounting evidence for an association of myosin V with the ER [Tabb et al., 1998; Wollert et al., 2002; Estrada et al., 2003], and similar data are emerging for myosin XI from studies of plants [Holweg and Nick, 2004]. In further support of myosin differential activity, yeast myosin V contains multiple receptors that specifically bind the vacuole and secretory vesicles, suggesting delivery to distinct places at different times [Pashkova et al., 2005]. In addition, recent evidence from plants demonstrates a high likelihood that myosins are binding specific proteins in the ER and Golgi to move these organelles along actin [Runions et al., 2006].

Because actin-dependent motion plays a pivotal role in the control of pollen tube growth, it might be expected to show an oscillatory component that relates to the well-known oscillation in growth rate. There are two studies that purport to show that actin oscillates in the pollen tube apex. Using green fluorescent protein (GFP)-talin to label actin, actin-associated fluorescence becomes more intense in anticipation of the next growth pulse [Fu et al., 2001; Gu et al., 2005]. However, these studies are fraught with difficulty since several emerging publications show that talin is not a faithful reporter for actin in plant cells [Ketelaar et al., 2004], including notably in pollen tubes where it fails to reliably reveal the cortical actin fringe [Wilsen et al., 2006]. In addition talin can cause the formation of aberrant actin structures and slow pollen tube growth.

Given the problem of monitoring actin itself, another approach is to examine the activity of actin. Because cytoplasmic streaming is dependent on the actin cytoskeleton, and because many organelles move along actin tracks, attempts have been made to correlate streaming with growth [Vidali et al., 2001]. In lily pollen tubes, the movement of particles ranged from 0.2 to 0.9 μm/s, but no correlation was found between these movements and growth rates [Vidali et al., 2001]. These studies may be confounded by the difficulty of following particles for extended periods of time. Also the particles could represent different organelles such as mitochondria or Golgi vesicle aggregations, which may move at different rates. Similar results were obtained in tobacco pollen tubes where all particles observed moved in an individual fashion [de Win et al., 1999]. The movement patterns do indicate a longitudinal differentiation, in which the particles exhibit Brownian-like motion in the clear zone, in contrast to a more vectorial and rapid movement in the shank. Although the various particles generally follow the reverse fountain cytoplasmic streaming pattern, de Win et al. [1999] conclude that

there is no bulk flow of the cytosol as there is in *Chara* and *Nitella* [Kuroda, 1990]. Again, these studies suffer because the identity of the particles is unknown.

Accordingly, we have focused herein on the movement of specific organelles in oscillating lily pollen tubes, using markers that selectively tag either the ER, mitochondria or the vacuole. The results clearly show that motion of the ER exhibits an oscillatory behavior that precedes the growth process. Additionally, we discovered that ER and mitochondria move similarly, but vacuole movement differs significantly. Because all organelles depend on the actin cytoskeleton, we propose that the vacuole is transported by a different myosin than the ER and mitochondria.

MATERIALS AND METHODS

Cell Culture

Lilium formosanum or *L. longiflorum* were grown in a medium containing 15 mM MES, 1.6 mM HBO_3, 1 mM KCl, 0.1 mM $CaCl_2$, 7% sucrose, pH 5.5. For plunge-freezing the level of sucrose was raised to 10%. For live studies, pollen tubes were first germinated for 45 min, plated in 0.7% low melting agar to ensure stabilized growth, and allowed to reach 700 μm in length. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich, St. Louis, MO.

Organelle Labeling

All dyes were purchased from Molecular Probes; Invitrogen Corporation, Carlsbad, CA.

ER Labeling. The ER was labeled with mGFP5-HDEL obtained from J. Runions and C. Hawes, (Oxford Brookes University), but originally produced by J. Haseloff [Haseloff et al., 1997]. For expression in lily pollen, mGFP5-HDEL was cloned into a pZmc13 vector using standard molecular techniques. Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) and ethanol precipitated to a final concentration of 1 μg/μl. Bombardment "bullets" were prepared by coating 3 mg tungsten particles (diameter 1.1 μm; Bio-Rad Laboratories, Hercules, CA) with 10 μg plasmid DNA according to manufacture's instruction. Microprojectile bombardment was achieved using a Bio-Rad helium-driven Biolistics PDS-1000 in conjunction with 1100-psi rupture discs. Approximately 10 mg of pollen were hydrated in 100 μl germination medium for 5 min before bombardment, and transferred to a 25-mm MF-Millipore membrane placed on top of moist Whatman filter paper in a petri dish. The microcarrier launch assembly was positioned in the second slot from the top with the hydrated pollen directly beneath it. Bombarded pollen grains were transferred to 1 mL germination medium and germinated for 45 min at which point they were plated as described above. The ER was also labeled with 250–500 nM cytochalasin D tetramethylrhodamine (TMR), and pollen tubes were imaged 5 min after dye addition.

Mitochondrial Labeling. Mitochondria were labeled with 15 μM (final concentration) Mitotracker Green FM and imaged 30 min after incubation.

Vacuole Labeling. The vacuole was labeled with 1 μM carboxy-(5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate) (carboxy-DCFDA), washed after 5 min with regular growth medium, and imaged 15–30 min later.

For double labeling experiments, cells were labeled with either carboxy-DCFDA or Mitotracker Green FM, allowed to incubate for 15–30 min, and then labeled with cytochalasin D TMR to stain the ER.

Image Acquisition

Imaging was performed on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Thornwood, NY) using the Argon 488 nm and HeNe 543 nm laser excitation, and a long pass 505 or 568 emission filter. For simultaneous green and red channel imaging, the multitracking function was utilized and each laser was activated one at a time, thus ensuring no cross-talk occurred between the two fluorochromes. The emission filters in this set-up included a bandpass filter 505–530 nm for the green channel, and a long pass 568 nm filter for the red channel. The supplemental movies are accelerated about 30× original speed.

Cryofixation/Immunolabeling

Cryofixation and immunolabeling were performed as described in Lovy-Wheeler et al. [2005]. Briefly, *L. longiflorum* was surface germinated on pollen tube growth medium for 2–4 h, picked up on 3% agar loops, and plunge frozen as described in [Lancelle et al., 1986]. Pollen tubes were freeze-substituted at −80°C for 36 h in dry acetone [Lancelle et al., 1986] but supplemented with 2–5% anhydrous glutaraldehyde. Samples were placed in a pre-cooled −80°C metal block, and allowed to come to room temperature over a period of 8 h. The loops containing the pollen tubes were rehydrated in 10% steps (15–30 min each) to 100% water, and then placed into 10 mM phosphate buffered saline (PBS). Pollen tubes were treated with 0.1% $NaBH_4$ for 15 min, then rinsed with PBS supplemented with 0.1% (w/v) Tween 20 for 15 min. Monoclonal mouse anti-actin antibody (C4-raised against chicken gizzard actin; Chemicon, Temecula, CA), and lily specific myosin XI antibodies from rabbit [Yokota et al., 1995] were applied sequentially. First the anti-actin antibody was applied at 4°C overnight, followed by a secondary goat anti-mouse Cy3 antibody (Jackson Immunoresearch Laboratories, West Grove, PA) which was applied for 3–4 h at 37°C. Next, the anti-myosin XI (170 kDa) [Yokota et al., 1995]

antibody was applied at 37°C for 4 h, followed by secondary antibody (Alexa Fluor 488 $F(ab')_2$ fragment of goat anti-rabbit IgG; Invitrogen Corporation) also at 37°C for 4 h. Samples were washed six times between each treatment using PBS supplemented with 0.05 % (w/v) Tween 20. Pollen tubes were mounted in 4% n-propyl gallate (90% glycerol, 10% PBS).

Actin and Microtubule Experiments

To test whether microtubules had any role in the organization of the vacuole, ER, or mitochondria, pollen tubes were treated with 2 μM oryzalin (2 mM oryzalin stock solution in DMSO). To test the involvement of the actin cytoskeleton in organelle movement, pollen tubes were treated with 2 nM latrunculin B. In the case of the vacuole, to tease out more details on growth inhibition and organelle movements, much lower concentrations of latrunculin B (down to 1 pM) were used.

