Endo/exocytosis in the pollen tube apex is differentially regulated by Ca\(^{2+}\) and GTPases

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Abstract

Pollen tube growth relies on an extremely fast delivery of new membrane and wall material to the apical region where growth takes place. Despite the obvious meaning of this fact, the mechanisms that control this process remain very much unknown. It has previously been shown that apical growth is regulated by cytosolic free calcium ([Ca\(^{2+}\)]\(_c\)) so it was decided to test how changes in [Ca\(^{2+}\)]\(_c\) affect endo/exocytosis in pollen tube growth and reorientation. The endo/exocytosis was assayed in living cells using confocal imaging of FM 1-43. It was found that growing pollen tubes exhibited a higher endo/exocytosis activity in the apical region whereas in non-growing cells FM 1-43 is uniformly distributed. During pollen tube reorientation, a spatial redistribution of exocytotic activity was observed with the highest fluorescence in the side to which the cell will bend. Localized increases in [Ca\(^{2+}\)]\(_c\) induced by photolysis of caged Ca\(^{2+}\) increased exocytosis. In order to find if [Ca\(^{2+}\)]\(_c\) changes were modulating endo/exocytosis directly or through a signalling cascade, tests were conducted to find how changes in GTP levels and GTPase activity (primary regulators of the secretory pathway) affect the apical [Ca\(^{2+}\)]\(_c\) gradient and endo/exocytosis. It was found that increases in GTP levels could promote exocytosis (and growth). Interestingly, the increase in [GTP] did not significantly affect [Ca\(^{2+}\)]\(_c\) distribution, thus suggesting that the apical endo/exocytosis is regulated in a concerted but differentiated manner by the Ca\(^{2+}\) gradient and the activity of GTPases. Rop GTPases are likely candidates to mediate the Ca\(^{2+}\)/GTP cross-talk as shown by knock-down experiments in growing pollen tubes.

Key words: Antisense, caged probes, calcium, endocytosis, exocytosis, GTP, pollen tubes, rop GTPase.

Introduction

Pollen tubes are among the most rapidly extending cells, growing up to 50 \(\mu\)m min\(^{-1}\). These growth rates are possible due to a highly polarized apical fusion of vesicles, which transport cell wall components to the growing tip (Steer and Steer, 1989). It was found some time ago that the quantity of membrane delivered by exocytosis was in excess for the pollen tube growth rate, suggesting an underlying recycling process (Picton and Steer, 1985). However, little is known about the molecular mechanisms involved in the regulation of exocytosis and membrane recycling in growing pollen tubes.

A tip-focused [Ca\(^{2+}\)]\(_c\) gradient is known to play a central role in the regulation of pollen tube growth and modulation of the [Ca\(^{2+}\)]\(_c\) concentration results in changes in the rate and direction of growth. (Pierson et al., 1994; Malhó and Trewavas, 1996). One of the targets of this Ca\(^{2+}\) signalling pathway was therefore claimed to be the secretory pathway (Malhó et al., 2000). However, up to this moment, only indirect evidence for this fact has been presented.

Picton and Steer (1985) have suggested that Ca\(^{2+}\) was also involved in the movement of vesicles towards the tip, fusion of vesicles with the plasma membrane, and the state of plasticity of the wall at the tip. Similar claims were made for GTP-binding proteins (Drigonová et al., 1996; Li et al., 1999; Lin et al., 1996). GTP-binding proteins act as molecular switch regulators, cycling between a GDP-bound (inactive) state and a GTP-bound (active) state and a large number of these G-proteins exert important functions in signalling transduction, namely in different trafficking steps on the secretory and endocytic pathways (Bar-Sagi
and Hall, 2000; Ridley, 2001). The Ras superfamily of small GTPases includes five families: Ras, Rab, Arf, Ran, and Rho. A Rab2 homologue, necessary for membrane traffic between ER and Golgi in mammalian cells (Tisdale et al., 1992), is present in Arabidopsis pollen grains (Moore et al., 1997) and was recently shown to be important for pollen tube growth (Cheung et al., 2002). Members of the Rho family with high homology to the Ras subfamily—Rop (Yang, 2002), have been implicated in the regulation of pollen tube growth and root hairs, possibly by interaction with the tip-focused [Ca$^{2+}$]$_c$ gradient (Lin et al., 1996; Lin and Yang, 1997; Molendijk et al., 2001) and with the phosphoinositol signalling pathway (Kost et al., 1999). It was therefore decided to investigate the effect of GTPase activity and [Ca$^{2+}$]$_c$ in membrane traffic in pollen tubes and to what extent, if any, the two signalling pathways are connected.

