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Vacuolar sorting receptors (VSRs) and secretory carrier membrane proteins (SCAMPs) are essential for pollen tube growth

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SUMMARY

Vacuolar sorting receptors (VSRs) are type-I integral membrane proteins that mediate biosynthetic protein traffic in the secretory pathway to the vacuole, whereas secretory carrier membrane proteins (SCAMPs) are type-IV membrane proteins localizing to the plasma membrane and early endosome (EE) or trans-Golgi network (TGN) in the plant endocytic pathway. As pollen tube growth is an extremely polarized and highly dynamic process, with intense anterograde and retrograde membrane trafficking, we have studied the dynamics and functional roles of VSR and SCAMP in pollen tube growth using lily (*Lilium longiflorum*) pollen as a model. Using newly cloned lily VSR and SCAMP cDNA (termed *LIVSR* and *LISCAMP*, respectively), as well as specific antibodies against VSR and SCAMP1 as tools, we have demonstrated that in growing lily pollen tubes: (i) transiently expressed GFP-VSR/GFP-LIVSR is located throughout the pollen tubes, excepting the apical clear-zone region, whereas GFP-LISCAMP is mainly concentrated in the tip region; (ii) VSRs are localized to the multivesicular body (MVB) and vacuole, whereas SCAMPs are localized to apical endocytic vesicles, TGN and vacuole; and (iii) microinjection of VSR or SCAMP antibodies and *LIVSR* small interfering RNAs (siRNAs) significantly reduced the growth rate of the lily pollen tubes. Taken together, both VSR and SCAMP are required for pollen tube growth, probably working together in regulating protein trafficking in the secretory and endocytic pathways, which need to be coordinated in order to support pollen tube elongation.

Keywords: vacuolar sorting receptor, secretory carrier membrane protein, lily pollen tube, secretory pathway, endocytosis pathway, apical inverted-cone zone.

INTRODUCTION

One of the fastest growing plant cells is the pollen tube. Growth occurs exclusively at the tip, to which the pollen cell directs most of its resources in a complex process comprising ion fluxes/gradients, a highly dynamic cytoskeleton, site-directed vesicle trafficking to and from the plasma membrane (PM), and, of course, cell wall synthesis (Taylor and Hepler, 1997; Malho *et al.*, 2006; Krichevsky *et al.*, 2007; Cheung and Wu, 2008). The growing pollen tube is therefore an ideal single-cell model system to study protein dynamics and function in the secretory and endocytic pathways in a plant. Some of the key players in these events have already been identified: for example, members of the Rho and Rab

family of GTPases, which localize to the growing tips and act as molecular switches regulating vesicle budding and fusion events in exo- and endocytosis (de Graaf *et al.*, 2005; Klahre and Kost, 2006; Nibau *et al.*, 2006; Samaj *et al.*, 2006; Brennwald and Rossi, 2007; Cheung and Wu, 2008; Lee and Yang, 2008; Cai and Cresti, 2009; Szumlanski and Nielsen, 2009). In addition, actin binding proteins (Xiang *et al.*, 2007; Wang *et al.*, 2008), and a proton ATPase (Certal *et al.*, 2008) are also important in pollen tube growth. However, much of the molecular machinery regulating protein transport in exo- and endocytosis in the growing pollen tube remains unknown.

In order to follow protein trafficking in the secretory and endocytic pathways in growing pollen tubes more closely, we have chosen to investigate the location and functionality of two different proteins that have been most valuable in defining the biosynthetic and endocytic pathways to the vacuole in other plant cells. Secretory carrier membrane proteins (SCAMPs) are integral membrane proteins with four transmembrane domains (TMDs) that are ubiquitously found in many eukaryotes, including nematodes, insects, fish, amphibia, mammals, and in both monocot and dicot plants, but not in yeast (Brand *et al.*, 1991; Fernandez-Chacon *et al.*, 2000). Studies on mammals suggest that SCAMPs localize to secretory granules and regulate exocytosis and endocytosis in exocrine gland cells showing regulated secretion (Fernandez-Chacon *et al.*, 2000; Liao *et al.*, 2008). In contrast, when rice (*Oryza sativa*) SCAMP1 (OsSCAMP1) and its YFP fusions were expressed in tobacco (*Nicotiana tabacum*) BY-2 cells, the fluorescent proteins were found to localize to the PM, to an EE, identified as the *trans*-Golgi network (TGN), and also to secretory vesicles (Lam *et al.*, 2007a,b, 2009; Toyooka *et al.*, 2009). Interestingly, SCAMPs also accumulate in the PM of the developing cell plate during cytokinesis (Lam *et al.*, 2008; Toyooka *et al.*, 2009). Although their function is not yet clear, SCAMPs are certainly a useful marker for the endocytic and secretory pathways in plant cells.

The other protein is BP-80, originally isolated from pea cotyledon clathrin-coated vesicles (CCVs) (Kirsch *et al.*, 1994), which is a member of the family of vacuolar sorting receptor (VSR) proteins, and mediates the sorting of soluble vacuolar cargo molecules (Paris and Neuhaus, 2002; Neuhaus and Paris, 2005). This is a type-I integral membrane protein with a single TMD and cytoplasmic tail (CT) (Ahmed *et al.*, 1997; Paris *et al.*, 1997). When expressed in tobacco cells, a fluorescently tagged BP-80 construct, comprised of the TMD and CT, with the lumenal cargo-binding domain being exchanged for GFP, co-localizes with endogenous VSR proteins at a multivesiculate, pre-vacuolar compartment (PVC) (Jiang and Rogers, 1998; Tse *et al.*, 2004, 2006). This demonstrates that the TMD and CT are sufficient for VSR targeting. Indeed, using a similar reporter system, all seven Arabidopsis VSRs (AtVSR1–AtVSR7) were found to co-localize with endogenous VSRs to the PVC in both tobacco BY-2 and Arabidopsis cells (Miao *et al.*, 2006, 2008). However, microarray analysis of gene expression in Arabidopsis (AtGenExpress) reveals that AtVSR1 and AtVSR3 are highly expressed in various tissues, except for pollen/flowers, whereas AtVSR2 is found mainly in pollen/flowers, and only shows low expression in other cell types/tissues.

We show here that the dynamics and subcellular localization of VSR and SCAMP in lily (*Lilium longiflorum*) pollen tubes are basically similar to those previously described in tobacco BY-2 cells. Significantly, whereas GFP-SCAMP constructs localized exclusively to the tip of the growing pollen tube, GFP-VSR constructs were found throughout the whole pollen tube, excepting the tip. Treatment with the actin-depolymerizing drug latrunculin B (LatB) demonstrated that the spatial and temporal dynamics of both GFP-tagged VSR and -SCAMP in growing pollen tubes are actin microfilament dependent. Microinjection of VSR and SCAMP antibodies as well as of *LIVSR* siRNAs severely inhibited the growth rate of the lily pollen tubes, pointing to essential roles for VSR and SCAMP in pollen tube growth.

RESULTS

Dynamics and distribution of VSR in germinating lily pollen tubes

We first examined the dynamics of VSR and SCAMP proteins *in vivo,* in order to obtain information on their possible functions in mediating pollen tube growth. As GFP-VSR fusion reporters with the TMD and CT of VSRs were found to co-localize with the endogenous VSR proteins (Jiang and Rogers, 1998; Tse *et al.*, 2004; Miao *et al.*, 2006, 2008), we employed a similar approach for studying VSRs in pollen tubes. We therefore cloned a full-length lily VSR cDNA (termed *LIVSR* in this study) via the 5′ and 3′ rapid amplification of cDNA ends (RACE) using cDNA derived from mRNA isolated from germinating lily pollen. We also generated a similar GFP-LIVSR construct under the control of the pollen-specific promoter *ZM13* (Wang *et al.*, 2008), which lacked the lumenal domain, and performed transient expression via particle bombardment. As a positive control, we also modified the original PVC marker GFP-BP-80 that co-localized with endogenous VSR proteins (Tse *et al.*, 2004) by replacing its *35S* promoter with the same pollen-specific promoter *ZM13*.