Cross Correlation Studies

Cross-correlation is a mathematical procedure that allows alignment between two different data sets of a series of information. We used this as a tool to compare the oscillation of ER motion with the oscillation of the growth rate. The goal was to determine the phase relationship between the two processes. Our application of cross-correlation analysis depends on slight irregularities in the periodicity and amplitude of both data sets, such as growth rate and fluorescence intensity. It is because of these irregularities that the similarities between two independent measures can be correlated and the lag between the two time series processes can be deciphered [Brillinger, 1981]. Oscillatory profiles of growth velocities and ER fluorescence values were cross-correlated using the convolution function from the stats library of the R computational environment [Maindonald and Braun, 2003]. The total fluorescence in boxed regions of the pollen tube apex was measured (indicated in Fig. 3). Boxed regions were kept the same distance from the apex during each frame. Growth velocities and ER movements were subjected to a Lowess smoothing function, to achieve base-line correction [Cleveland, 1981].

RESULTS

Labeling of the ER

Initially we used mGFP5-HDEL [Haseloff et al., 1997] to label the ER. This method has been proven in many systems to provide a specific label for ER in living cells, and when properly executed permits visualization for hours; a confocal laser scanning microscopy (CLSM) image is shown in Fig. 1 (see Supplemental Movie 1 to watch ER dynamics). The ER is present throughout the shank of the pollen tube but also within the clear zone of the apex. Although the ER seems uniformly distributed throughout the cytoplasm of lily pollen tubes, measures of fluorescence intensity indicate that it is more concentrated in the subapical region, perhaps due to the reversal of streaming in this area. Its distribution closely follows a reverse fountain pattern of cytoplasmic streaming, except that it travels closer to the apex than is seen for the typical reverse fountain motion when examining amyloplasts. These images are similar to those published by Parton et al. using this construct [2003].

By chance, when testing another dye, namely cytochalasin D TMR, we discovered that it too labeled the ER. Initially we had been interested in its prescreened role as a selective stain for actin plus ends. However, it soon became obvious to us that this reagent was binding to or being sequestered in a non-actin compartment. We performed double labeling studies with mGFP5-HDEL and cytochalasin D TMR, and found that the cytochalasin D TMR, after just a few minutes, produced a signal that overlapped to a great extent with the mGFP5-HDEL signal in growing lily pollen tubes (Fig. 2). There is almost complete colocalization in the apical domain except for low background staining in the cytoplasm, as is visible in the top panel of Fig. 2. The punctate pattern further down the shank of the pollen tube (bottom panel of Fig. 2) may be due to cytochalasin D TMR also loading into Golgi dictyosomes since these can appear punctate [Cheung et al., 2002].

This fortuitous observation encouraged us the use cytochalasin D TMR for much of our studies on ER motion. Whereas transient expression of mGFP5-HDEL is recognized as a faithful marker of the ER, there are substantial problems with its use. Low expression efficiency and difficulty in controlling expression levels hinder the use of these constructs when collecting time series data. In addition, by the time growing pollen tubes transiently express these constructs, they are usually quite long and often too fragile to provide reliable time series information. By contrast, cytochalasin D TMR is a permeant dye that effectively stains cells with very high efficiency ($\sim$100%). Moreover, when used at 500 nM, cytochalasin D TMR has no effect on pollen tube growth.

In a further characterization we applied cytochalasin D TMR to pig kidney epithelial (LLCPK) cells that were expressing GFP-actin (in collaboration with Patricia Wadsworth). As in the pollen tube, most of the dye colocalized with the ER (data not shown) and not with the actin, leading us to conclude that cytochalasin D TMR does not stain actin plus ends as advertised, but sequesters into the ER. Cytochalasin D TMR therefore becomes an effective and non-toxic marker for the ER, requiring neither bombardment nor long waiting for expression, as needed with mGFP5-HDEL.

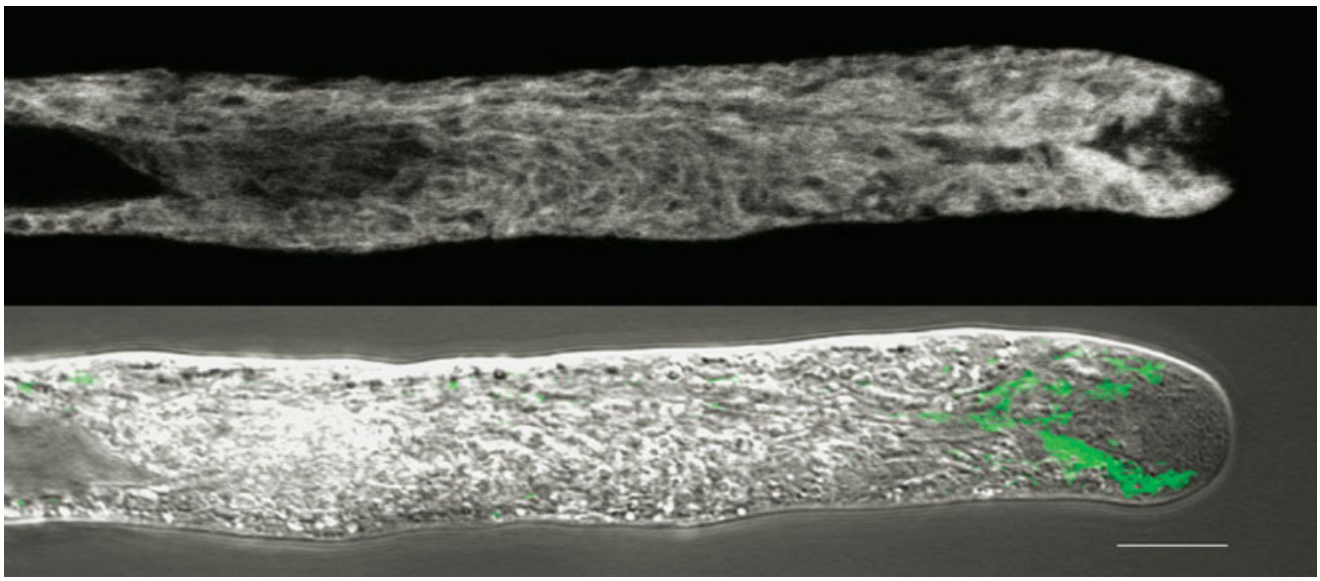

Fig. 1. Endoplasmic reticulum labeling in lily pollen tubes expressing mGFP5-HDEL. Top panel is a CLSM image and the bottom panel shows a differential interference contrast (DIC) image overlay with the image above; the ER is shown in green. Bar = 10 μm. See supplemental movie 1 to visualize ER dynamics; images were taken every 6 s.