For this purpose, the fluorescent probe FM 1-43 has been used. This dye has been found increasingly useful in exploring the endo- and exocytosis mechanisms in a variety of biological models (Smith and Betz, 1996). FM 1-43 is a non-toxic, water-soluble dye, virtually non-fluorescent in aqueous medium, and is believed to insert into the outer leaflet of the surface membrane where it becomes fluorescent. Since the dye stains membranes in an activity-dependent manner, it has proved to be useful for studies of vesicle recycling, exocytosis and endocytosis (Cochilla et al., 1999). Therefore variations in the apical fluorescence of FM 1-43 were monitored while manipulating Ca$^{2+}$ and GTP levels with caged-probes. It was found that both activation of GTPase enzymes and increase in [Ca$^{2+}$]$_c$ stimulate secretion. Similarly, antisense inhibition of Rop GTPases diminish secretion. Furthermore, evidence was obtained for the existence of a concerted but differentiated regulation of secretion by GTPase activity and apical [Ca$^{2+}$]$_c$.

**Materials and methods**

**Plant material**

Pollen of *Agapanthus umbellatus* L’Her was harvested and stored at −80 °C. Pollen was germinated in *vitro* as described previously (Malhö et al., 1994), using a modified version of the Brewbaker and Kwack (1963) medium: 2.5% sucrose, 0.01% H$_3$BO$_3$, 0.02% MgCl$_2$, 0.02% CaCl$_2$, and 0.02% KCl, pH 6.0 at 27 °C.

**FM 1-43 labelling and imaging**

After 1 h germination, pollen tubes were labelled with FM 1-43 (Molecular Probes). The dye was added to the germination medium to a final concentration of 0.2 μM. This value was chosen because it resulted in high levels of labelling but no toxicity for pollen tubes; cells grew normally for >2 h in medium with such concentration of extracellular FM 1-43. Time-course images of FM 1-43 fluorescence were acquired with a Bio-Rad MCR-600 (Microscience Ltd, Hemel Hempstead, UK) confocal laser scanning microscope (CLSM), attached to an Olympus BX-51 upright microscope, using the BHS filter (excitation: 488 nm; dichroic reflector: 510 nm; barrier filter: 515 nm). Fluorescence was quantified in terms of average pixel intensity using the histogram command of COMOS/MPL software (Bio-Rad).

**Modulation of [Ca$^{2+}$]$_c$ and GTP levels**

Pollen tubes were loaded by pressure microinjection as described in Camacho et al. (2000) with one of the following probes (the concentration refers to the solution injected): (1) caged Ca$^{2+}$ (NITR-5 tetrasodium, 0.5 mM, Calbiochem) and the guanine nucleotide analogues (2) GDPβS (1 mM, Sigma-Aldrich) and (3) [S-(DMSNPE)-caged] GTP-γ-S (1 mM, Molecular Probes). The concentration of the solutions was chosen so that pollen tube growth was not arrested upon microinjection and release. Ca$^{2+}$ and GTPβS were released by exposing pollen tubes to a 2 s of UV light pulse produced by a 100 W mercury lamp via a 400 nm dichroic mirror and an Iris diaphragm. Controls involved exposing non-loaded tubes to the same amount of UV light (Malhö and Trewavas, 1996).

**[Ca$^{2+}$]$_c$ imaging**

Pollen tubes were microinjected with the Ca$^{2+}$-sensitive dye Calcium Green-1 and the Ca$^{2+}$-insensitive Rhodamine B, both linked to a 10 kDa dextran (1 mM, Molecular Probes). [Ca$^{2+}$]$_c$ ratio imaging was performed by CLSM in the dual channel mode (excitation: 488/568 nm; 520/630 nm double dichroic; barrier filter of 522 nm; barrier filter 2 of 585 nm). Fluorescence ratio images were calculated with the TCSM/MPL software (Bio-Rad) as described before (Camacho et al., 2000) and then quantified in terms of average pixel intensity (0–255 scale for 8 bit images). For calculation of [Ca$^{2+}$]$_c$, values, an in vitro calibration was used (Camacho et al., 2000).

