Dynamics of the apical vesicle accumulation and the rate of growth are related in individual pollen tubes

R. M. Parton*, S. Fischer-Parton, M. K. Watahiki and A. J. Trewavas
Institute of Cell and Molecular Biology, University of Edinburgh, Mayfield Road, Edinburgh, EH9 3JH, UK
*Author for correspondence (e-mail: rparton@srv0.bio.ed.ac.uk)

Accepted 12 April 2001

SUMMARY

Regulated secretory vesicle delivery, vesicle fusion and rapid membrane recycling are all contentious issues with respect to tip growth in plant, fungal and animal cells. To examine the organisation and dynamics of membrane movements at the growing pollen tube apex and address the question of their relationship to growth, we have used the membrane stain FM4-64 both as a structural marker and as a quantitative assay. Labelling of living Lilium Longiflorum pollen tubes by FM4-64 resulted in a distinct staining pattern in the tube apex, which corresponds spatially to the previously identified cone-shaped ‘apical clear zone’ containing secretory vesicles. Dye uptake could be inhibited by sodium azide and followed a strict temporal sequence from the plasma membrane to a population of small (1-2 μm diameter) discrete internal structures, with subsequent appearance of dye in the apical region and ultimately in vacuolar membranes. Washout of the dye rapidly removed the plasma membrane staining, which was followed by a gradual decline in the apical fluorescence over more than an hour. Injected aqueous FM4-64 solution showed a relatively even distribution within the pollen tube. Association of FM4-64 with apical secretory vesicles was supported by the effects of the inhibitors Brefeldin-A and Cytochalasin-D, which are known to affect the localisation and number of such vesicles, on the FM4-64 staining pattern. Examination of the dynamics of FM4-64 labelling in the pollen tube tip by time-lapse observation, supported by fluorescence-recovery-after-photobleaching (FRAP) analysis, suggested the possibility of distinct pathways of bulk membrane movement both towards and, significantly, away from the apex. Quantitative analysis of FM4-64 distribution in the apex revealed that fluctuations in fluorescence 5 to 10 μm subapically, and to a lesser extent the apical 3 μm, could be related to the periodic oscillation in pollen tube growth rate. This data reveals a quantitative relationship between FM4-64 staining and growth rate within an individual tube.

Key words: Tip growth, Oscillation, Pollen tube, Polarity, Vesicles, FM4-64

INTRODUCTION

Pollen tube extension is an excellent example of polarised growth and provides an ideal model system for understanding the processes of organisation and regulation involved. Central to this growth, and to the control of growth rate and orientation, are the processes of localised delivery to and secretion (exocytosis) of cell wall material and membrane at the tube apex (Messerli et al., 2000; Li et al., 1999; Roy et al., 1999; Holdaway-Clarke et al., 1997; Malhó and Trewavas, 1996; Derksen et al., 1995; Steer and Steer, 1989).

At the pollen tube tip Golgi-derived secretory vesicles containing cell wall materials are accumulated in a particular region of the pollen tube apex (defined in Lancelle and Hepler, 1992, as the apical clear zone) prior to fusion with the apical plasma membrane (Heslop-Harrison, 1987; Picton and Steer, 1982; Picton and Steer, 1983; Van Der Woude et al., 1971). Studies to date on pollen tube growth suggest that secretory vesicle production and delivery to the clear zone are not regulated with respect to the average growth rate (Roy et al., 1998; Picton and Steer, 1981; Picton and Steer, 1982; Picton and Steer, 1983). Furthermore, it has been estimated that membrane incorporation at the apex exceeds the increase in membrane area required for tip extension and hence a requirement for active membrane recovery from the tip was proposed (Steer and Steer, 1989). Membrane uptake in the pollen tube tip (endocytosis) has been supported by the discovery of clathrin-coated vesicles (Derksen et al., 1995), the localisation of clathrin (Blackbourn and Jackson, 1996) and uptake of cell impermeant fluorescent markers (O’Driscoll et al., 1993). Whether membrane uptake is localised at the extreme apex or in a region behind the clear zone is still undecided.