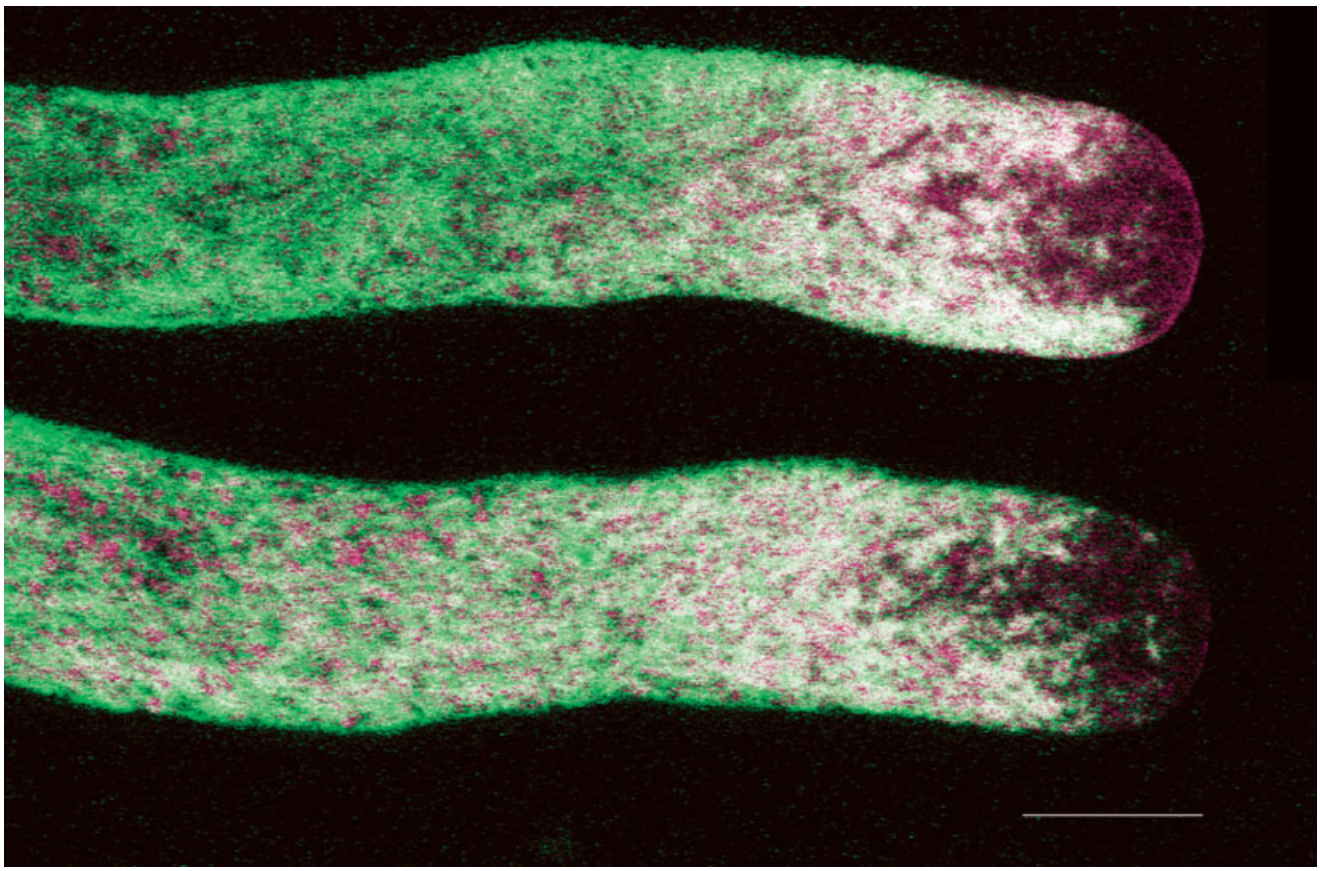

Fig. 2. Endoplasmic reticulum marker mGFP5-HDEL (green) colocalization with 500 nM cytochalasin D TMR (magenta). Top panel shows colocalization after 2 min of cytochalasin D TMR addition, and the bottom panel is the same tube imaged 4 min after treatment. Bar = 10 μm.

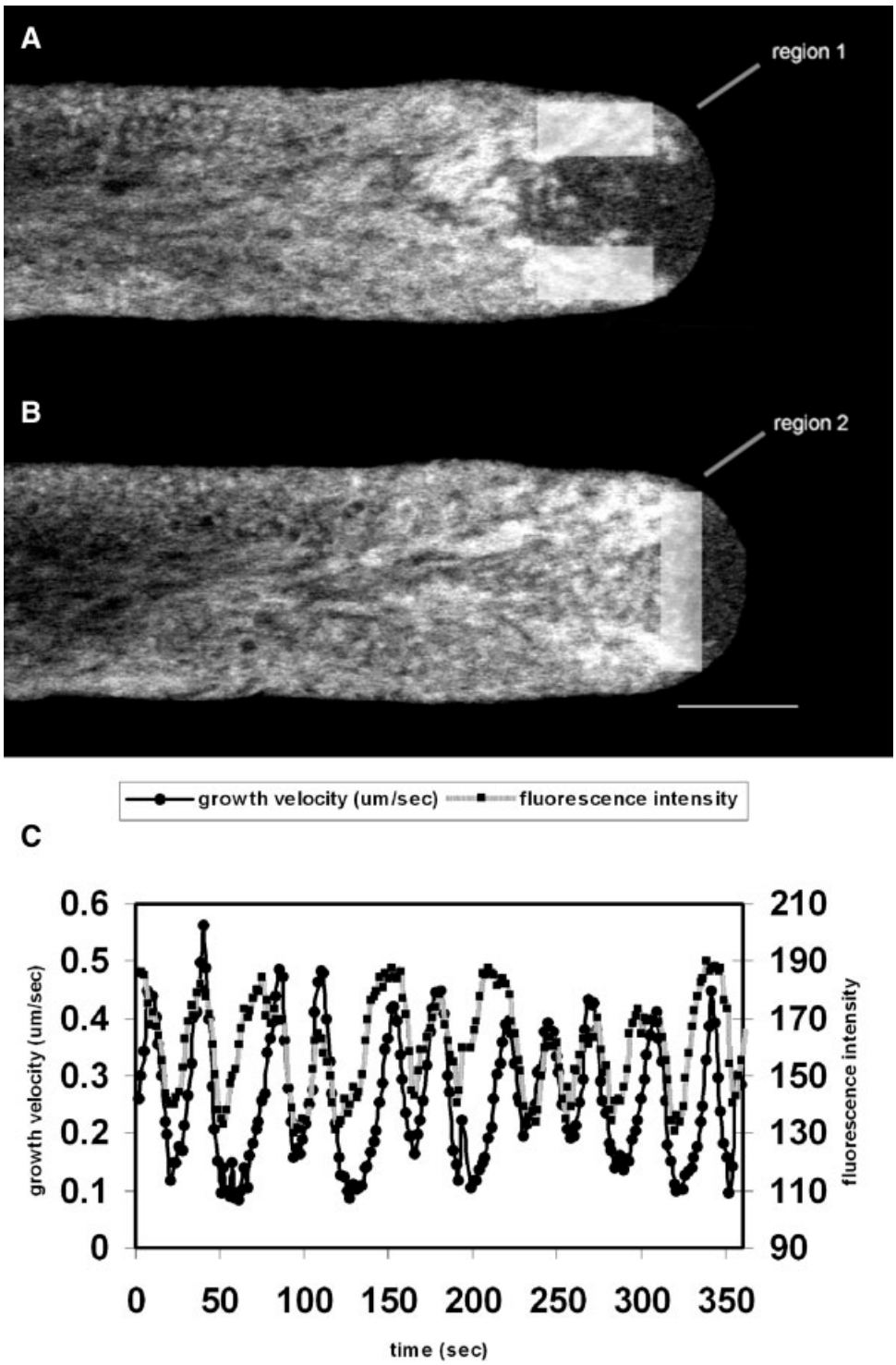

Fig. 3. ER morphology and distribution changes in lily pollen tubes labeled with 500 nM cytochalasin D TMR. Boxed regions were measured for fluorescence intensity to be used in cross-correlation analysis. Top panel shows the ER first moving into the apex along the cortex (region 1), and the bottom panel shows the platform formation as marked by region 2. Graph illustrates how the platform (region 2) oscillates relative to pollen tube growth rates. Bar = 10 μm.

ER Distribution Changes in the Pollen Tube Apex During Growth

The ER in the apex of pollen tubes moves through two prevailing morphological configurations in an oscillatory manner. One configuration (Fig. 3A) is an open funnel where the ER in the apical domain is mostly cortical and outlines the inverted cone of vesicles at the apex. The other configuration (Fig. 3B) is made evident by the funnel folding in on itself and filling the inverted cone region of the apical domain. A cross-section of these two morphologies within 10 μm of the apex would look in the first instance like a hollow ring (corresponding to Fig. 3A),