When transiently expressed in growing lily pollen tubes, both the GFP-BP-80 and the GFP-LIVSR signals were found to be present throughout the pollen tube, except in the apical inverted-cone region of the clear zone (Figure 1b,c). In contrast, control cytosolic GFP signals were found to be evenly distributed in the growing pollen tube (Figure 1a). Interestingly, the clear zone at the tip matched perfectly with the distribution of the internalized endocytic marker FM4-64

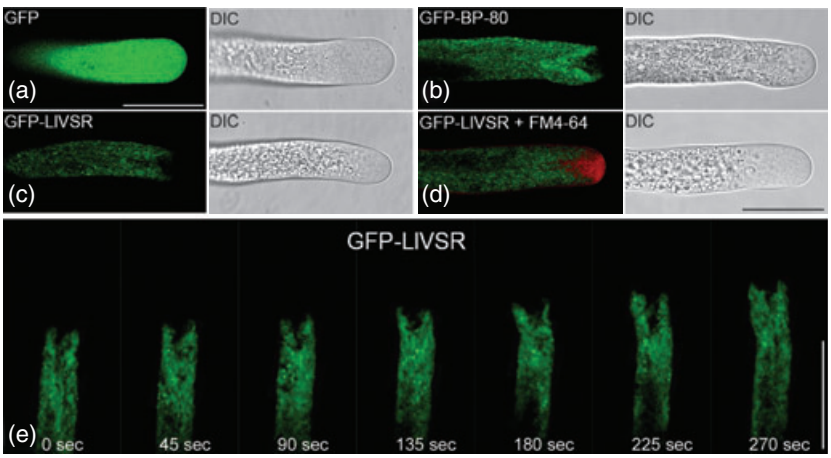

Figure 1. Dynamics of GFP-VSR in germinating lily pollen tubes.
Germinating pollen were transfected with various GFP fusions, as indicated, via bombardment for transient expression, followed by confocal imaging. Representative confocal images are pollen tubes expressing free GFP protein (a), GFP-BP-80 (b), GFP-LIVSR (c) and GFP-LIVSR, with subsequent FM4-64 dye uptake (d).
(e) Time-course confocal images of a growing lily pollen tube expressing GFP-LIVSR. Scale bars: 25 μm. AUTHOR: Scale bars: 25 μm. Please confirm bars in (a), (b) and (c) are all 25 μm.

(Figure 1d). The clear zone consistently lacked the GFP-LIVSR signal during pollen tube growth (Figure 1e). In contrast to the diffuse cytosolic GFP signal (Figure 1a), signals from GFP-VSR fusions (Figure 1b–e) showed a punctate pattern, with the punctae moving towards the apex in the pollen cortex, and then returning back through the center of the tube (Figure 1e and Video Clip S1). However, for reasons still unclear, the transient expression of these VSR constructs also produced a somewhat diffuse background, which was clearly distinct from the typical ER network in a pollen tube (Figure S2). In addition, the GFP-tagged ER marker was only excluded from the very tip of the apex, but GFP-LIVSR was missing from the much larger v-shape region of the apex (Figure 1). These data would suggest that endocytosis and the trafficking of VSR-labeled PVCs are kept strictly separate from each other in the apical inverted-cone region.

Dynamics and distribution of SCAMP in germinating lily pollen tubes

As both YFP-SCAMP1 and SCAMP1-YFP fusion constructs were found to co-localize with the endogenous SCAMP1 proteins to both PM and TGN in tobacco BY-2 cells (Lam *et al.*, 2007a,b), we decided to use a similar reporter system for studying SCAMP in pollen tubes. We first cloned a full-length lily SCAMP cDNA (termed *LISCAMP* in this study) via 5′ and 3′ RACE using cDNA derived from mRNA isolated from germinating lily pollen. We also cloned and used the rice SCAMP6 as another control in this study. We then placed both GFP-LISCAMP and GFP-OsSCAMP6 constructs under the control of the pollen-specific promoter *ZM13* for particle bombardment. When transiently expressed in lily pollen tubes, both GFP-OsSCAMP6 and GFP-LISCAMP were found to be predominantly localized in the apical clear-zone region, which is also enriched with the internalized endocytic marker FM4-64, leading to a merged image for both signals (Figure 2a,b). This is a result consistent with the possible role of SCAMPs in endocytosis. However, some less intense GFP signals representing punctate organelles (possibly TGNs) were also found to be present throughout the whole of the growing pollen tube (Figure 2a–c). The tip-localized GFP-SCAMP signals were also obvious in time-lapse confocal images collected from a growing pollen tube expressing GFP-LISCAMP (Figure 2c), where the GFP signals remained concentrated at the elongating tip region (Figure 2d). In addition, the GFP-SCAMP-labeled apical inverted-cone zone region continually streams backwards, like the tail of a comet (Video Clip S2).

To decide whether there is a direct relationship between VSR and SCAMP in pollen, we co-expressed GFP-LIVSR together with RFP-LISCAMP and followed their dynamics in the same growing pollen tube. As shown in Figure 3, GFP-LIVSR and RFP-LISCAMP showed clearly distinct distributions in the growing pollen tube: RFP-LISCAMP was predominantly present in the apical region, whereas GFP-LIVSR is missing at this location (Figure 3a). Such a distinct but coordinated dynamic distribution between GFP-LIVSR and RFP-LISCAMP remained constant as the pollen tube continuously elongated (Figure 3b and Video Clip S3). Thus, the distribution and dynamics of the GFP/RFP-SCAMP fusions are very different to that of the GFP-VSR fusions, indicating their participation in different trafficking routes.

Nature of the 'clear zone' at the tip of germinating lily pollen tubes

In order to characterize further the nature of the compartments labeled by GFP-LIVSR and GFP-LISCAMP, respectively, in germinating pollen tubes, we applied three drugs, wortmannin, brefeldin A (BFA) and LatB. As mentioned above, wortmannin typically induces the vacuolation of GFP-tagged PVCs. Lat B at low concentrations (2.5 nM) is known to specifically depolymerize short actin microfilament bundles present in the clear-zone region, whereas long and fine actin microfilaments behind the apical region and throughout the whole pollen tube are not affected (Gibbon *et al.*, 1999; Vidali *et al.*, 2001; Chen *et al.*, 2006, 2007).