How the balance between secretory vesicle delivery, membrane recovery and growth rate is organised has yet to be fully understood, especially with the discovery that tip extension rate in the pollen tube is not constant but varies in an oscillatory fashion (Feijó et al., 2001). A correlation between oscillatory fluctuations in growth rate and oscillatory fluctuations in the magnitude of the tip-focused calcium gradient has been established; the latter was proposed as the mechanism regulating secretory vesicle delivery at the apex (Messerli et al., 2000; Holdaway-Clarke et al., 1997).

Work in a variety of experimental systems has led to a better understanding of the mechanisms of exocytotic and endocytic pathways (Zheng and Yang, 2000; Edwardson, 1998; Pelham, 1997). However, understanding the dynamics of membrane delivery to and recycling from the apical plasma membrane in
actively tip-growing systems has to some extent been hampered by the lack of an adequate means to visualise and track these processes in action. Dyes such as FM4-64 \([N-\(3\)-triethylammoniumpropyl\)-4-(6-(4-(diethylamino) phenyl hexatrienyl) pyridinium dibromide] promise the possibility of a new perspective on such dynamic activities at the growing apex. Such amphiphilic fluorescent dyes have been developed with animal cell systems as tools for the study of membrane trafficking in living cells (Bet et al., 1996). FM4-64 acts as a membrane marker, being only weakly fluorescent in water with a quantum yield that increases dramatically when associated with membranes (Bet z et al., 1996). This dye has been used in yeast to follow membrane uptake and transport to the vacuole (Vida and Emr, 1995). More recently, the application of FM4-64 to track membrane trafficking in tip growing plant and fungal cells has emerged (Belanger and Quatrano, 2000; Fischer-Parton et al., 2000; Hoffmann and Mendgen, 1998).

A possible problem with the use of FM-dyes is the ambiguity in their mode of internalisation by certain cell types and subsequently their internal localisation (Fischer-Parton et al., 2000; Nishikawa and Sasaki, 1996). We have addressed this issue in pollen tubes and present evidence of an endocytic uptake mechanism and localisation to membrane vesicles at the pollen tube apex. Analysis of FM4-64 staining in growing pollen tubes reveals a distinct apical staining pattern, dynamic movements of FM4-64 stained material and a quantitative relationship with growth rate in individual tubes. The significance of these observations is discussed in relation to the role of the apical vesicle accumulation in pollen tube extension.

MATERIALS AND METHODS

Chemicals and materials

Chemicals for culture media were obtained from BDH Chemical Co. (Poole, UK) or Fisher Scientific (Loughborough, UK) unless otherwise stated. Dyes and inhibitors were obtained from Molecular Probes Europe (Leiden, Netherlands) and were prepared as directed by the manufacturer.

Plant material

Pollen of \(L.\) longiflorum was collected from freshly cut flowers obtained locally. Anthers were dried at room temperature for two days then vortexed vigorously to release the pollen, which was aliquoted, frozen and stored at \(-80^\circ\) C.

Pollen culture and handling

Pollen was imbibed for 5 minutes in culture medium (modified from Feijó et al., 1999): 1.6 mM \(H_2BO_3\), 2.0 mM \(CaCl_2\), 1.0 mM KCl, 5% sucrose, 0.05 mM MES before being transferred to thin layers of 0.2% Gellangum (from Wako, Osaka, Japan) solidified culture medium. Thin gel layers (optimised for imaging) were prepared on washed No. 2686 \(L.\) longiflorum pollen tubes

RESULTS

FM4-64 displays a distinct staining pattern in \(L.\) longiflorum pollen tubes

In median confocal optical section (Fig. 1A), FM4-64 consistently produced a distinct bright peripheral staining and bright, V-shaped apical staining. The brightness of the peripheral staining was dependent upon the availability of extracellular dye and diminished with increasing time after dye application. Peripheral staining was shown to be plasma-membrane-associated, not cell-wall associated, by plasmolysis in the presence of FM4-64 (Fig. 2). Growth of \(L.\) longiflorum pollen tubes was unaffected by the presence of FM4-64 at the concentrations used (continuing at normal growth rates of 5-25 \(\mu\)m/minute) and, although growth rate diminished with time, it continued for up to 48 hours. Pollen tubes of \(N.\) tabacum, \(N.\) Plumbaginifolia and \(A.\) umbellatus showed similar FM4-64 staining patterns to \(L.\) longiflorum (data not shown).