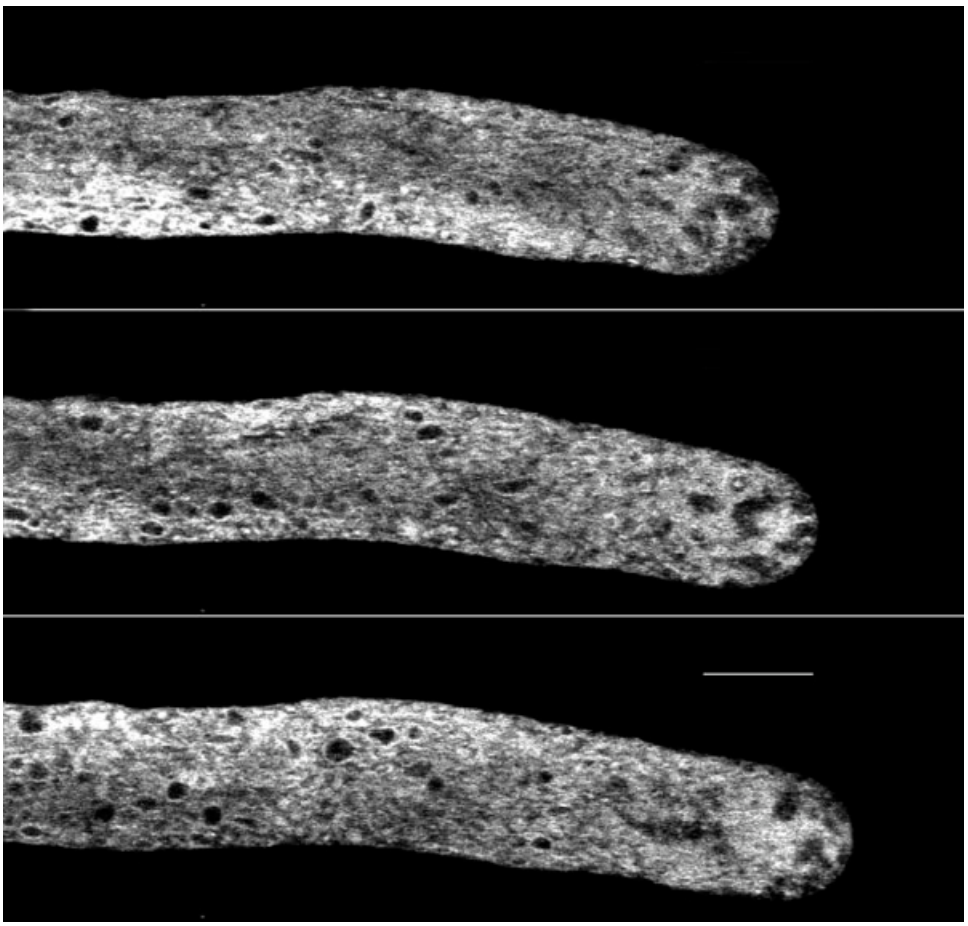

Fig. 4. ER distribution after treatment with 2 nM latrunculin B. Lily pollen tube was first labeled with cytochalasin D TMR, then treated with latrunculin B. Growth persisted at 20% regular velocity, and the ER moved all the way into the apex. Images are 1 min apart. Bar = 10 μm.

and in the second instance a filled circle, or a platform (corresponding to Fig. 3B). All pollen tubes oscillating in growth expressed a cortical ER accumulation in the shape of a funnel alternating with the formation of a platform of ER perpendicular to the axis of growth. When the fluorescence intensity is monitored over time in the regions shown in Figs. 3A and 3B, it is clear that the movement of the ER is oscillating relative to growth, as shown in Fig. 3C. This is significant because for the first time, the movement of an organelle can be correlated to growth oscillations.

The movement of the ER is dependent on the actin cytoskeleton. When lily pollen tubes are treated with 2 nM latrunculin B, the ER morphology is severely affected. Not only does it move all the way into the apex, as seen in Fig. 4, but its pattern of streaming changes from reverse fountain to circular. Although the speed of streaming of individual particles is unaffected, the growth rate is inhibited by 80%, as observed previously [Cárdenas, unpublished; Vidali et al., 2001].

Cross-Correlation of ER Accumulations in the Apex Versus Growth Velocity in Lily Pollen Tubes

To study the morphological changes in ER during pollen tube growth, fluorescence intensity of the above described funnel and platform regions was cross-correlated with growth velocity to determine whether the formation of either of these structures anticipates or follows maximal growth rates. The formation of the platform of (region 2) showed a clear correlation with growth velocity, as visible in Fig. 3C. In the 11 oscillating pollen tubes studied, the ER platform formed 3 s before maximal growth rates (see Table I). Additionally, the ER cortical funnel (region 1) preceded growth and by an average of 4 s. The signal of the ER funnel was not as easily defined as the ER platform (region 2) and thus 5 instead of 11 cells were measured.

Mitochondria Accumulate at the Subapex of Lily Pollen Tubes

Mitochondria were found to accumulate in the subapical region of lily pollen tubes labeled with Mitotracker Green FM (Fig. 5). To quantitatively examine this asymmetric distribution we divided the pollen tube in 10-μm increments along its length and measured the fluorescence intensity in each region. As clearly seen in the bar graph there are only a few mitochondria in the most apical region. Mitochondrial density increases markedly in the region 10–20 μm behind the apex, and thereafter drops off more gradually ($n = 5$). This mito-

chondrial enrichment is probably important due to the high demand for energy at the growing region of the cell. Mitochondrial movements are similar to those of the ER. These organelles travel all the way into the clear zone along the cortex, and fold inward close to the apex in a pattern very similar to that of the ER (Fig. 6; see Supplemental Movie 2 to visualize ER and mitochondrial dynamics). Among the mitochondria that start to flow rearward, several appear to be caught in eddy currents where they reverse direction and again flow forward. There is thus a constant supply of mitochondria close to the apex. Upon treatment with 2 nM latrunculin B, mitochondria move all the way into the apical domain and lose their organization and enrichment in the subapex, emphasizing the importance of the actin cytoskeleton in this organization (data not shown).

TABLE I. ER Accumulation in the Apex of Pollen Tubes Relative to Growth Velocity

Growth rate (μm/s)	Region 1 relative to growth (s)	Region 2 relative to growth (s)	Period (s)
0.211	–	−4	37
0.193	–	−6	38
0.340	–	−4	26
0.29	–	−2	27
0.253	–	−5	30
0.235	–	−4	27
0.308	−3	−1	22
0.29	−4	−1	32
0.344	−5	−4	25
0.257	−4	−2	36
0.287	−4	−3	27
0.27 ± 0.05	−4 ± 1	−3 ± 2	30 ± 5

Each row represents one cell and displays the growth rate, cross-correlation results of the phase relationship to growth (negative number means that the process occurs before maximal growth rates), and the periodicity displayed by growth oscillations. Last line of the table shows the average and standard deviation of each column.

Vacuole Distribution is Different From ER and Mitochondria, but Still Depends on the Actin Cytoskeleton

The vacuole in plants has been imaged in various ways including with dyes such as FM1-43 [Ruthardt et al., 2005], and GFP fusion proteins such as AtVam3p [Kutsuna et al., 2003; Kutsuna and Hasezawa, 2005] and δ-TIP [Hicks et al., 2004], which have revealed it to have a tubular structure in pollen tubes and during mitosis in live tobacco cells. We have found that carboxy-DCFDA is a good marker for the vacuole in lily pollen tubes. Fine, thread-like vacuolar strands are distributed throughout the shank of the pollen tube (Fig. 7). However, the vacuole does not permeate the clear zone as do the ER and mitochondria (Fig. 8). Although its morphology is constantly changing, its motion is different from that observed for the ER and mitochondria. Its thread-like