**Data analysis**

Numerical analysis of the fluorescence images was performed using COMOS commands. Growth rates were calculated using Image-Pro® Plus 4.0 software (Media Cybernetics).

**Cloning of a Rop gene fragment**

Two degenerated oligonucleotides (R1- 5'-GTG GAC/T GGI GCI GTG GNN AAA/G AC-3' and R2- 5'-TA A/GTC C/TTT C/TTG ICC IGC NTT G/A/GTC-3'), in which I is inosine and N represents all four nucleotides) were used as PCR primers (Yang and Watson, 1993). Total RNA was isolated from germinated pollen by the phenol/SDS method and reverse transcribed using primer R1. The single-stranded cDNA was used as the template for PCR and amplification with primers R1 and R2. The amplification product (around 165 bp long) was cloned using the pT7Blue3-Perfectly BluntÔ Cloning Kit (Novagen) and sequenced for both strands using the ABI PRISMÔ Cloning Kit (PE Applied Biosystems). cDNA sequences were analysed using BLASTN software (Altschul et al., 1997).

The 3' end of Rop gene was obtained by 3'RACE (Frohman et al., 1988). The first strand of cDNA was synthesized using primer QR (5'-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GC (dT)$_3$-3') (Bespalova et al., 1998) and used as template for PCR amplification. Nested PCR was performed. The first PCR used the primers R1 and Q0 (5'-CCA GTG AGC AGA GTG ACG AGG ACT CGA GCT CAA GC (dT)$_3$-3') and the second amplification the primers RacF (5'-TCG TAC ACC AGC AAC ACT TTT -3') and Qi (5'-ACG AGG ACT CGA GCT CAA GC-3'). Cloning, sequencing and analysis of the amplification product were performed as described before.

**Knock-down of a Rop gene expression**

The expression of the Rop gene was reduced by loading pollen with antisense oligonucleotides (AS-ODN) complementary to the cloned AulRop cDNA fragment. The Rop2-AS antisense primer, that yielded the best results, was 16 bp long and modified in the last four bases of both the 5' and 3' terminus with phosphorothioate.
bonds, as indicated in lowercase: 5'-tga gCA TGC AGG tct t-3'.

Delivery of the AS-ODN to pollen was accomplished by combining the AS-ODN with the cationic lipid Cytofectin GS 3815 (Glen Research, Sterling, VA, USA). The cytofectin vesicles were prepared according to supplier’s instructions and mixed with AS-ODN to a final concentration of 15 μg ml⁻¹ lipid and 30 μM AS-ODN (Moutinho et al., 2001). After 15 min incubation at room temperature, the cytofectin/AS-ODN complex was diluted with 45 μl of growth medium and pollen was germinated in this supplemented medium. The controls performed were the absence of the cytofectin and/or of the AS-ODN, as well as transfection with cytofectin and sense ODN complex (Moutinho et al., 2001). Transmitted light images were captured through the CSLM fibre optic device. Length, diameter and growth rates were calculated for 20 pollen tubes per assay using Image-Pro® Plus 4.0 software (Media Cybernetics).

Results

FM 1-43 dye is a valid marker for studying endo/exocytosis

In order to understand how the endo/exocytosis is regulated in growing pollen tubes, the use of FM 1-43 as a marker to study membrane recycling in *Agapanthus umbellatus* was optimized. An external dye concentration of 0.2 μM was chosen, as it did not affect growth rate (data not shown) and yielded a good fluorescent signal.

FM 1-43 is highly fluorescent in a membrane environment. However, its permanent charge prevents the dye from passively crossing the membrane and so the loading into the cell is limited by an uptake from endocytic vesicles (Cochilla et al., 1999). Staining of the pollen tubes plasma membrane started immediately after the addition of FM 1-43 dye to the germination medium. This was followed by internalization of the dye, mainly in the tip region, and within a few minutes the typical staining pattern of the FM 1-43 dye was observed: a bright staining in all the apical region that extends to the sub-apical region with an inverted cone shape (Fig. 1A). The most fluorescent region corresponds to the so-called clear zone, a region of large organelle exclusion filled with secretory vesicles (Lancelle and Hepler, 1992). The staining pattern was smooth without large hotspots indicating that, mostly, near- and sub-resolution organelles (e.g. vesicles) were labelled. In non-growing pollen tubes, the fluorescence hotspot in the apical region was absent and fluorescence was almost confined to the plasma membrane without being internalized (Fig. 1B).