Apical FM4-64 staining of \(L.\) longiflorum pollen tubes coincided approximately with the area known commonly as the ‘apical clear zone’, described previously (Lancelle and Hepler, 1992) as a vesicle rich V-shaped region of the apex excluding large organelles (Fig. 1; Fig. 3). Careful analysis of many median optical section confocal images of FM4-64 fluorescence in growing tubes consistently revealed a pattern of a sharply defined high signal in the extreme apical 3-5 \(\mu\)m region. Beyond this region, FM4-64 staining was generally lower and varied considerably in both fluorescence signal and distribution (Fig. 1; Fig. 3). Examination of FM4-64 staining in three dimensions by rapid confocal optical sectioning
Vesicle accumulation and growth rate in pollen through different focal planes (Z-series) showed the radial symmetry of pollen tubes. Rather than a simple cone shape, FM4-64 staining defined a distinct, bright, lens-shaped apical region with a dimmer, much less distinct region of staining more subapically, often extending considerably away from the apex as a narrow 'tail' (Fig. 1; Fig. 3). In many images an area of reduced staining could be observed that separated the extreme apical region and more subapical regions of staining.

**FM4-64 shows time- and energy-dependent internalisation**

Uptake of FM4-64 into *L. longiflorum* pollen tubes followed a strict time sequence (Fig. 4; Fig. 5). Immediately after dye application, staining associated with the plasma membrane was observed. Within 1-2 minutes, clear dye internalisation could be discerned that was associated with small, near-spherical structures located behind the apex (Fig. 4B,C). These structures moved towards the tip with the cytoplasmic streaming but tended to be excluded from the extreme apical region; they persisted as long as high concentrations of external...
dye were present (indicated by bright peripheral staining). During the following 3-10 minutes a fainter, more diffuse, internal staining could be seen, initially behind the extreme apex but soon spreading to the whole apical 20-25 μm region. Only after 10-15 minutes did the typical FM4-64 staining (described in Fig. 1) become apparent (Fig. 4H-J; Fig. 5A,B). Staining intensity reached a relatively stable state ~30 minutes after dye application. By this time peripheral staining was reduced relative to the internal fluorescence and the early staining spherical structures were more difficult to distinguish. Further application of dye at this point increased peripheral staining and resulted in a ‘reappearance’ of the spherical structures seen earlier (data not shown). It is likely that the free external FM4-64 concentration is reduced with increasing time after application as the dye becomes sequestered or diluted.

From 30-60 minutes, the staining pattern was indistinguishable from that achieved with L. longiflorum pollen tubes cultured for 4-7 hours after FM4-64 applied to imbibed spores (see Materials and Methods; Fig. 1A) and did not appreciably differ over the following 12 hours. By ca. 24 hours a distinct staining of subapical structures, possibly vacuolar membranes, had developed (Fig. 5C,D). The apical staining pattern, although somewhat fainter, was still visible, whereas the peripheral

(plasma membrane) staining could not be easily distinguished from internal fluorescence levels.

Introduction of FM4-64 into pollen tubes by microinjection produced a different staining pattern to that described above (Fig. 6). Injected dye spread slowly from the site of injection to give a general fluorescence with no distinctive localisation. No bright peripheral staining was seen. Despite the absence of external dye, the internal dye staining persisted, diminishing only gradually with time (>30 minutes) after injection.

Uptake of FM4-64 was inhibited in a concentration-dependent manner by the metabolic inhibitor sodium azide (Fig. 7). Concentrations of azide that arrested growth but did not halt cytoplasmic streaming allowed uptake to progress at a reduced rate (Fig. 7A), whereas higher concentrations (Fig. 7B), which inhibited both growth and streaming, effectively halted uptake.