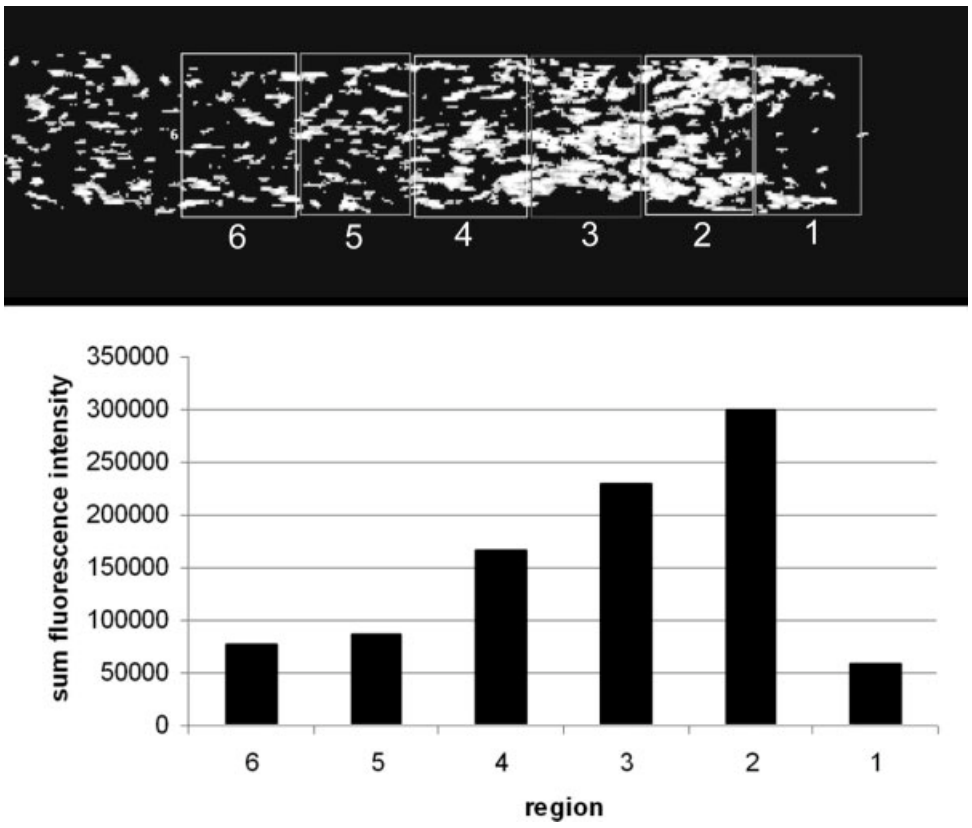

Fig. 5. Mitochondrial distribution in lily pollen tubes stained with Mitotracker green. The sum of fluorescence intensity was measured in six regions as shown above.

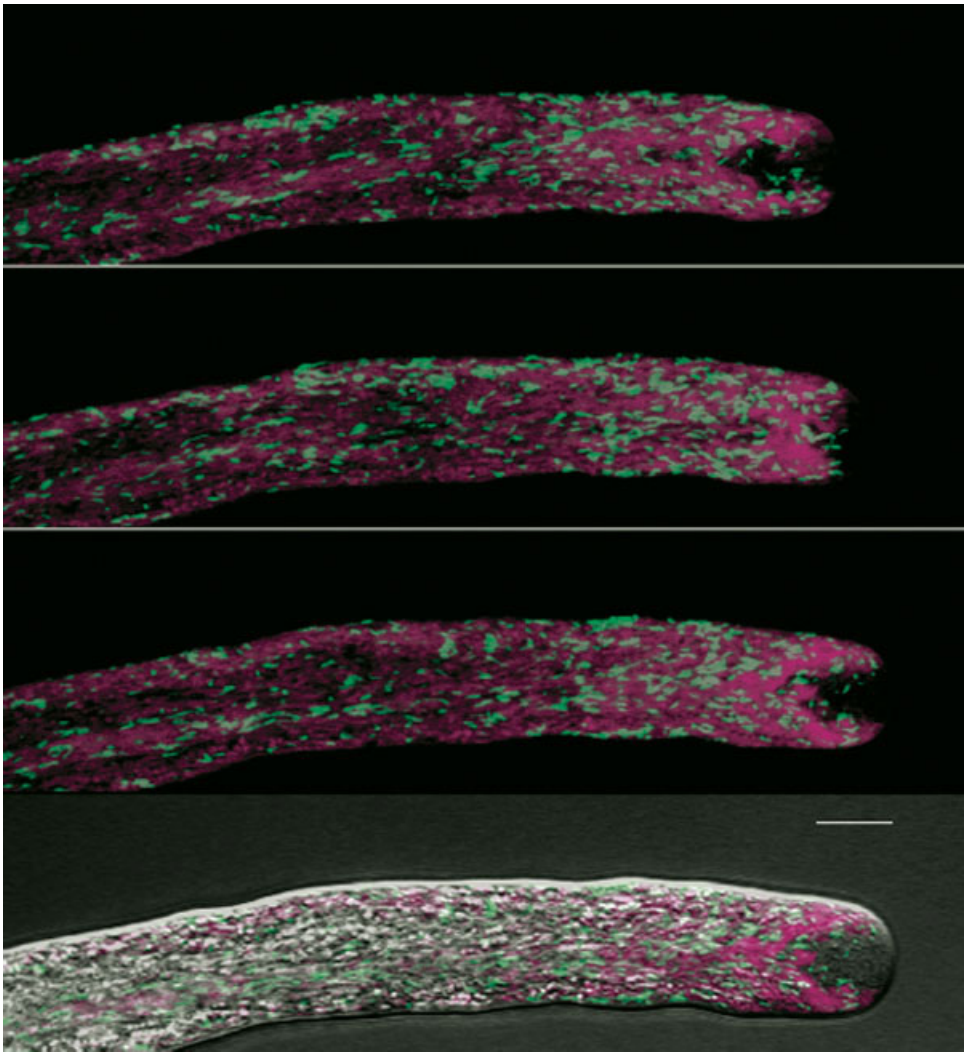

Fig. 6. Mitochondrial (Mitotracker Green, colored green) and ER (cytochalasin D TMR, colored magenta) labeling in lily pollen tubes. CLSM images are 12 s apart, and show that the distribution of both organelles changes similarly. The bottom panel is an overlay of DIC and the image above it. Bar = 10 μm. See supplemental movie 2 to visualize simultaneous mitochondrial and ER dynamics; images were taken every 3 s.

tubules turn inward but do not form a dense aggregation as do the ER and mitochondria (see Supplemental Movie 3 to visualize vacuole and ER dynamics).

Like the ER and mitochondria, vacuole movement depends on the actin cytoskeleton. When lily pollen tubes are treated with 1 nM latrunculin B, vacuole morphology and motion are significantly affected. As pollen tube growth is inhibited, the polarized cytoplasm in the apex quickly becomes disorganized. To slow down these deleterious effects and try to understand them in more detail, we treated pollen tubes with lower amounts of latrunculin B. Even 1 pM latrunculin B disrupted the apical zonation of the cytoplasm, and inhibited growth (Fig. 9). The long tubular structure of the vacuole changes from being axial to clustered and kinked, as if becoming entangled after latrunculin treatment. The vacuole permeates the clear zone, traveling close to the extreme apex. Although streaming continues, the pattern becomes more circular rather than the typical reverse fountain.

Microtubules Are Not Involved in the Organization of ER, Mitochondria or the Vacuole

Despite many studies on the role of microtubules in lily pollen tube growth, their function is unclear [see Cai and Cresti, 2006]. The presence of this uncertainty may come from many studies being performed on young pollen tubes grown in vitro, because studies on pollen tube growth in culture for a longer period suggest that microtubules are involved in controlling the movement of the generative cell and vegetative nucleus [Joos et al., 1994]. The subapex of lily pollen tubes contains a microtubule fringe [Foissner et al., 2002; Lovy-Wheeler et al., 2005]. Evidence that the positioning of mitochondria in tobacco cells is maintained by actin filaments and microtubules [Van Gestel et al., 2002], suggest that microtubules might be playing a role in retaining mitochondria in the subapex. However, upon treatment with 2 μM oryzalin for 30 min, there is no difference in ER or mitochondrial distribution even