Pretreatment of pollen tubes with 100 μM sodium azide impaired dye uptake. At this concentration growth stops, but cytoplasmic streaming remains active. FM 1-43 associated with the plasma membrane, but was not internalized (Fig. 1C). This control excludes passive diffusion of dye into the cell (Parton et al., 2001) confirming the involvement of an endocytic mechanism in the internalization of FM 1-43. Dye release by exocytosis also took place as revealed by washout experiments. If FM 1-43 was removed from the medium, the labelling pattern was maintained, but fluorescence in the cell slowly decreased; the rate of this process varies from cell to cell with fluorescence persisting from 30 min to >90 min (data not shown).

**FM 1-43 as a marker of polar growth**

FM 1-43 was tested for its ability to act as a marker for the prediction of a new site of polarization. In pollen tubes with isodiametrical growth (e.g. induced by a series of electrical pulses; Malhó et al., 2000), FM 1-43 fluorescence was observed around all the apical region (Fig. 2). However, in pollen tubes that stopped and later recovered apical growth, a fluorescence hotspot became visible in the area where the new growing tip was about to emerge (Fig. 3A–D); when growth fully resumed, the typical inverted cone shaped apical staining of FM 1-43 was again observed (Fig. 3E, F). This fluorescence redistribution is likely to be a combination of new internalized dye and movement of previously stained vesicles.

A similar result was found when the pollen tubes reoriented their growth. Just prior to reorientation, FM 1-43 apical staining became asymmetric with the highest...
fluorescence associated to the side of the dome to which the tube will bend (Fig. 4B, C, F, G). When the tube ceases to curve, the apical hotspot was again re-centred in the growing tip (Fig. 4D–H).

Cytosolic calcium modulates endo/exocytosis

To understand the role of \([Ca^{2+}]_c\) in the processes of endo/exocytosis, apical FM 1-43 fluorescence was monitored while manipulating \([Ca^{2+}]_c\) levels. The \([Ca^{2+}]_c\) levels were increased in the tube cytoplasm by releasing caged Ca\(^{2+}\) (NITR-5). This allowed the monitoring of changes in the secretory process just before and after the increase in \([Ca^{2+}]_c\).

The increase of \([Ca^{2+}]_c\) upon release did not alter significantly the growth rates (\(n=9\); Table 1). One-third of the tubes slowed down the growth slightly (to about 97% of the original growth rate), while the remaining two-thirds increased the growth rate by 3% (results not shown). Despite the almost stable growth rate, apical FM 1-43 fluorescence was affected by the increase in \([Ca^{2+}]_c\). 75% of the pollen tubes had a decrease in apical fluorescence intensity (Fig. 5A). This reduction averaged 22% relative to the fluorescence value recorded prior to release; in the control cells, FM 1-43 fluorescence remained constant before and after the UV pulse (data not shown). These data indicate that changes in \([Ca^{2+}]_c\) which do not significantly affect growth rates may still affect the endo/exocytosis mechanism suggesting the existence of a different growth regulator mechanism.

Changes in GTPase activity lead to alterations in endo/exocytosis

In yeast and animal cells, several GTPases have been shown to modulate both endo- and/or exocytosis and it was decided to test if they play a similar role in pollen tubes. One way to modulate GTPase activity is by binding the GTPase enzymes to the guanine nucleotide analogues GTP\(^\gamma\)S and GDP\(^\beta\)S. The non-hydrolysable analogue of GTP (GTP\(^\gamma\)S) keeps G proteins (both ras-like monomeric and heteromeric) in a permanently active conformation, while the analogue of GDP (GDP\(^\beta\)S) renders the enzymes inactive. Therefore, the guanine nucleotide analogues were microinjected in pollen tubes preloaded with FM 1-43.