Removal of FM4-64 from pollen tubes could be achieved by continuous perfusion with dye-free medium (Fig. 8). Dye was rapidly removed from the PM but persisted for >2 hours within the apical region, declining slowly with time. Removal of dye from different regions of internal staining all showed similar kinetics of decline in fluorescence intensity (Fig. 8B).
Vesicle accumulation and growth rate in pollen

Inhibitors affecting tip growth redistribute the FM4-64 staining pattern

Two well-characterised inhibitors known to affect tip growth in pollen tubes, in particular the number and distribution of secretory vesicles, were tested on FM4-64-loaded tubes to assess the effect on dye distribution.

Brefeldin A (BFA) treatment consistently disrupted the FM4-64 staining pattern and arrested growth in L. longiflorum pollen tubes (Fig. 9). The timing of growth arrest was dependent on the concentration applied, with higher concentrations acting faster (Fig. 9B). The decline in growth with inhibitor concentrations ≥3.6 μM was generally rapid after onset and coincided with the disappearance of the bright apical FM4-64 staining (Fig. 9B; 3.5-5.5 minutes). Brefeldin A was co-applied with the BFA-Bodipy FL conjugate, which acted as a useful marker of BFA uptake after application. Staining with this dye was very rapid, appearing within the first minute after application (data not shown). After the disappearance of bright apical FM4-64 staining, a weaker accumulation of stained material emerged at the apex (Fig. 9B, from 6.5 minutes), matching reports of the development of an apical aggregation of membrane material (Rutten and Knuiman, 1993).

Cytochalasin D (0.5-1.5 μM) treatment of L. longiflorum pollen tubes produced distorted and reduced growth within 2 minutes of application (Fig. 10). Co-incident with this was the loss of the normal cytoplasmic streaming pattern and both rapid and severe disruption in the pattern of FM4-64 staining. Dissociation and dissipation of FM4-64 staining rapidly progressed throughout the apex (Fig. 10; 25-55 seconds). Within 5-10 minutes after application, FM4-64 staining had largely redistributed from the apex and became scattered in patches over more subapical regions. By this time growth was almost completely arrested and streaming in the apical compartment was reduced to discontinuous and erratic movements.

FM4-64 reveals bulk membrane flow away from the apex

The pathway of cytoplasmic streaming is well known in pollen tubes (Heslop-Harrison, 1987) and follows a ‘reverse fountain’, modelled in Fig. 11J from time-lapse imaging (data not shown) of the trajectories of large lipid storage vacuoles, amyloplasts and fluorescently labelled mitochondria. By studying time-lapse confocal image sequences of FM4-64-stained growing pollen tubes at rates of between one and four frames per second, the movements of FM4-64-stained material could also be seen. Most obvious was the progression of ‘brighter’ patches of FM4-64-stained material from close

---

Fig. 6. Injection of aqueous FM4-64 into a L. longiflorum pollen tube. Insert shows the bright field image at 1/3 size. (A) Tip region; (B) subapical region. Bar, 15 μm.

Fig. 7. Effects of sodium azide on FM4-64 uptake by L. longiflorum pollen tubes. (A) 30 minutes after applying 2 μM dye to a tube pre-treated for 2 minutes with 500 μM sodium azide. (B) Similar treatment as in A with 1 mM sodium azide pre-treatment. Dye was applied in the continued presence of inhibitor. Inserts show bright field images at 1/3 size. Bar, 15 μm.

Fig. 8. FM4-64-washout time course from a growing pollen tube of L. longiflorum preloaded with dye for 3 hours. Washout was achieved by continuous perfusion with dye-free medium. (A) Selected images showing the pattern of decline in fluorescence; times relative to the start of perfusion are given in minutes. (B) Plots of normalised fluorescence intensity within four regions, defined in A by the areas a-d: (a) extreme apex, (b) subapical region, (c) plasma membrane (plotted as c minus d) and (d) periphery. Bar, 15 μm.
behind the extreme apical 3-5 μm stained region away from the tip along the reverse cytoplasmic flow at the centre of the tube, which could be followed over several frames (Fig. 11A-C,J-K). An apparent ‘cycling’ of patches of FM4-64-stained material from >10 μm from the extreme apex back towards the extreme apical region was also occasionally seen, appearing as an ‘eddy’ in the general movement away from the tip (Fig. 11D-K). Movement of individual vesicles towards the apex could not be seen using confocal fluorescence imaging; individual vesicles were subresolution but would contribute to the low-level background fluorescence outside the brightly stained apical V-shaped region. However, in video sequences small patches of FM4-64-stained material could be clearly seen moving along the tube periphery towards the apex.