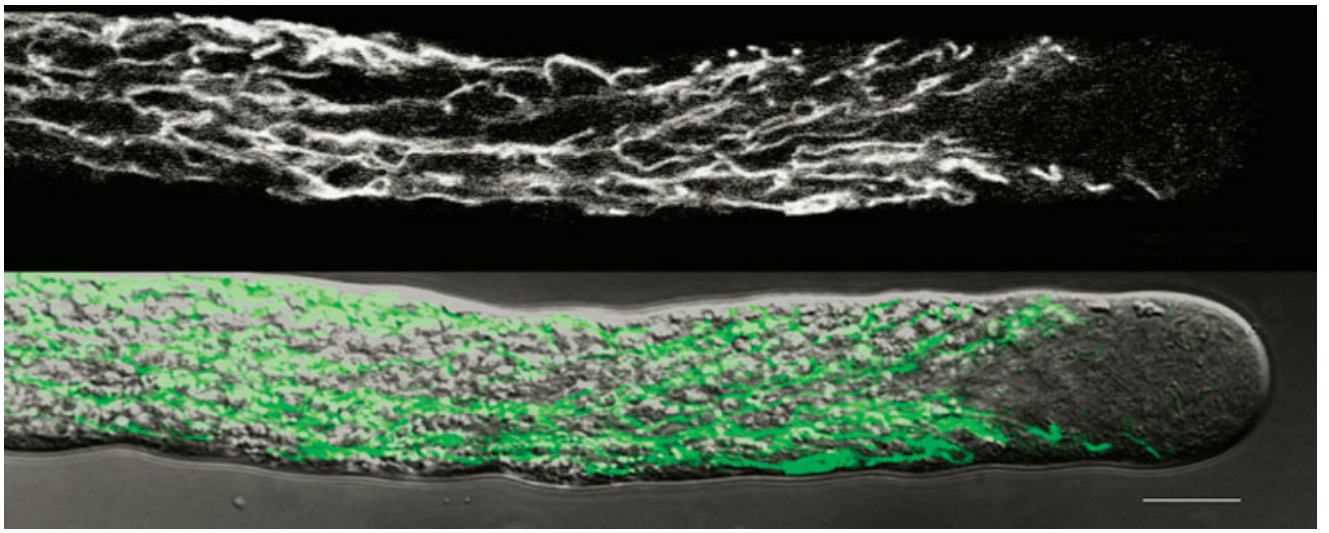

Fig. 7. Vacuole distribution in lily pollen tubes labeled with carboxy-DCFDA. Top image shows a CLSM image of the midplane slice of a lily pollen tube, and the bottom panel shows an overlay of the top panel with a differential interference contrast (DIC) image. Bar = 10 μm.

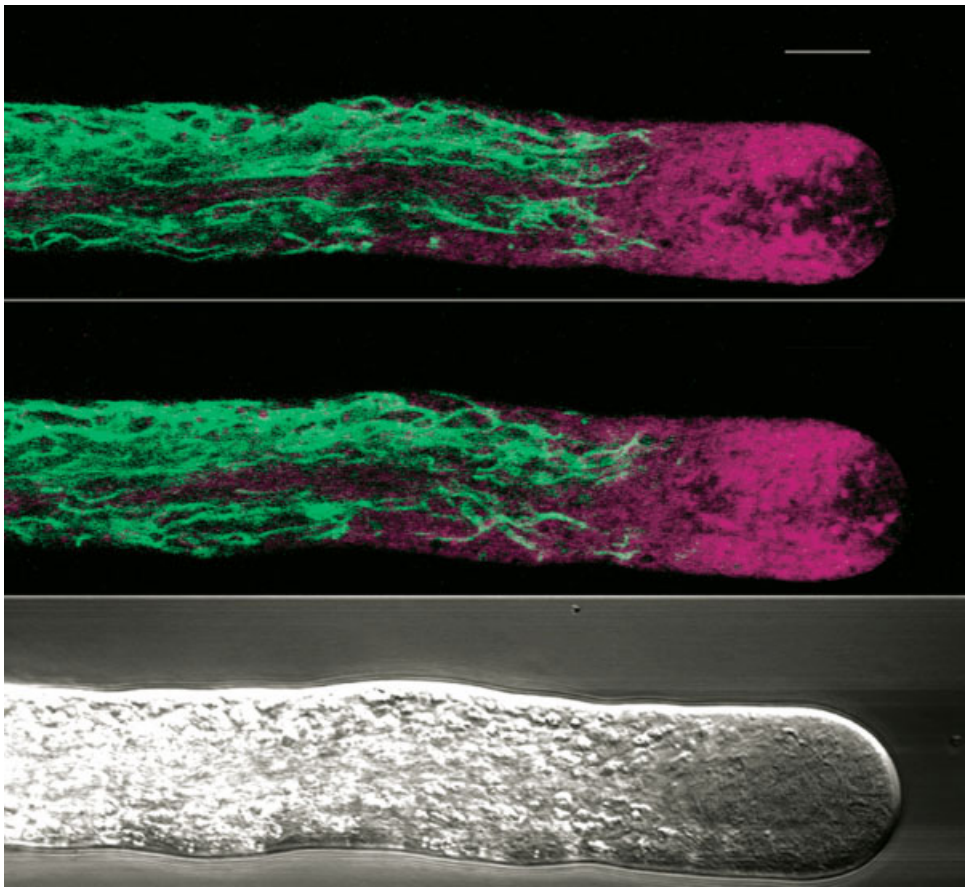

Fig. 8. Vacuole (carboxy-DCFDA, colored green) and ER (cytochalasin D TMR colored magenta) dual labeling in live lily pollen tubes. Top two panels are CLSM images 10 s apart and the bottom panel is a DIC image of the middle panel. The vacuole distribution differs significantly from the ER, and outlines the inverted clear zone area on the outside, whereas the ER is located throughout the clear zone and changes morphology concomitant with growth. Bar = 10 μm. See supplemental movie 3 to visualize simultaneous vacuole and ER dynamics; images were taken every 10 s.

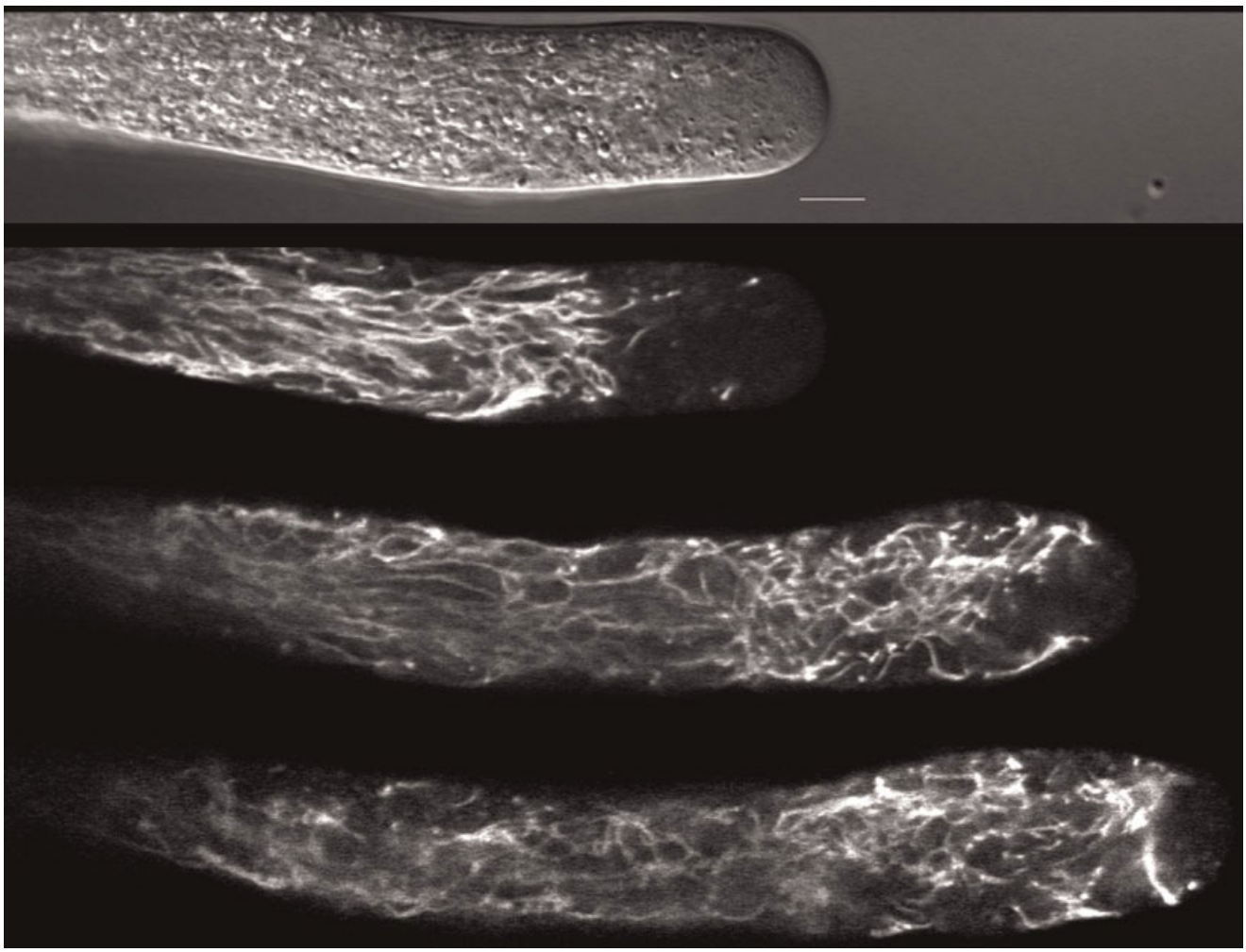

Fig. 9. Vacuole morphology (carboxy-DCFDA labeling) in growing lily pollen tubes treated with 1 pM latrunculin B. Top two panels show a DIC image and a CLSM midplane image of vacuole labeling in a control pollen tube. The next two panels show CLSM images of the same pollen tube treated with latrunculin B 2.50 min later, and 5.15 after treatment. Growth is severely inhibited, and vacuole morphology and cytoplasmic streaming are severely altered. Bar = 10 μm.

though 1 μM oryzalin destroys microtubules within 5 min (data not shown).