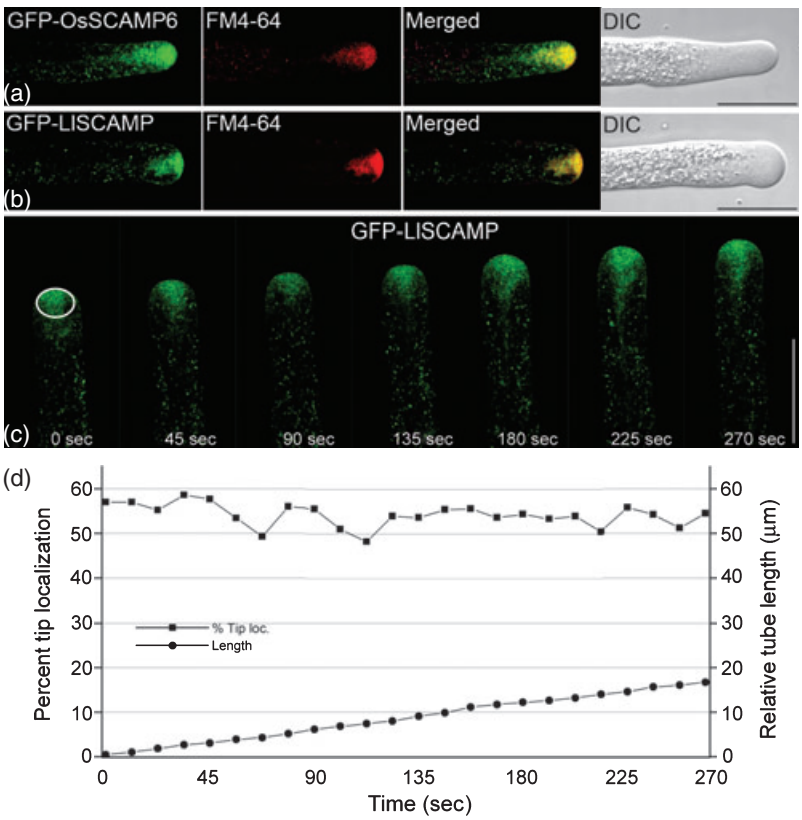

Figure 2. Dynamics of GFP-SCAMP in germinating lily pollen tubes.
(a) and (b) Co-localization of GFP-OsSCAMP6 or GFP-LISCAMP with FM4-64 in germinated lily pollen tubes expressing the reporters. Scale bars: 25 μm.
(c) Time-lapse conofcal images of a pollen tube expressing GFP-LISCAMP. Scale bar: 25 μm.
(d) The relationship between the percentage of tip-localized GFP-LISCAMP and the relative length of the growing pollen tube.

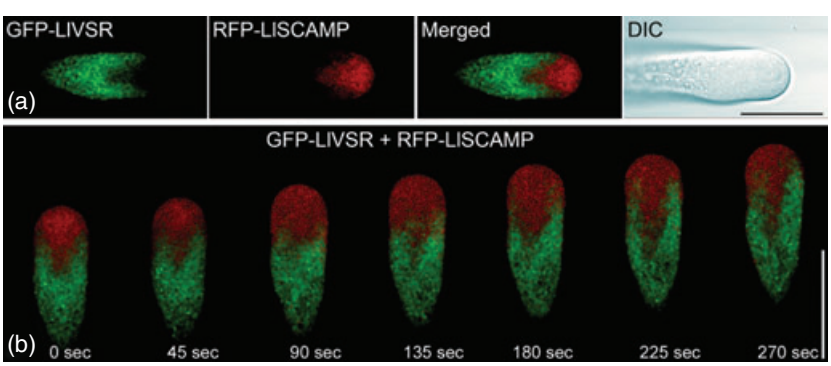

Figure 3. Dynamics of GFP-LIVSR and RFP-LISCAMP upon co-expression in a growing lily pollen tube.
(a) Relationship between GFP-LIVSR and RFP-LISCAMP upon co-expression in a growing lily pollen tube.
(b) Time-lapse confocal images of a pollen tube co-expressing GFP-LIVSR and RFP-LISCAMP. Scale bar: 25 μm.

Moreover, Lat B at low concentrations does not stop cytoplasmic streaming, although further pollen tube growth is inhibited.

Treatment of bombarded lily pollen tubes for 15 min with wortmannin (8.25 μM) induced the cytoplasmic GFP-LIVSR punctae to form ring-like structures (Figure 4a), with on average about 10–12 such ring-like structures per whole growing pollen tube. The addition of 2.5 nM Lat B for 15 min to growing lily pollen tubes expressing the GFP-LIVSR caused the punctate GFP signals to invade the apical inverted-cone region. Pollen tube growth was slowed down, with the tube beginning to twist below the tip (Figure 4b). Nevertheless, the GFP-LIVSR punctae remained motile, moving towards the apex and returning through the tube center (data not shown). On the other hand, Lat B-induced disruption of the inverted-cone region at the tip prevented the endocytic uptake of FM4-64 (Figure 4c). This inhibition was reversible after washing out the drug for 10–15 min, leading to the reconstitution of the apical inverted-cone region (Figure 4d). These results indicate that: (i) the dynamics of GFP-LIVSR trafficking is independent of the short actin microfilament bundles at the apex of the pollen tube; and (ii) the short actin microfilament bundles in the tip region are essential for maintaining the structure of the apical inverted-cone zone, and for the endocytic processes occurring at the tip of the pollen tube.

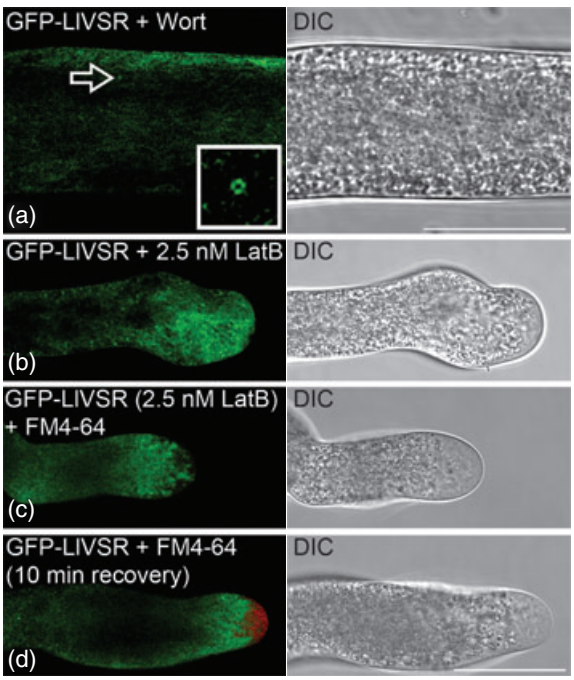

Figure 4. Dynamics of GFP-LIVSR in response to drug treatments.
Upon transformation of GFP-LIVSR via bombardment, the transformed lily pollens were germinated and (a) treated with 8.25 μM wortmannin for 15 min before confocal imaging, or (b) treated with 2.5 nM Latrunculin B (LatB) for 15 min before confocal imaging.
(c) Further uptake with FM4-64 after treatment with 2.5 nM Lat B before confocal imaging.
(d) Lat B was washed off and there was a 10-min recovery period before FM4-64 uptake and confocal imaging. DIC, differential interference contrast. Scale bars: 10 μm in (a); 25 μm in (b), (c) and (d).

We also studied the effects of BFA and Lat B on the dynamics and distribution of GFP-LISCAMP in germinating pollen tubes. BFA has previously been shown to block the secretion of cell wall material in pollen tubes, resulting in growth arrest and in the re-organization of secretion vesicles and endosomal compartments at the pollen tube apex (Rutten and Knuiman, 1993; Wang *et al.*, 2005). In fact, a 10-min exposure to BFA at 10 μg ml^{-1} already causes changes at the tip of the lily pollen tube, as monitored by the uptake of FM4-64 (Figure 5a). Upon treatment with BFA, the FM4-64-labeled inverted-cone region became less organized, and a large subapical aggregate was formed (Parton *et al.*, 2001, 2003). However, treatment with BFA at 10 μg ml^{-1} for 30 min or longer caused the BFA aggregates to be gradually disrupted, and the FM4-64 distribution in the apical area of the tube became diffuse and less well organized (Figure 5b). Similar results were obtained when the same BFA treatments were carried out in pollen tubes expressing GFP-LISCAMP (Figure 5c,d,f). Here, GFP-LISCAMP-positive BFA-induced aggregation was obvious in the tip region, with aggregates present throughout the whole tube within 10 min of treatment with BFA (Figure 5c). The subapical-localized BFA-induced aggregate was gradually disrupted, changing to a diffuse pattern in less than 10 min (Figure 5f), and eventually changed into a cytosolic pattern lacking tip localization within 30 min of treatment with BFA (Figure 5d). Nevertheless, the cytosolic BFA-induced aggregates marked by GFP-LISCAMP remained visible and mobile.