To track the movements of FM4-64-stained material, the technique of FRAP was employed. Irradiating the region 20-30 μm behind the tip resulted in an area of bleaching, which was invariably displaced away from the apex along the pathway of reverse flow, in agreement with the earlier time-course observations (Fig. 12A,B). Moderate photobleaching treatment of regions along each flank behind the apex (lateral region) produced a rapid transient reduction in the already weak fluorescence of that region. It also subsequently caused a transient reduction in the fluorescence intensity within the bright apical 3-5 μm region and in more subapical regions, without stopping growth (Fig. 12C,D). Irradiating growing tubes within the extreme apical 5 μm region, with laser intensities sufficient to cause bleaching in other regions (or at the apex of growth-arrested tubes), failed to produce distinct bleached ‘spots’.

**FM4-64 staining fluctuations suggests a possible correlation with growth fluctuations in individual pollen tubes**

The typical FM4-64 staining pattern was only seen in normally growing pollen tubes. Staining pattern was similar in both young (<700 μm long) and old (>1000 μm long) tubes and over a range of growth rates from 5 to >20 μm/minute. No obvious correlation between growth rate and staining pattern within populations of tubes was found. In non-growing or stressed pollen tubes the apical FM4-64 stained region was variously disorganised or absent (data not shown).

In individual pollen tubes >3 hours after germination, the growth rate fluctuated in a regular oscillatory fashion (Fig. 13A) with a period between 35 and 45 seconds. Quantitative analysis of FM4-64 staining intensity in median section confocal fluorescence images (see Fig. 1; Fig. 13E) taken at regular time intervals revealed that the FM4-64 fluorescence signal also varied in a regular oscillatory manner with a similar period to the oscillatory growth-rate fluctuations (Fig. 13A-D). When examined as video clips, time-lapse images showed that the previously described movements of material from behind the apical 3-5 μm region accounted for the fluctuations in FM4-64 signal variation, which in turn could be related to the fluctuations in growth rate. The most obvious variation in fluorescence was found in the region between 5 and 15 μm behind the extreme apex. The apical 1-3 μm sample area showed a low-amplitude, less-clearly-defined variation in fluorescence. Slight differences in magnitude of the growth-rate fluctuations could be related to variations in peak fluorescence intensity. This suggested that the phase of the oscillation in FM4-64 fluorescence was shifted relative to the

**Fig. 9.** Time course of changes in growth rate and dye distribution of FM4-64-loaded *L. longiflorum* pollen tubes treated with BFA. (A) Confocal fluorescence images of a pollen tube at different times (in minutes) following treatment with 3.6 μM BFA. (B) Typical growth rates for tubes treated with 0.36, 3.6, 18 and 36 μM BFA. An arrow indicates addition of BFA. Bar, 15 μm.

<table>
<thead>
<tr>
<th>BFA concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ 36.0 μM</td>
</tr>
<tr>
<td>▲ 18.0 μM</td>
</tr>
<tr>
<td>● 3.6 μM</td>
</tr>
<tr>
<td>▼ 1.8 μM</td>
</tr>
</tbody>
</table>

**Fig. 10.** The effects of Cytochalasin D treatment on the FM4-64 staining pattern of an *L. longiflorum* pollen tube; times are in seconds. Bar, 15 μm.
Fig. 11. Movement of FM4-64-stained material in the apex of a *L. longiflorum* pollen tube (average growth rate 21.9 μm/minute). (A-C) Median section confocal images taken 2 seconds apart showing bulk movement of FM4-64-stained material from a region ~5 μm behind the apex away from the tip along the path of cytoplasmic streaming (arrows). (D-I) Images from the same time series, taken 1 second apart, showing movement of FM4-64-stained material from a region ~25 μm subapically back towards the apex (arrows). Diagrammatic interpretation of the ‘reverse fountain’ path of cytoplasmic streaming in a growing *L. longiflorum* pollen tube (J) (see main text), and of the movement of FM4-64-stained material based upon time-sequence imaging (see A-I) and FRAP experiments (see text for details) (K). Bar, 20 μm.