The caged release of GTP\(^\gamma\)S in the cytoplasm led to a significant increase in pollen tube growth rate with an average increase of 18% (\(n=19\); Table 1). By contrast, a microinjection of the inactivator GDP\(^\beta\)S led to a decrease in growth rate of about 29% (\(n=5\); Table 1). Photoinactivation of caged GTP\(^\gamma\)S led, in 88% of the cases, to a decrease in FM 1-43 apical fluorescence (Table 1). The decrease in fluorescence occurred in a sustained but sometimes (40% of the cases) quite abrupt way (Fig. 5B). The effect of GDP\(^\beta\)S could not be tested in this respect because a caged version was not available. These results strongly suggested that the process of

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**Fig. 3.** FM 1-43 staining distribution in a pollen tube that stopped and recovers its polarity. (A) Apex shortly after growth arrest; previous direction of growth (left side) is still the most fluorescent region. (B) Apex swells and a new growing apex starts to form; a fluorescence hotspot appears where the new growing tip is about to emerge (indicated by arrow) while fluorescence in the former apex decreases. (C) The polarized fusion events continue and (D) give rise to a new growing tip. (E, F) The new tip exhibits the typical inverted cone shape. Bar=10 \(\mu\)m.

**Fig. 4.** FM 1-43 staining distribution in a pollen tube changing growth direction. When the pollen tube is growing straight (A, E), FM 1-43 fluorescence is almost symmetrical, within the apical dome. Upon reorientation, a larger recruitment of vesicles to one side of the apical region occurs (B, C, F, G) and new cell wall and membrane is added in an asymmetrical way. Once a new axis of polarity has been established, the typical FM 1-43 apical staining is recovered (D, H). (I) The magnified apex of (G) (circled region) showing detail of the asymmetric distribution. The diagrams (J-L) illustrate the hypothesized distribution of the secretory vesicles in the apex of a reorienting pollen tube. Bar=10 \(\mu\)m.
endo/exocytosis in growing pollen tubes was controlled by GTPases.

\[ \text{Ca}^{2+} \]c and GTPases cross-talk

From the results described above, it was concluded that both GTPases and \([\text{Ca}^{2+}]_c\) are involved in the control of growth and membrane recycling in pollen tubes. In an attempt to unravel if both GTPases and \([\text{Ca}^{2+}]_c\) interact physiologically, caged GTP\(\gamma\)S was released in cells preloaded with the \(\text{Ca}^{2+}\)-sensitive dye Calcium Green-1 and the \(\text{Ca}^{2+}\)-insensitive dye Rhodamine B, both linked to a 10 kDa dextran molecule. This dye combination for \([\text{Ca}^{2+}]_c\) imaging has been previously assessed (Camacho et al., 2000) and found to be the one which produced more consistent results. Along with an increase in growth rate, release of GTP\(\gamma\)S induced an apical \([\text{Ca}^{2+}]_c\) decrease in 78% of the pollen tubes (\(n=16\); Fig. 6).

Identification of a Rop homologue and knock-down by antisense assay

In order to identify a GTPase involved in apical growth, PCR cloning and 3'\(\text{RACE}\) techniques were used to identify cDNAs encoding putative Rop proteins from germinated pollen. From the isolated clones, a fragment containing ~800 bp encoding an open reading frame of 187 aa including the stop codon was chosen. Analysis of the sequences using BLASTN program revealed the cDNA was 92% identical to the Rac subfamily of Rho GTPases from other monocot plants (barley, rice and maize). It has the three typical GTP binding sites, an effector region and

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**Table 1. Effect of NITR-5 (\(n=9\)), GDP\(\beta\)S (\(n=5\)) and GTP\(\gamma\)S (\(n=19\)) in pollen tube growth rate and on the average apical fluorescence of FM 1-43 dye (0–255 scale)**

In brackets is the percentage of variation relative to the initial value. Standard deviation values for the FM 1-43 fluorescence are not shown since these are independent.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Growth rate ((\mu)m s(^{-1}))</th>
<th>FM 1-43 apical fluorescence</th>
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<tr>
<td></td>
<td>Before</td>
<td>After</td>
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<tr>
<td>NITR-5 (Caged (\text{Ca}^{2+}))</td>
<td>0.36 ± 0.05</td>
<td>0.36 ± 0.05</td>
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<tr>
<td>Caged GTP(\gamma)S</td>
<td>0.33 ± 0.07</td>
<td>0.39 ± 0.07 (−18.18%)</td>
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<tr>
<td>GDP(\beta)S</td>
<td>0.35 ± 0.05</td>
<td>0.25 ± 0.06 (−29.38%)</td>
</tr>
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**Fig. 5.** Correlation between pollen tube growth and FM 1-43 apical staining before and after release of (A) caged \(\text{Ca}^{2+}\) and (B) caged GTP\(\gamma\)S; the UV pulse corresponding to the caged photolysis is indicated by a vertical bar. (A) Though the average growth rate is kept approximately unchanged, the increase in \([\text{Ca}^{2+}]_c\) results in a significant decrease of apical fluorescence. (B) The release of GTP\(\gamma\)S leads to an increase of the growth rate and decrease of the FM 1-43 fluorescence. Dotted line: growth rate (\(\mu\)m s\(^{-1}\)); solid line: FM 1-43 fluorescence (average intensity in a 0–255 scale).