When the pollen tube was treated with 2.5 nM Lat B for 15 min to depolymerize the short actin microfilament bundles in the tip of the pollen tube, the apical localization of GFP-LISCAMP was also disrupted. However, the punctate GFP signals representing endosomal compartments remained highly dynamic, although the growth of the pollen tube was stopped (Figure 5e). These results again demonstrate that the dynamics and maintenance of apical-localized GFP-LISCAMP is dependent on the short actin microfilament bundles.

Subcellular localization of VSR and SCAMP1 in germinating lily pollen tubes

To investigate further the subcelluar localization of VSRs and SCAMPs in germinating lily pollen, we next performed immunogold electron microscopy (EM) studies using affinity-purified anti-SCAMP1 and anti-VSR (VSRat-1 and anti-BP-80 CT) antibodies (Tse *et al.*, 2004; Lam *et al.*, 2007a). To test the specificity of these antibodies, western blot analysis was first carried out on proteins isolated from germinated (45 min) lily pollen using these antibodies. As shown in Figure 6, both VSRat-1 and BP-80 CT antibodies detected a major protein band at about 80 kDa in the membrane fraction, as well as a very weak protein band at around 50 kDa that was probably a result of proteolytic activity. Similarly, SCAMP1 antibodies also detected a major protein band at about 30 kDa in the same fraction, with a very weak protein band at around 50 kDa. These results indicate that immunologically related homologs for VSR and SCAMP1 are present in germinating lily pollen tubes. Thus, these antibodies are likely to be specific for the detection of endogenous VSR and SCAMP proteins in germinating lily pollen tubes.

We performed immunogold EM on ultrathin sections prepared from high-pressure frozen/freeze-substituted lily pollen tubes with VSRAt-1 and SCAMP antibodies to determine the nature of the VSR- and SCAMP1-labeled organelles. VSR antibodies were observed to label the putative PVC/multivesicular body (MVB) (Figure 7a–c) and small vacuole-like structures (Figure 7d,e).

Similarly, SCAMP1 antibodies also labeled the TGN (Figure 8d) and vacuole-like structures (Figure 8e). In addition, SCAMP1 antibodies also specifically labeled small vesicles that were highly enriched in the apical region of the tube (Figure 8a–c), supporting the observation for the tip localization of SCAMP-GFP in transiently expressing pollen tubes. Based on the virtual lack of background labeling, the

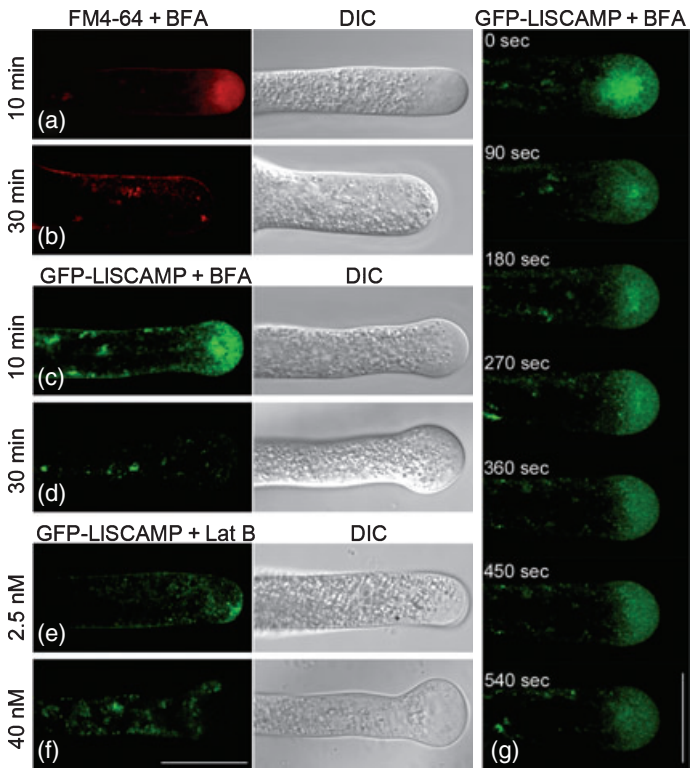

Figure 5. Dynamics of GFP-LISCAMP in response to drug treatments.
(a) and (b) Germinated lily pollen tubes were subjected to FM4-64 dye uptake, followed by a 10-min brefeldin A (BFA) treatment at 10 μg ml^{-1}, followed by another 10- or 30-min incubation, as indicated, prior to conofcal imaging.
(c) and (d) Germinated lily pollen tubes expressing GFP-LISCAMP were treated with BFA at 10 μg ml^{-1} for 10 and 30 min prior to confocal imaging.
(e) and (f) Germinated lily pollen tubes expressing GFP-LISCAMP were treated with 2.5 nM (e) or 40 nM (f) Lat B for 15 min before confocal imaging.
(g) Germinated lily pollen tubes expressing GFP-LISCAMP were treated with BFA at 10 μg ml^{-1} for 30 min prior to time-lapse confocal imaging at the indicated times, from 0 to 540 sec. Scale bar: 25 μm.

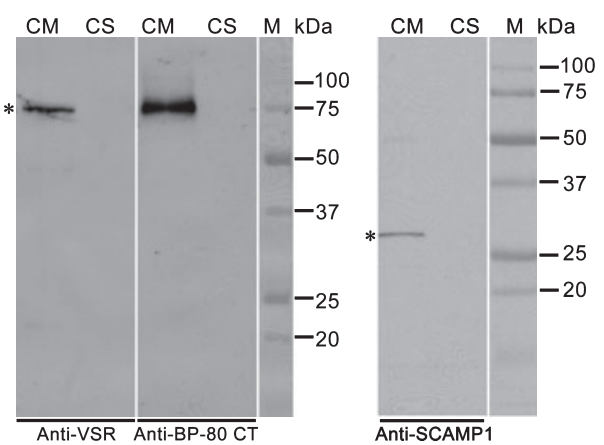

Figure 6. Western blot analysis of VSR and SCAMP1 proteins in germinating lily pollen.
Cell soluble (CS) and total cell membrane (CM) proteins were isolated from germinating lily pollen, followed by protein separation via SDS-PAGE, and western blot detection using VSRat-1, BP-80 CT and SCAMP1 antibodies, as indicated. The asterisk indicates positions of the target proteins. M, molecular weight marker in kDa.