Because the microtubule fringe [Foissner et al., 2002; Lovy-Wheeler et al., 2005], and the vacuole reside in the same region, and because previous studies had indicated that microtubule depolymerization leads to cytoplasmic rearrangement in some pollen tubes, e.g., tobacco [Joos et al., 1994], we carefully tested whether the position of the vacuole in lily would move when the microtubules were depolymerized with oryzalin. At 1 μM oryzalin, microtubules are rapidly depolymerized (data not shown); however, after several minutes in 2 μM oryzalin, there was no change in vacuolar morphology as seen in Fig. 10. Some tubes have been cultured for hours in oryzalin, without causing a change in the normal organization of the vacuole (data not shown).

Localization of Lily-Specific Myosin XI

Because all of the above mentioned organelles are dependent on the actin cytoskeleton, it becomes important to see the distribution of myosin. We performed immunolabeling with an antibody made specifically against lily pollen tube myosin XI. The technique involved plunge-freezing in liquid propane, freeze-substituted in −80°C acetone, fixation and rehydration as described previously [Lovy-Wheeler et al., 2005]. These cells were double labeled with anti-actin and lily-specific anti-myosin 170-kDa antibodies. The images reveal that the lily-specific 170 kDa (myosin XI) primarily labels a cap region ahead of the actin fringe (Fig. 11), but instead of being present primarily in the cortex like the actin, it densely labels the apical domain of the pollen tube throughout its thickness. Labeling continues behind the

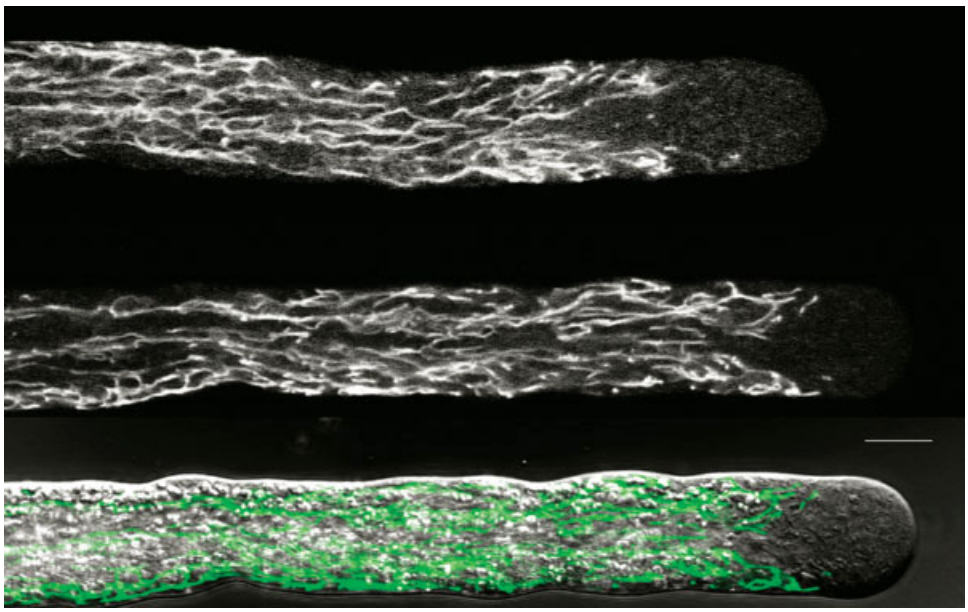

Fig. 10. Vacuolar morphology (visualized with carboxy-DCFDA) in lily pollen tubes before and after 2 μM oryzalin treatment. Top panel is a CLSM image of a control pollen tube showing vacuolar labeling; middle panel is the same tube as above after 8 min of oryzalin treatment. The bottom panel shows an overlay of the middle panel merged with the DIC image; vacuole morphology and motility are unaffected. Bar = 10 μm.

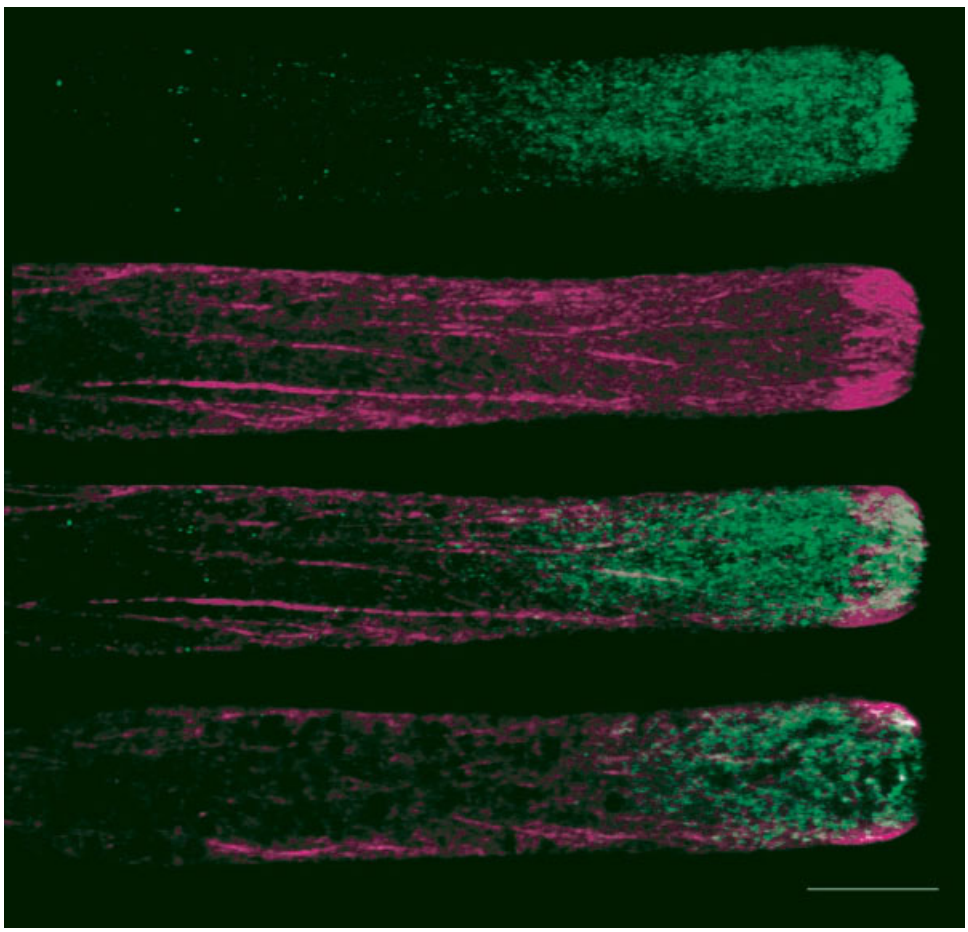

Fig. 11. Actin (magenta) and myosin (green) immunolabeling in plunge-frozen pollen tubes of lily. First three panels are CLSM projections of a lily pollen tube optically sliced every 0.2 μm. Top two panels are myosin and actin immunolabeling, the third panel is a merged image of the two. Bottom panel is a single midplane slice of the actin and myosin immunolabel. Bar = 10 μm.

fringe into the shank of the tube, with the degree of staining declining. As shown previously by Yokota et al. [1995], it is clear from CLSM imaging (Fig. 11) that myosin accumulates in the apical domain. Additionally, we now have the resolution to see that the myosin XI occurs primarily at the front end of the actin fringe. In contrast to the cortical actin fringe, myosin is localized throughout the thickness of the tube in the apical domain, where it may be bound to vesicles and other membranes such as those of the ER and mitochondria. This labeling pattern is highly suggestive that this myosin XI is responsible for carrying vesicles to the apex.