**Fig. 6.** Correlation between pollen tube growth and apical \([\text{Ca}^{2+}]_c\) before and after release of caged GTP\(\gamma\)S; the UV pulse corresponding to the caged photolysis is indicated by a vertical bar. The increase of GTPase activity induced by GTP\(\gamma\)S leads to an increase in the growth rate and a slight decrease of \([\text{Ca}^{2+}]_c\). Dotted line: growth rate (\(\mu\)m s\(^{-1}\)); solid line: \([\text{Ca}^{2+}]_c\) fluorescence (average ratio intensity in a 0–255 scale).
the typical C-terminal CXXL motif, required for geranylation (boxes, Fig. 7).

Based on the cloned cDNA sequences, antisense oligonucleotides were designed transiently to inhibit the expression of the Rop protein and to investigate the effect of its knockdown in the growth of pollen tubes (Moutinho et al., 2001). During the first hour of germination, antisense-treated pollen tubes were similar to controls (absence of the cytofectin and/or of the AS-ODN; transfection with cytofectin and sense ODN complex), presenting an average growth rate of 0.37 ± 0.03 μm s⁻¹ and a tube diameter of 10.4 ± 0.43 μm. However, after that period, the antisense effect started to become visible in cells treated with RopAS2 oligonucleotides. Pollen tubes slowed down growth rate (0.10 ± 0.01 μm s⁻¹) and increased tip diameter (16.4 ± 3.0 μm) in contrast to the normal phenotype exhibited in the controls performed (Fig. 8A, B).

Antisense-treated pollen tubes also took up FM 1-43 dye from the germination medium, but at a much slower rate when compared to control cells (~10 min instead of a few seconds). Although fluorescence was still higher at the tip, the hotspot occupied the entire abnormally extended apex and was more confined to the membrane region (Fig. 8C, D).

In agreement with previous observations, the antisense-treated, but still growing, pollen tubes, revealed a highly localized tip-focused [Ca²⁺] gradient (Fig. 8E) which was also more confined to the membrane region than normal cells (Camacho et al., 2000). In pollen tubes more severely affected by the antisense treatment (e.g. totally lost apical growth), this [Ca²⁺] gradient could no longer be observed. Thus, the spatial definition of the [Ca²⁺] gradient seems also to depend on Rop protein expression.

**Discussion**

**The use of FM 1-43 to study membrane trafficking**

FM 1-43 proved to be a valid probe for monitoring the processes of endo/exocytosis in A. umbellatus pollen tubes. The rapid uptake suggests an extremely high rate of endocytosis and membrane traffic, as expected in such fast-growing cells. The dye uptake was inhibited by the metabolic inhibitor sodium azide confirming that FM 1-43 does not accumulate into the cytoplasm by diffusion. The staining pattern also agrees with an endocytic uptake of the dye as revealed by the higher fluorescence intensity at the tip of the growing tube. When growth is arrested and the clear cap dissipated, an indication that vesicles no longer accumulate in the apex (Lancelle and Hepler, 1992), the fluorescence pattern changes to a uniform distribution which again suggests that the dye is loaded (and released) into vesicles. The apical accumulation is in agreement with previous studies that show that both endo- and exocytosis occur in this region of the cell (Steer and Steer, 1989; O’Driscoll et al., 1993; Derksen et al., 1995; Parton et al., 2001).