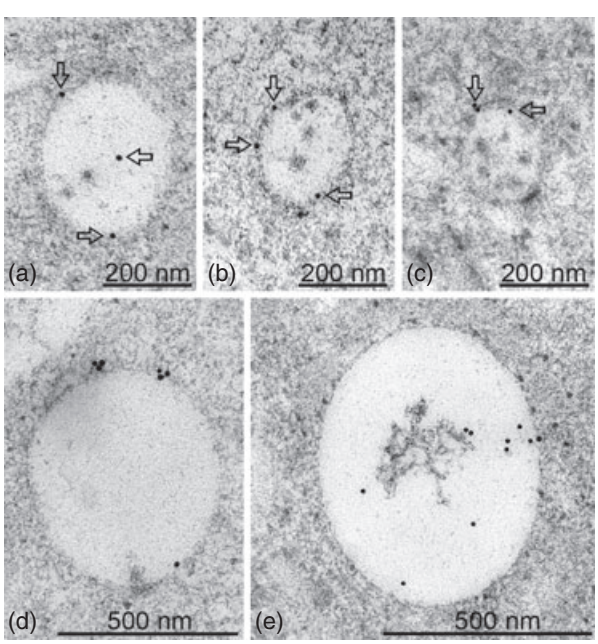

Figure 7. Immunogold electron microscopy localization of VSR in lily pollen tubes.
Ultrathin sections prepared from high-pressure freezing/freeze-substitute pollen samples were labeled with VSRat-1 antibodies. Arrows indicate examples of gold particles on putative pre-vacuolar compartment/multivesicular body (a, b and c), and vacuole-like structure (d and e). Scale bars: 500 nm in (d) and (e); 200 nm in (a), (b) and (c).

immunogold EM labeling was deemed to be highly specific (data not shown).

A statistical analysis on the immunogold EM VSR- and SCAMP1-labeled sections for the distribution of gold

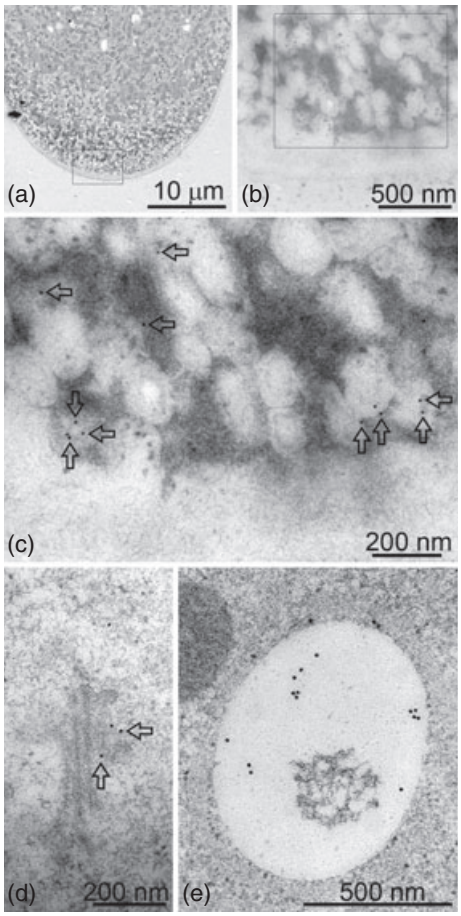

Figure 8. Immunogold electron microscopy localization of SCAMP1 in lily pollen tubes.
Ultrathin sections prepared from the high-pressure freezing/freeze-substitute pollen tube tip were labeled with SCAMP1 antibodies.
(a) Overview of the ultrathin section across the tip of a lily pollen tube used for labeling.
(b) The enlarged apical region of the pollen tube from (a), as indicated.
(c) Enlarged area from (b) showing localization of gold particles on the vesicles, indicated with arrows. Arrows also indicate examples of gold particles on the trans-Golgi network (d) and vacuole-like structure (e). Scale bars: 10 μm in (a); 500 nm in (b) and (e); and 200 nm in (c) ans (d).

particles in various organelles was also carried out. As shown in Table 1, gold particles on VSR-labeled sections were mainly found over the PVC/MVB and small vacuoles, with an average of 3.08 and 6.36 gold particles per organelle, respectively, with little background labeling in the cytosol or over mitochondria. Similarly, in SCAMP1-labeled sections, gold particles were mainly found over the TGN and small vacuoles, with an average of 3.48 and 6.68 gold particles per organelle, respectively, whereas close to one gold particle was present over each of the small vesicles in the tip region. Little background labeling was observed in the cytosol and over mitochondria (Table 2).

Taken together, these results indicate that in germinating lily pollen tubes VSRs localize to the PVC/MVB and small vacuolar structures, whereas SCAMP1 was found in small vesicles in the apical region, TGN and vacuoles. In regard to the labeling of the internal structures, these results are consistent with PVC-localization of VSR and TGN-localization of SCAMP1, as found in previous studies (Tse *et al.*, 2004; Lam *et al.*, 2007a) and in the dynamic live cell distribution of LIVSR and LISCAMP in this study. However, the additional localization of SCAMPs to the apical vesicles seem to be a pollen-specific feature, with possible functional implications (see Discussion).

Table 1 Distribution of gold particles (GPs) for vacuolar sorting receptor antibodies in immunogold electron microscopy labeling of germinating lily pollen tubes

Organelle	GP no.	Organelle no.	GP per organelle
MVB/PVC[a]	77	25	3.08**
Small vacuole	159	25	6.36**
Mitochondria	4	25	0.16**

[a]Multivesicular body/pre-vacuolar compartment.
**Significant differences between two organelles were analyzed using a one-sided paired Student's *t*-test ($P < 0.01$). Data were collected and analyzed from seven independent labeling experiments.

Table 2 Distribution of gold particles (GPs) for SCAMP1 antibodies in immunogold electron microscopy labeling of germinating lily pollen tubes

Organelle	GP no.	Organelle no.	GP per organelle
TGN[a]	87	25	3.48**
Small vacuole	167	25	6.68**
Tip-endocytic vesicles	24	25	0.96**
Mitochondria	3	25	0.12**

[a]Trans-Golgi network.
**Significant differences between two organelles were analyzed using a one-sides paired Student's *t*-test ($P < 0.01$). Data were collected and analyzed from seven independent labeling experiments.

Microinjection of VSR and SCAMP antibodies strongly inhibits pollen tube growth

To investigate further the functional roles of VSRs and SCAMPs in pollen tube growth, we performed microinjection experiments with antibodies, a technique that has been previously shown to be effective in addressing the function of proteins during pollen tube growth (Lin and Yang, 1997). We therefore microinjected VSR and SCAMP antibodies into growing pollen tubes cultured on low-melting agarose pollen germination medium, followed by observation of the subsequent tube growth. As shown in Figure 9a,b, microinjection of either BP-80 CT or SCAMP1 antibodies into pollen tubes significantly reduced the growth rate of the

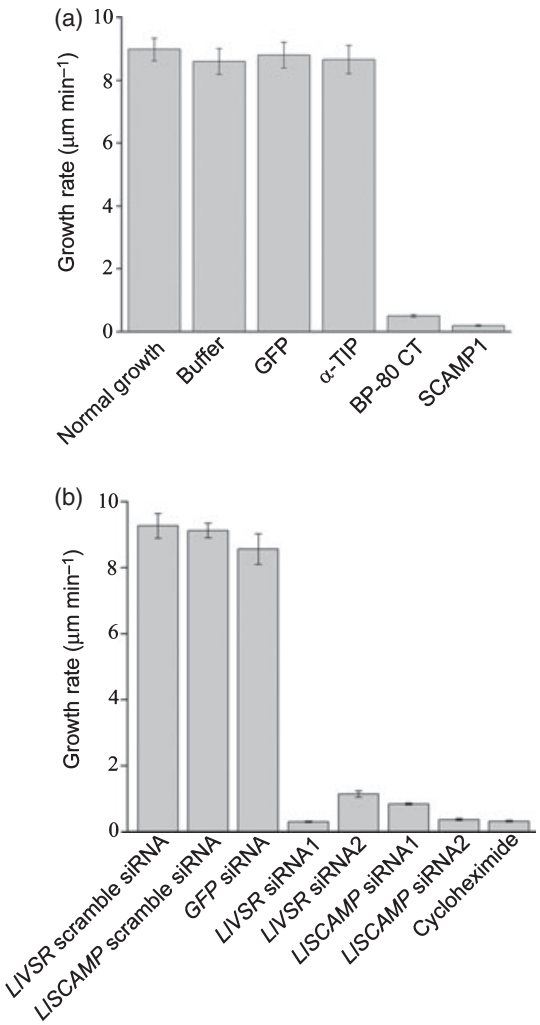

Figure 9. Effects of antibody and small interfering RNAs (siRNA) injection on lily pollen tube growth.