Fig. 12. Fluorescence recovery after photobleaching (FRAP) analysis showing the direction of movement of FM4-64-stained material around the pollen tube. (A,B) Movement from the apex down the centre of the cell to more subapical regions. (A) Time sequence of confocal fluorescence images; time relative to bleaching is in seconds. The original area of bleaching is shown as the boxed area. (B) Pixel intensity plots along a central transect through the images in A. The original bleached zone is indicated as the boxed area. The relative position of the original bleached zone to the apex is shown as the dashed boxed area; graphs have been aligned from the cell apex. (C,D) Movement from subapical peripheral regions into the apex. (C) Time sequence of confocal fluorescence images; the first image indicates the lateral bleaching areas (box a). (D) Graphs of fluorescence intensity against time for the areas indicated a-c. The relative position of the sample areas to the apex, shown in the first image of (C), was maintained throughout. The grey shaded area indicates time of bleaching. Bars, 15 μm.
variation in growth rate, with the peak FM4-64 fluorescence preceding peak growth rate by 5-10 seconds. It was also noted that ‘isolation’ of the apical 3-5 µm region is most distinctly defined from the more subapical FM4-64 staining (see Results and F). (E) Sample areas A-C: apical 1-3 µm, 5 µm and 10 µm subapically, respectively. (F) Graphs of pixel values along a central transect of the pollen tube are shown (as in Fig. 1) for images covering the growth period outlined by the cross-hatched area in A. The maximum and minimum rate growth phases of the period are indicated.

Growth on high sucrose medium (15%) caused a reproducible reduction in growth and slower cytoplasmic streaming (as described by Li et al., 1996), a shorter apical clear zone and also an appreciable alteration in the apical FM4-64 staining pattern (Fig. 14A). FM4-64 staining distribution was significantly reduced, less extended subapically (lacking a significant tail) and showed far less distinct ‘reverse’ movement of material from behind the apex into more subapical regions.

Growth on high calcium medium (25-10 mM) caused delayed germination and slow growth (<1 to ~5 µm/minute) with thickening of the apical cell wall (Fig. 14B). Despite the considerably slowed growth, FM4-64 staining under these conditions did not differ greatly from that observed with normal growth.

**DISCUSSION**

In this study we investigated dynamic membrane movements at the pollen tube apex in relation to tip growth, based upon FM4-64 staining. In order to establish FM4-64 staining as a marker of membrane movement, the pathway of FM4-64 internalisation and its distribution within the pollen tube were investigated. Subsequent analysis of FM4-64 staining pattern in the growing pollen tube revealed a quantitative relationship with oscillations in growth rate.

**Endocytic internalisation of FM4-64 in the pollen tube**

In the current study we used FM4-64 as a tracer of membrane movement in the pollen tube apex. In other cell systems this and other related FM-dyes have been used to follow both vesicle trafficking and endocytosis (Cochilla et al., 1999).