FM 1-43 was also found to be a marker for polar growth. In pollen tubes exhibiting isodiametrical growth, fluorescence was approximately uniform all over the apical region. However, if apical growth was recovered, the region of the new growing tip showed a fluorescence hotspot. A similar observation was made when a pollen tube changed its growth direction. It has been hypothesized that this event results in relocation of the secretory vesicles to one side of the dome (Malho et al., 2000). Indeed, it was observed that the side of the dome to which the cell bent became more fluorescent, thus suggesting vesicle relocation and an asymmetric distribution of the fusion events.

**Fig. 7.** Nucleotide and deduced amino acid sequence of the cDNA clone AuRop. Numbers on the right refer to the nucleotide sequence. Numbers on the left refer to the amino acid sequence. Amino acids are numbered following the Rop protein complete sequence. The DNA sequences corresponding to the degenerated PCR primers are underlined with dashed lines and to the 3’RACE primer is underlined with a dash-dot line. The antisense oligonucleotide DNA sequence is in bold.
The cargo, which is released upon vesicle fusion, provides the material for the construction of the new cell wall needed for growth (not simply elongation of existing wall).

Cytosolic calcium controls secretion of cell wall material?

The results of FM 1-43 fluorescence upon reorientation of the pollen tube growth axis strongly resembled those obtained by the authors when \([\text{Ca}^{2+}]_c\) was mapped during this process (Malhó and Trewavas, 1996). This suggests that \([\text{Ca}^{2+}]_c\) is a regulator of the coupling between growth and endo/exocytosis. Indirect support for this statement can be found in older literature. Reiss and Herth (1978) found that chlortetracycline disturbed the apical cytoplasmic gradient in pollen tubes, and induced the formation of abnormal wall thickenings composed of aggregates of the dictyosome-derived wall precursor bodies. These authors attribute the effect to the chelation of \(\text{Ca}^{2+}\) and the consequent disruption of exocytosis. But the same authors (Reiss and Herth, 1979) also found that A23187, a calcium ionophore, induced thickening of the wall at the tip by the accretion of the precursor bodies.

However, these data also suggest that cell growth is not strictly dependent on a \(\text{Ca}^{2+}\)-mediated stimulation of exocytosis. This is in agreement with the results of Roy et al. (1999) using the Yariv reagent, thus suggesting that a \(\text{Ca}^{2+}\)-dependent exocytosis serves mainly to secrete cell wall components. This explains why rises in apical \(\text{Ca}^{2+}\) alone led to augmented secretion, reorientation of the growth axis, but not increase in growth rates. A tight connection between \([\text{Ca}^{2+}]_c\) and cell wall material have also been suggested by Holdaway-Clarke et al. (1997). These authors found that during oscillatory growth, peaks of fast growth were not coincident with peaks of high \([\text{Ca}^{2+}]_c\). In addition, they proposed a model where the intracellular \([\text{Ca}^{2+}]_c\) gradient determines the rate of deposition of wall material.

GTPases control growth and membrane retrieval?

Modulation of GTP levels was shown to have a strong effect on pollen tube growth. The non-hydrolysable analogues of guanine nucleotide interfere in biological processes by modulating the activity of GTPase enzymes and it was observed that GDP\(\beta\)S decreased growth rate, while GTP\(\gamma\)S had the opposite effect. Similar results were obtained by Ma et al. (1999) in lily pollen tubes and confirm the importance of GTPases for apical growth (Lin et al., 1996; Lin and Yang, 1997).

GTP levels were also shown to affect endo/exocytosis making this signalling pathway a likely candidate to mediate secretion and growth. The release of GTP\(\gamma\)S, thus an increase in GTPase activity, caused FM 1-43 fluorescence to decrease. This can be due to a decrease in endocytosis rate (lower dye uptake) or an increase in exocytosis (higher dye release). Given the rapid rate of fluorescence decrease (when compared to the dye washout experiments), the latter is a more likely explanation. Exocytosis seems to be more dependent on the active state of GTPases rather then on the cycling between the GTP–GDP bond state (Zheng and Yang, 2000). Therefore, while GDP\(\beta\)S has a negative effect on exocytosis by rendering GTPases inactive, GTP\(\gamma\)S has a positive effect.