Growing lily pollen tubes were microinjected with various antibodies (BP-80 CT, SCAMP1, GFP and α-TIP), together with control buffer, as indicated, and various siRNAs (*LIVSR* siRNA, *LISCAMP* siRNA, Scramble siRNA and *GFP* siRNA), and with 100 nM cycloheximide as another control. After 5 min of recovery, micropipette tips were slowly removed from the pollen tube, and the pollen tube length was measured 10 min later after injection.

(a) Statistical analysis of growth rate versus antibody injection using data from at least 10 independent experiments, and expressed as mean values ± SEs.

(b) Statistical analysis of growth rate versus siRNAs or 100 nM cycloheximide injection using data from at least 10 independent experiments, and expressed as mean values ± SEs.

pollen tubes within 10 min of the completion of the microinjection, as compared with normal lily pollen tubes (Figure 9a). These negative effects were specific for these two antibodies, as when identical microinjection experiments were carried out using buffer, GFP or α tonoplast intrinsic protein (TIP) antibodies, the injected pollen tubes retained normal growth rates (Figure 9a). These observations were supported by a statistical analysis of data collated from multiple replicate experiments (Figure 9a). Although they give no direct information on their relative functions and dynamics, these results clearly underline the importance of VSR and SCAMP proteins for pollen tube growth.

Microinjection of siRNA of LIVSR and LISCAMP strongly inhibits pollen tube growth

Direct delivery of synthesized siRNA into cells to inhibit gene expression is the fastest and most direct way to downregulate a gene of interest (Elbashir *et al.*, 2001a,b; Harborth *et al.*, 2001; Meins *et al.*, 2005; Vaughn and Martienssen, 2005; Bonnet *et al.*, 2006; Vermeulen *et al.*, 2007; Ossowski *et al.*, 2008), and has become a reliable tool to study the functions of specific genes in mammalian cells (Caplen *et al.*, 2001; Harborth *et al.*, 2001; Vermeulen *et al.*, 2007). In contrast, direct siRNA-induced gene silencing in plants has been technically difficult to perform because of the presence of the cell wall (Meins *et al.*, 2005; Bonnet *et al.*, 2006). However, as we have just demonstrated through control experiments, growing pollen tubes can be successfully microinjected without necessarily perturbing their function. Thus, to provide further support for the hypothesis that LIVSR and LISCAMP are essential for lily pollen tube growth, we injected *LIVSR* siRNAs and *LISCAMP* siRNAs into growing lily pollen tubes to specifically knock-down the expression of the *LIVSR* and *LISCAMP* genes. As shown in Figure 9b, the injection of either *LIVSR* siRNA1 and *LIVSR* siRNA2 or *LISCAMP* siRNA1 and *LISCAMP* siRNA2 dramatically reduced the growth rate of the pollen tubes, as did microinjection of the general protein synthesis inhibitor cycloheximide (Figure 9b). This inhibition was specific as injection of the control scramble siRNA and *GFP* siRNA sample did not slow down pollen tube growth (Figure 9b). These observations were supported by a statistical analysis of data collated from multiple replicate experiments (Figure 9b).

DISCUSSION

The growing pollen tube provides an excellent single cell model system to study mechanisms of growth regulation, polarity and periodic behavior. The apical clear zone of a growing pollen tube is densely and exclusively occupied by transport vesicles, where both endocytosis and exocytosis are highly active to meet the demands of vigorous and rapid membrane expansion, as well as cell wall synthesis (Taylor and Hepler, 1997; Hepler *et al.*, 2001; Krichevsky *et al.*, 2007; Cheung and Wu, 2008). In this study, we have taken the advantage of this system to study the dynamics and functional roles of VSRs and SCAMPs, two integral membrane proteins that are believed to play important roles in the plant secretory and endocytic pathways, respectively (Jiang *et al.*, 2000; Tse *et al.*, 2004; Lam *et al.*, 2007a; Robinson *et al.*, 2008).

VSRs and SCAMPs are required for the growth of the lily pollen tube

Microinjection of molecules such as proteins and antibodies has been established as a reliable and direct gain-of-function or loss-of-function strategy for functional studies on proteins in mammalian cells, in particular during embryo development (Hogan *et al.*, 2008; Gusel'nikova and Pastukhov, 2009; Rosen *et al.*, 2009; Wei and Seemann, 2009). Similarly, *in vivo* downregulation of a specific gene by transforming gene-specific targeting of siRNA via microinjection is also an established and relatively fast method to study the functions of specific genes in mammalian cells (Elbashir *et al.*, 2001a; Harborth *et al.*, 2001; Meins *et al.*, 2005; Vaughn and Martienssen, 2005; Bonnet *et al.*, 2006; Vermeulen *et al.*, 2007; Ossowski *et al.*, 2008). On the other hand, microinjection is technically challenging, and has rarely been used in plants because of the cell wall, turgor and in general because of the smaller size of plant cells. However, lily pollen has been occasionally used in microinjection studies because of the relatively large size of the pollen tube, making it easy for micromanipulation, as compared with the pollen of other plant species such as Arabidopsis or tobacco. In fact, the first example for microinjection was performed using RopIP antibodies to study the function of a tip-localized Rho-type GTPase (Rop) in controlling pollen tube growth (Lin and Yang, 1997).

In this study, we microinjected two affinity-purified polyclonal antibodies into growing lily pollen tubes: one generated against a synthetic peptide corresponding to the CT of the VSR BP-80 (Paris *et al.*, 1997; Tse *et al.*, 2004), and another against a synthetic peptide corresponding to the N-terminal NPF (Asn-Pro-Phe) repeats and the second conserved loop (predicted to locate in the cytosolic side) of rice SCAMP1 (Lam *et al.*, 2007a). Injection of either antibody specifically inhibited the growth of the lily pollen tube, because other control antibodies, including GFP and α-TIP, were without effect (Figure 9a). A similar result was obtained when *LIVSR* siRNAs and *LISCAMP* siRNAs were used in identical microinjection experiments (Figure 9b). These results came as a surprise in particular for VSR, because they indicate that this receptor must be recycled with a very short half-life, meaning that it requires continuous expression to maintain its transport activity. At first glance, this seems unlikely because in normal growing plant cells VSRs are believed to recycle many times, but for rapidly growing pollen tubes the situation may be different, requiring newly synthesized VSRs to keep up the rapid growth. In this regard it should be emphasized that the 'minimal invasive' nature of these antibody injections was apparent under the microscope, as they did not significantly perturb the 'reverse fountain' cytoplasmic streaming at the tip of the tube (data not shown). Together, these results strongly indicate that functional VSRs and SCAMPs are essential for lily pollen tube growth.