DISCUSSION

Lily pollen tubes can grow to be several centimeters (10–15 cm) in length, and regardless of the bidirectional and vigorous cytoplasmic streaming, all of their intracellular components are retained in an asymmetric and polarized organization. This organization is orchestrated by the actin cytoskeleton, because the addition of latrunculin B, but not oryzalin, perturbs the positioning of the ER, mitochondria, and vacuole. We recognize that these studies stand in contrast to those on tobacco, which show that pollen tubes treated with colchicine or propham exhibit cytoplasmic disorganization [Joos et al., 1994]. It is possible that species difference explain these discrepancies, however, it is important to emphasize that several other studies with anti-microtubule agents on different pollen tube systems have failed to elicit marked effects on tube structure, growth or intracellular motility [Derksen et al., 1985; Heslop-Harrison and Heslop-Harrison, 1988].

Sequential imaging of the ER in particular, reveals a periodic pattern of motion that is correlated with oscillatory pollen tube growth. Specifically, the ER flows forward along the cortex forming a funnel, and periodically folds inward forming a platform. Through cross-correlation analysis, we have found that the formation of the ER platform leads the growth process by 3 s; additional data on five cells indicate that ER moves apically along the cell cortex even earlier and that this forward motion transforms into the platform structure. It is additionally pertinent that mitochondria move like the ER (Fig. 6), and that they are present within the ER platform, which anticipates growth. These observations stand in marked contrast to previous studies that were unable to show a correlation between particle motions and the growth process [de Win et al., 1999; Vidali et al., 2001]. Taken together these results reveal an activity of actin, possibly combined with the activity of myosin, that precedes and anticipates the increase in the growth rate, and thus may be part of the sequence of events that serve as primary regulators of growth. We speculate that the forward motion of the ER may indicate a similar temporal surge in vesicles that is needed for cell elongation.

Although all three organelles examined, ER, mitochondria, and vacuole, are transported along actin, it is noteworthy that ER and mitochondria show a different pattern of motion than that of the vacuole. The latter does not move noticeably into the clear zone, but instead only reaches the subapical area similar to the distribution of the amyloplasts. In this regard it is important to emphasize, based on numerous Nomarski DIC observations, that the position and motion of the amyloplasts are very similar to the vacuole. And while both vacuole and amyloplasts coincide with the position of the microtubule cytoskeleton at the base of the clear zone, microtubule inhibitors have absolutely no effect on their motion, whereas they are profoundly affected by the anti-actin inhibitor, latrunculin-B. We assume that the position and motion of the vacuole and amyloplasts are also controlled by myosin, but one that differs from that controlling ER and mitochondria. We also include the idea that the inability of the vacuole and amyloplasts to move into the clear zone may be due to a physical filtering mechanism at the base of the clear zone [Heslop-Harrison and Heslop-Harrison, 1990].

Because myosin XI is a plus-end directed motor [Shimmen and Yokota, 2004], the direction of movement of the ER allows us to predict the polarity of the actin filaments. The ER moves toward the apex along the edges of the pollen tube, likely to the end of the cortical actin fringe [Lovy-Wheeler et al., 2005], suggesting that actin plus ends are also facing the tip. At the tip of the clear zone, the ER turns inward and therefore the actin polarity also presumably changes with the barbed ends directed toward the grain; this configuration gives rise to the typical reverse fountain streaming pattern. In support of this contention we note the work on root hairs of *Hydrocharis*, which also exhibits reverse fountain streaming. Here, using myosin subfragment-1 binding, Tominaga et al. [2000] have shown that actin plus ends face the tip along the cortical regions, and reverse polarity in the middle of the root hair.

There is considerable evidence supporting the idea that myosin binds to the ER, and thus may be responsible for generating bulk cytoplasmic streaming in pollen tubes [Williamson, 1993; Shimmen and Yokota, 2004]. One of the myosin XI isoforms, of which there are 13 in *Arabidopsis* [Reddy and Day, 2001], has recently been found to bind and move the ER [Holweg and Nick, 2004]. Electron microscopy studies are consistent with this observation since they have revealed the ER to be closely associated with actin [Lichtscheidl et al., 1990]. But in addition the ER may also be responsible for cytoplasmic arrangement, insofar as it could have several specific domains to its own structure that perform unique

functions [Staehelin, 1997; Levine and Rabouille, 2005]. Thus the ER, through its myosin dependent motion, may define the position and motion of other organelles. In the pollen tube our data could hold partially for ER-mitochondrial associations because these organelles exhibit very similar movement patterns, but not for an ER-vacuole association.

In addition to their association with ER, there are myosin XI isoforms that appear to associate with mitochondria and plastids [Wang and Pesacreta, 2004], peroxisomes [Hashimoto et al., 2005], and vesicles [Yokota et al., 1995]. Myosin V, the closest class of myosins to the plant myosin XI, is important for vacuole inheritance and movement in yeast [Ishikawa et al., 2003], and thus it is conceivable that a myosin XI isoform would have similar activity in plants. The lily specific myosin XI characterized by [Yokota et al., 1995] and examined here is most enriched at the front edge of the actin fringe, but also creates a cap. When taken together these observations are consistent with vesicular labeling in the apical region. Both in the tip and further back the myosin XI labeling does not always overlap with actin filaments, indicating a likely association with membranous organelles.

In any discussion of motility in the pollen tube apex it is essential to consider a role for calcium. The tip-focused gradient, found in the extreme apex of the tube, is sufficiently high (10 μM) [Messerli and Robinson, 1997] that it will inhibit the activity of myosin XI, which is a calcium sensitive motor. The high calcium would also be expected to fragment actin, possibly working through gelsolin [Yokota et al., 1999; Huang et al., 2004]. The inactivation of myosins together with the fragmentation of the actin tracks, would be expected to cause changes in ER and mitochondrial motion and distribution. On the other hand ER and mitochondria sequester calcium [Solovyova and Verkhratsky, 2002; Wilsen, 2005] and thus their presence in the apex may be essential for calcium homeostasis in the pollen tube. It is important to note that calcium levels in the apex of pollen tubes oscillate in concentration during oscillatory growth [Pierson et al., 1996; Holdaway-Clarke et al., 1997; Holdaway-Clarke and Hepler, 2003], and therefore that these ion changes could affect ER and mitochondrial motion. However, the data provided herein argues to the contrary, namely that the factors that cause ER and mitochondrial motion modulate changes in calcium, rather than the changes in calcium modulating motility. Cross-correlation analysis thus reveals that the peak calcium concentration follows peaks growth rate [Messerli et al., 2000], whereas apical ER motion, as shown herein, precedes and anticipates growth.

Based on published information we can begin to construct a scheme that explains apical motion of ER and mitochondria, and ultimately the regulation of oscillatory growth. While all the underlying factors are clearly not known there are several that fit together in a cohesive unit. Firstly, we draw attention to the generation of the alkaline band, which also anticipates growth and may contribute to actin turnover through the pH sensitive actin binding protein, ADF [Lovy-Wheeler et al., 2006]. The resulting remodeling and stimulated polymerization of the cortical actin fringe occupies a key link between tube growth and the actin cytoskeleton [Gibbon et al., 1999; Vidali et al., 2001]. Here we would further argue that the polymerization of the actin fringe allows the forward motion of myosin and its bound cargo (including the ER and mitochondria). Secondly, recent evidence shows that Rops, a family of small G proteins, occupy a position on the plasma membrane near the pollen tube apex, and importantly they oscillate, with evidence for a component that anticipates growth [Hwang et al., 2005]. Rops control actin dynamics, and because actin also exhibits oscillatory behavior that precedes growth [Fu et al., 2001], this further supports the idea that actin polymerization may play a primary role in the control of pollen tube growth. Finally, energy levels, as revealed through imaging of NAD(P)H, oscillate [Cárdenas et al., 2006]. At least one component (possibly $NADP^+$) anticipates growth and its presence may be coupled to ATP production. ATP occupies a pivotal position in all the circumstances noted above; it will be particularly important to all the motile processes described herein because myosin is an ATPase, and because actin turnover will be sensitive to ATP/ADP ratios.

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