Selective exposure to elevated \(\text{Ca}^{2+}\) alone is not sufficient to explain the selectivity of membrane retrieval (Smith et al., 2000). It is therefore possible that GTPases play an active role in exocytosis by coupling the actin cytoskeleton to the sequential steps underlying membrane trafficking at the site of exocytosis. This suggestion finds support in the results of Fu et al. (2001) who reported that F-actin dynamics in the tip of pollen tubes is GTPase-dependent. GTPases were reported to modulate not only the actin cytoskeleton, but also phosphoinositol metabolism (Kost et al., 1999) and protein kinase activity (Nagata and Hall, 1996), both known to affect apical growth (Malhó, 1998; Moutinho et al., 1998).
A Rop protein affects endo/exocytosis and apical growth

A large number of members of the Rho family of small GTPases fit the model described above, as they behave as simple on–off switches: when bound to GTP, they transmit extracellular signals to downstream effectors, whereas in the GDP state, signal propagation does not occur (Symons and Settleman, 2000). Possible candidates in pollen tubes are rac-like proteins (Rops). Rac proteins were shown to regulate a late step in exocytosis in neurons (Doussau et al., 2000), chromaffin (Gasman et al., 1999) and mast cells (Hong-Geller and Cerione, 2000). In pollen, expression of dominant negative Rac inhibited tube growth (Kost et al., 1999), whereas expression of constitutive active Rop induced ectopic growth (Li et al., 1999) suggesting that it is the active protein that promotes apical growth. These data using antisense oligonucleotides to reduce Rap expression support this assumption. These experiments revealed (1) that perturbation of polar growth can be accomplished by reducing the expression of Rap proteins; (2) provided that growth is not totally disrupted, a [Ca\(^{2+}\)]\(_c\) gradient and, therefore, Ca\(^{2+}\) influx, can be maintained; (3) that Rop inhibition results in a substantial decrease in the rate of FM 1-43 uptake, but not significant changes in the fluorescence pattern. Taken together these data suggest that the Rop protein is involved in endo/exocytosis and membrane retrieval, but may not be crucial for secretory vesicle targeting.

Rapid endocytosis in pollen tubes?

Endocytosis has been generally associated with clathrin coats, but electron microscope studies have shown that clathrin coated vesicles are not abundant in the pollen tube apex (Lancelle and Hepler, 1992). Recent data, however, has revealed the existence of a different form of endocytosis which do not require clathrin coats (Nichols and Lippincott-Schwartz, 2001).

The results obtained in pollen tubes favours the existence of a rapid endocytosis mechanism. This type of endocytosis is a Ca\(^{2+}\)-dependent process, coupled to exocytosis, that requires GTP hydrolysis and dynamin but not clathrin. Dynamin is a GTPase involved in the pulling and detachment of the endocytic vesicle from the plasma membrane. This protein, already identified in plant pulling and detachment of the endocytic vesicle from the membrane retrieval, but may not be crucial for secretory vesicle targeting.

[A\(^{2+}\)]\(_c\) and GTP control endo/exocytosis in a concerted but differential way

One striking feature that emerged from recent data on GTPases is the extensive cross-talk and co-operation that exists between GTPase-regulated signal transduction pathways (Bar-Sagi and Hall, 2000). Using microinjection of specific antibodies, Li et al. (1999) previously reported an interaction between Ca\(^{2+}\) and the expression of a Rop 1 protein. These authors demonstrated that inhibition of Rop1At proteins modifies Ca\(^{2+}\) influx, but could not determine inhibition of the Ca\(^{2+}\) influx altogether (which would require electrophysiological methods). It was actually found that growth inhibition was reversed by higher extracellular Ca\(^{2+}\), which means that influx was still possible. The apparent discrepancy between those data and that of the present study may simply be a matter of technique and interpretation. It is unlikely that any Rop protein will have the same effect on Ca\(^{2+}\) dynamics and growth rates. Furthermore, inhibition (or knock-out) of any protein crucial for tip growth will certainly affect the Ca\(^{2+}\) gradient either directly or through a signalling loop. This study’s results support a model in which Ca\(^{2+}\) and Rop GTPases act differentially but in a concerted form in the sequential regulation of pollen tube secretion and membrane retrieval. Although there may be several different interpretations to these data, it is suggested that the most likely explanation is that Ca\(^{2+}\) plays a major role in the secretion of cell wall components while Rop GTPases appear to play a key role in fusion of docked vesicles and endocytosis. It is clear, though, that the exact position of these elements in the signalling cascade controlling pollen tube growth and orientation is still uncertain and requires further investigations.

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References


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