What is the possible mode of action for these two microinjected antibodies in inhibiting the growth of the pollen tube? The BP-80 CT antibodies recognize the CT of the receptor, which is conserved in VSR family proteins (Tse *et al.*, 2004), whereas SCAMP1 antibodies also detect a highly conserved cytosolic region of the SCAMP family protein (Lam *et al.*, 2007a). These antibodies will probably recognize domains in these molecules that might be predicted to interact with other proteins essential for their proper function. For example, the YMPL motif of the VSR CT may interact with adaptor proteins (APs) in the formation of the CCV (Jiang and Rogers, 1998; Sanderfoot *et al.*, 1998), where the binding of the microinjected antibodies to the VSR CT would abolish its normal interaction with other proteins essential for its function. A similar scenario can be envisaged for SCAMPs. As a result, the sorting function of VSRs in the secretory pathway (Jiang and Rogers, 2003) or the putative role of SCAMPs in mediating endocytosis (Lam *et al.*, 2007b, 2008) would be prevented. Following this line of argument, we consider that it is more than likely that both VSR-mediated protein trafficking and SCAMP-mediated endocytosis are essential for pollen tube growth.

Western blot analysis showed that both VSRat-1 and BP-80 CT antibodies detected a major protein band about 80 kDa, whereas OsSCAMP1 antibodies detected a major protein band about 30 kDa in the membrane protein fractions of germinating lily pollen (Figure 6). We would maintain that these antibodies detected their corresponding lily homologs because the molecular weights based on the newly cloned LIVSR and LISCAMP are also predicted to be 80 and 30 kDa, respectively (Figure S1). A microarray analysis of gene expression in Arabidopsis (AtGenExpress) reveals that only AtVSR2 is highly expressed in pollen/flowers, and shows low expression in other cell types/tissues, suggesting a specific involvement of AtVSR2 in Arabidopsis pollen tube pollen growth. It is reasonable to assume that the situation in lily is similar, firstly because microinjection of LIVSR siRNAs inhibited the growth of the lily pollen tube, and secondly, LIVSR was cloned using cDNA derived from mRNA isolated from growing lily pollen tubes.

Dynamics and distinct distribution of VSR and SCAMP in growing lily pollen tube

Central to the control of growth rate and orientation of the pollen tube are the processes of localized delivery and exocytosis of cell wall materials and membrane at the pollen tip (Cheung and Wu, 2008; Zonia and Munnik, 2008). It has been established that membrane incorporation at the apex of the pollen tube greatly exceeds the increase in membrane area required for tip extension, setting a requirement for active recovery from the tip through endocytosis. That endocytosis is particularly active at the tip and within the clear zone of the pollen is dramatically visualized in internalization studies using styryl dyes like FM4-64 (Parton *et al.*, 2001, 2003; Zonia

and Munnik, 2008; Wei and Seemann, 2009). It is therefore generally accepted that tip-growing cells need balanced vesicle-mediated exo- and endocytic events at the tip to regulate the quantity of PM at the apices of these cells (Parton *et al.*, 2001; Samaj *et al.*, 2005; Cheung and Wu, 2008).

When GFP-LIVSR or GFP-BP-80 and GFP-LISCAMP or GFP-OsSCAMP6 were transiently expressed in growing pollen tubes, they were found to have distinct but different localizations. GFP-VSR was found to be present in most of the pollen tube except in the tip-growth clear zone. In contrast, GFP-SCAMP was mainly concentrated at the tip, co-localizing with the internalized endocytic marker FM4-64 (Figures 1–3). As GFP-VSR and GFP-SCAMP faithfully reflect the localization of the endogenous VSR and SCAMP proteins in other plant cells (Tse *et al.*, 2004; Lam *et al.*, 2007a), such a distinct distribution of GFP-VSR versus GFP-SCAMP in the growing lily pollen tube is likely to represent spatially different vesicle-trafficking pathways.

The FM4-64-labeled tip region of a growing pollen tube consists of Golgi-derived and endocytic membrane vesicles (Cheung and Wu, 2008). As GFP-SCAMP (Figures 2 and 3) is predominantly localized to the same tip region in growing lily pollen tubes, it suggests that SCAMPs are important in mediating endocytosis in these cells. Furthermore, support for an involvement of SCAMPs in endocytosis is given by BFA treatment, which caused GFP-LISCAMP to form aggregates in the subapical region of a growing pollen tube (Figure 5), a perturbation typically representing the accumulation of endocytic vesicles in the inverted-cone region (Cheung and Wu, 2008).

Functional implications of the distinct distributions and dynamics of VSRs versus SCAMPs in pollen tube growth

Pollen tube is one of the fastest growing cell types, which as a consequence invokes high demands on membrane flow to and from the PM. In comparison with other plant cells, the secretion and endocytosis in the growing pollen tube are site-directed, and are therefore much more active and vigorous. Transport vesicles move via cytoplasmic streaming towards the pollen tip apex, where they fuse with the PM, releasing their contents into the apoplast, before some of their membrane is rapidly retrieved through endocytosis to allow for a new round of transportation. In this way, the pollen tube can maintain both its fast expansion and keep its polarity (Taylor and Hepler, 1997; Hepler *et al.*, 2001; Samaj *et al.*, 2006; Krichevsky *et al.*, 2007; Cheung and Wu, 2008). Although the transportation of vesicles during exocytosis or endocytosis is cytoskeleton dependent (Vidali *et al.*, 2001; Samaj *et al.*, 2006; Xiang *et al.*, 2007; Wang *et al.*, 2008; Zonia and Munnik, 2008), these two opposing processes are closely related, but occur in two distinct regions of the pollen tube. Whereas endocytosis occurs at the pollen tube apex region, exocytosis seems to occur in a zone distal to the tip (Samaj *et al.*, 2005; Cheung and Wu, 2008).

In this study, the distribution of GFP-tagged VSR and SCAMP proteins co-expressed in the same germinating pollen tube were found to be distinct and well coordinated with each other: the SCAMP signals were mainly found in the apical tip region, whereas the VSR signals were mainly present in the elongated tube, but were missing from the tip. As GFP-tagged VSR and SCAMP are reliable markers representing protein trafficking in the plant secretory and endocytic pathways, resepctively, in plant cells (Li *et al.*, 2002; Jiang and Rogers, 2003; Tse *et al.*, 2004; Lam *et al.*, 2007a,b), such a distinct distribution of VSRs versus SCAMPs in growing pollen tubes suggests that secretion-driven exocytosis may support pollen tube growth within a region 3–5 μm away from the pollen apex, whereas the SCAMPs are involved in endocytic events at the tip of the pollen tube. Thus, the growth rate of the pollen tube is the consequence of the fine-tuning of vesicle fusion and fission at the tip.

What role do VSRs play in exocytosis during pollen tube growth? Perhaps only indirectly, in that secretion can only be effective when the correct vacuolar trafficking occurs. However, we would like to note that even though GFP-tagged VSR proteins were missing from the tip region of the growing lily pollen tube, VSR-positive vesicles were frequently observed in close proximity to the PM in the elongating region of a growing pollen tube (data not shown). Moreover, in our ongoing immunofluorescent and immunogold EM studies using VSR antibodies on chemically fixed pollen tubes, VSRs have in fact been seen to localize to the PM (data not shown). We believe that the PM-localized VSRs are eventually internalized from the PM to the PVC via early endosomal compartments. To address more closely the possible roles of VSRs in exocytosis, and SCAMPs in endocytosis, as well as their inter-relationships, we are now in the process of examining the dynamics and possible cross-talk between VSR-tagged and SCAMP-tagged transport vesicles in the same growing pollen tube.