Our data showed a distinct intracellular localisation of FM4-64 staining, which corresponds strikingly, in both location and distribution, to the accumulation of exocytotic vesicles containing cell wall material within the apical clear zone (defined by EM studies of different pollen tube species (Derksen et al., 1995; Lancelle and Hepler, 1992)).
Examining the time-course of dye uptake suggested that dye is internalised from the plasma membrane by a pathway involving particular endomembrane compartments: an early uptake compartment (possibly endosomes or the Golgi apparatus), apical vesicle membranes and ultimately the vacuolar membrane. A similar sequence of uptake has been observed in filamentous fungi (Fischer-Parton et al., 2000) and in yeast, where endocytic uptake of the dye has been established (Vida and Emr, 1995). In animal cells localisation of membrane marker dyes internalised by endocytosis to particular endomembranes by endogenous ‘sorting’ mechanisms has been reported (Murkerjee et al., 1999). Such a mechanism acting to direct FM-dye to particular endomembranes could account for the apparently restricted localisation observed in pollen tubes. Furthermore, the apex and apical vesicle cloud are sites of considerable membrane flux, whereas the vacuole membrane would be expected to be less ‘active’ explaining its slower FM4-64 accumulation.

Here we have shown that FM4-64 entry does not proceed by unfacilitated diffusion (Fig. 7). It has, however, been suggested that internalisation of FM-dyes may be mediated by alternative mechanisms, specifically, the activity of ‘flippases’ (Fischer-Parton et al., 2000) or mechanosensitive cation channels (Nishikawa and Sasaki, 1996). Both of these mechanisms could allow ‘free’ dye in the cytoplasm and dissociate dye distribution from vesicle trafficking. Injection of free dye into the cell (Fig. 6) produced a drastically different intracellular localisation compared to external application, arguing against such entry mechanisms. To date there is no evidence that flippase enzymes are able to translocate FM-dyes across the lipid bilayer. Dye entry by a mechanosensitive cation channel (Nishikawa and Sasaki, 1996) could also be argued against on the grounds that: (1) contrary to earlier work (Nishikawa and Sasaki, 1996), high external divalent cation concentration did not inhibit dye uptake (Fig. 14B,C); and (2) in the pollen tube such channels are most likely to be active at the pollen tube apex, yet the dye uptake sequence showed that dye internalisation proceeded from sites behind the apex. Washout of dye from a loaded pollen tube (Fig. 8) shows that the V-shaped apical labelling is not the product of continual dye influx from the extreme apex, as this staining pattern persisted well after external dye removal.

**Exocytotic versus endocytic vesicles at the pollen tube apex**

The predominant FM4-64 labelling in the pollen tube coincides with a region that consists largely of Golgi-derived membrane vesicles (Lancelle and Hepler, 1992). Thus, the observed staining pattern would be consistent with subapical endocytic uptake of the dye and redistribution through trafficking of stained vesicles predominantly to the apex, arguably the main region of membrane flux in the growing tube. FM-dye has previously been found to be associated with exocytotic vesicles in other tip growing cells (Fischer-Parton et al., 2000; Belanger and Quatrano, 2000; Hoffmann and Mendgen, 1998; Betz et al., 1996).

Brefeldin A, which inhibits anterograde ER to Golgi traffic, and Cytochalasin D, which disrupts the actin cytoskeleton (Geitman et al., 1996 and references cited therein; Rutten and Knuiman, 1993; Picton and Steer, 1981), have effects on the number and distribution of secretory vesicles in pollen tubes and are well characterised by electron microscopy. These inhibitors produced characteristic effects on FM4-64 staining pattern (Fig. 9; Fig. 10) consistent with labelling Golgi-derived secretory vesicles.

Although current observations support the contention that FM4-64 labels secretory vesicles in the pollen tube apex, reporting their distribution and the extent of their accumulation, it is also possible that FM4-64 is present in endocytic vesicles. Endocytosis is now recognised as a requirement for pollen tube tip growth (Steer and Steer, 1989) and evidence to show this is already accumulating (Blackbourn and Jackson, 1996; Derksen et al., 1995; O’Driscoll and Steer, 1993).

Endocytosis of membrane could offer an explanation for the observed movement of FM4-64-stained material away from the tip. However, this explanation does not fit in with current observations on the sequence of dye internalisation, dye washout or, equally significantly, the effects of restricting growth rate with high external sucrose or calcium conditions. According to previous studies (Roy et al., 1998; Picton and Steer, 1983; 1982), vesicle production and delivery to the apex occur at the same rates in normally growing tubes and under conditions of slow growth. This implies that the rate of membrane recycling would be increased under slow growth conditions. However, when we applied the two different restrictive growth conditions (Fig. 14; high calcium or sucrose), FM4-64 staining at the apex was either unchanged or diminished. The lack of increased FM4-64 staining under these conditions suggests that endocytosed material does not contribute significantly to the pool of FM4-64-stained material observed moving away from the apex.

More recently, the concept of rapid membrane recycling by a ‘kiss and run’ mechanism has emerged from work on nerve terminals (Stevens and Williams, 2000). In this model, secretory vesicles do not completely integrate with the target membrane but release their contents through a short-lived fusion pore before detaching. This mechanism of rapid endocytosis is suggested to ‘avoid’ uptake of FM-series endocytic markers because of the short lifetime of the fusion pore, which exposes the vesicle membrane only briefly to the external pool of dye (Cousin and Robinson, 2000; Stevens and...
Williams, 2000). Such a mechanism could conceivably allow rapid endocytosis of membrane at the pollen tube apex without it being reported by FM4-64 staining of the endocytic vesicles.

**Dynamics of the apical vesicle accumulation are related to the rate of growth**

Examination of many FM4-64-loaded pollen tubes showed a clear relationship between the V-shaped apical staining pattern and normal growth. However, no obvious relationship could be found between the different growth speeds of ‘healthy’ individuals within a population and FM4-64 staining pattern.

The apparent presence of distinct regions of FM4-64 staining within the V-shaped (cone shaped in three dimensions) apical region of secretory vesicles (Fig. 1; Fig. 3; Fig. 11; Fig. 12) and the movements of material within these regions observed here (Fig. 11; Fig. 12) suggest both an underlying structural basis and a relevance to the process of tip growth. The region occupying the apical 3-5 μm region of the tip shows a superficial correspondence to the region of high calcium at the pollen tube apex defined by Pierson et al. (Pierson et al., 1996) and is believed to regulate vesicle fusion to the apex. Also reported is the existence of an actin ring near the apex of growing tobacco pollen tubes (Kost et al., 1998), which would define such an apical region within the overall V-shaped apical vesicle cloud region.

The importance of our observations of apparently distinct regions in the apical FM4-64 staining and distinct patterns of movement of FM4-64 staining is revealed in the further observation that a strict quantitative relationship exists for individual growing cells between FM4-64 signal and the regular oscillatory growth-rate fluctuations that are a feature of *L. longiflorum* pollen tube growth (Fig. 13). Interestingly, more subapical regions of FM4-64 staining gave the most dynamic variation in signal and showed the clearest relationship to the growth rate fluctuation (Fig. 13A-D).

The exact correlation between FM4-64 staining and growth fluctuations still remains to be determined by statistical analysis. However, it appears that peak growth rates coincide with a decline in the FM4-64 signal immediately behind the apical 3-5 μm region (Fig. 13F). This corresponds to a particular phase of the movement of FM4-64-stained material from behind the apical 3-5 μm apical region to more subapical regions (see Fig. 11).

Any model that seeks to establish the relationship between the dynamics of the FM4-64 apical staining pattern and oscillatory growth of individual tubes needs to take account of the observations of a possible underlying structural organisation to the apical FM4-64 staining and the distinct pattern of movement of material reported here. We speculate that the observed movement of material may be simply the ‘cycling’ of an excess of secretory vesicles delivered to the apex in between the rounds of maximum tip extension. It is possible that at the maximum rate of tip extension the consumption of vesicles is high. Correspondingly, at the lower growth rate fewer vesicles are used, which results in an ‘overspill’ of the excess secretory vesicles, which pass back into the cytoplasmic stream to be eventually returned to the apex. This model fits in with the current understanding that the rate at which secretory vesicles are produced and delivered to the tip is not regulated with respect to the rate of tip extension. It also implies that the apical 3-5 μm region is a pool of limited capacity and as such may act to regulate the rate of vesicle delivery to the apex.

The research was supported by BBSRC postdoctoral fellowships (to RMP and SF-P) and an HFSP postdoctoral fellowship (to MKW).

**REFERENCES**