EXPERIMENTAL PROCEDURES

Plant materials, pollen tube germination and chemicals

Lily flowers were purchased from a local market. Mature pollen grains were collected from anthers, and after air drying were stored at 4°C before use. For germination, pollen grains were suspended in a medium containing 10% sucrose, 1.3 mM H_3BO_3, 2.9 mM KNO_3, 9.9 mM $CaCl_2$, pH 5.8, at 27.5°C for 45 min. Stock solutions of wortmannin (2.5 mM in DMSO; Sigma-Aldrich, http://www.sigma-aldrich.com), BFA (1 mM in DMSO; Sigma-Aldrich) and Lat B (1 mM in DMSO; Sigma-Aldrich) were aliquoted and stored at −20°C.

Cloning of LIVSR and LISCAMP genes and transient plasmids construction

Lily pollen tubes were germinated for 45 min, and total RNA was extracted from each sample using the Qiagen RNeasy Kit

(http://www.qiagen.com). cDNA was synthesized from each RNA sample using the Invitrogen SuperScript III first-strand synthesis system for RT-PCR using the oligo(dT) primer (http://www.invitrogen.com), according to the kit's instructions. A Clontech SMART RACE cDNA Amplification Kit (http://www.clontech.com) was used to clone the full-length *VSR* and *SCAMP* genes from lily germinating pollen tubes. Based on the conserved amino acid alignment between Arabidopsis *SCAMPs* and rice *SCAMPs,* two primers (forward primer, 5′-ATGAGAAC TGAGAGTGCTTTG-3′; reverse primer, 5′-GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT-3′) were used to obtain the 3′ end of *LISCAMP.* Based on the known 3′ end gene sequence, another two primers (the forward primer was provided and used according to the kit's instructions; reverse primer, 5′-GTGCTGCCTCG CGCTTCAACTCTGCAGC-3′) were used to obtain the 5′ end of *LISCAMP.* The *LISCAMP* gene was then amplified from lily germinating pollen tube cDNA with two primers with restriction enzyme *Xba*I/*Nco*I sites (forward primer, 5′-GGGTCT-AGAA TGGCGGGCCGCTACGACAGC-3′; reverse primer, 5′-GGGCCATGGTAATGTTGCC CTCAAGGCACCAC-3′), and then the *LISCAMP* gene was subcloned into *ZM13pro-GFP* transient expression vector with the same restriction sites. A similar strategy was also used to clone the *LIVSR* gene from germinating lily pollen tubes, based on the alignment of Arabidopsis *VSRs*, rice *VSRs*, *BP-80* and pumpkin (*Cucurbita maxima*) *PV70.* The *LIVSR* gene spacer with both TMD and CT regions was then amplified with two primers with restriction enzyme *Xba*I/*Nco*I sites (forward primer, 5′-GGGTCTAGAACATGCATCAGTAAAAAACC-3′; reverse primer, 5′-GGGCCATGGTCATATATCGCCATGCGATACG-3′), and then the *LISCAMP* gene was subcloned into *ZM13pro-GFP* transient expression vector with the same restriction sites.

Particle bombardment of pollen

Ten anthers were harvested from two lily flowers and transferred into 20 ml pollen germination medium. After vortexing to release the pollen grains into the medium, 20 ml of the pollen suspension was vacuum filtrated onto a pre-wetted filter paper. The filter paper covered with the pollen grains was then immediately transferred surface-up onto 2% agar in an 85-mm-diameter Petri dish. For the transient expression of proteins in pollen tubes, pollen grains were bombarded with gold particles as previously described (Kost *et al.*, 1998; Wang *et al.*, 2008). Pollen grains were bombarded on filter paper three times at three different positions. Immediately after the bombardment, the pollen grains were then washed down from the filter paper with germination buffer into a 50-ml conical tube. Bombarded lily pollen was allowed to germinate in a 27.5°C shaker at 80 rpm for 2–6 h before observation of the fluorescent signals.

EM of resin-embedded germinating pollen

The general procedures for transmission EM sample preparation and thin sectioning of samples were essentially as previously described (Tse *et al.*, 2004; Lam *et al.*, 2007a). For high-pressure freezing, the germinating 45-min lily pollen tubes were harvested by filtering, and were frozen immediately in a high-pressure freezing apparatus (EMP2; Leica, http://www.leica.com). Immunogold labeling was carried out using VSR, BP-80 CT and SCAMP1 antibodies at 40 μg ml^{-1} and gold-coupled secondary antibodies at 1 : 50 dilution. Post-stained sections were examined in a Hitachi H-7650 transmission EM with a CCD camera (Hitachi High-Tech, http://www.hitachi-hitec.com) operating at 80 kV.

Protein extraction, protein gel and immunoblotting

Harvested lily pollen grains were germinated in pollen germination medium for 2 h. Germinated lily pollens were ground into a powder in liquid nitrogen, followed by isolation into soluble and membrane fractions using extraction buffer (Tris–HCl 50 mM, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and 5 mg ml^{-1} leupeptin), and then western blot analysis was performed with various antibodies at 4 mg ml^{-1}, as previously described (Tse *et al.*, 2004; Lam *et al.*, 2007a).

Design and synthesis of LIVSR siRNAs

LIVSR siRNA was designed based on its *LIVSR* gene sequence. Double-strand 21-nt siRNA was designed with 3′ overhang TT dinucleotides, and was synthesized by Sigma-Aldrich. To achieve an effective RNAi, one additional siRNA sequence targeting the same *LIVSR* mRNA was designed to ensure that it reduced *LIVSR* gene expression by comparable levels. Moreover, we used one negative control of scramble siRNA with the same nucleotide composition as *LIVSR* siRNA1, but in which the nucleotide sequence of the gene-specific siRNA was scrambled, and thereby lacked any significant sequence homology with other genes. To make sure the designed siRNA specifically targeted the *LIVSR* gene, we compared the potential target sites in the plant genome database, and eliminated from consideration any target sequences of homology genes or with other coding sequences in the database. Also, one more *GFP* siRNA was included as another negative control, commercially available from Invitrogen, and proved to be effective in downregulating *GFP* expression (Caplen *et al.*, 2001). The sequences of siRNAs are as follows: *LIVSR* siRNA1 (sense, 5′-UAGGCAAUAC-AGAGGUAAATT-3′; anti-sense, 5′-UUUACCUCUGUAUU GCCU-ATT-3′); *LIVSR* siRNA2 (sense, 5′-GUACCGAAUCAGGAGUUACTT-3′; anti-sense, 5′-GUAACUCCUGAUUCGGUACTT-3′); *LIVSR* scramble siRNA (sense, 5′-GACGAAGGUACGAAUAUAATT-3′; anti-sense, 5′-GUAACUCCUGAUUCGGUACTT-3′); *LISCAMP* siRNA1 (sense, 5′-GGGCUGGAAUUGUUAUAGATT-3′; anti-sense, 5′-UCUAUAA-CAAUUCCAGCCCTT-3′); *LISCAMP* siRNA2 (sense, 5′-CCUGGAG-CUUAUGUGUUAUTT-3′; anti-sense, 5′-AUAACACAUAAGCUCCA-GGTT-3′); *LISCAMP* scramble siRNA (sense, 5′-GAGCAUGGAU-GUGUAUGUATT-3′; anti-sense, 5′-CTCGUACCUACTCAUACAUTT-3′) and *GFP* siRNA (sense, 5′-GCAAGCUGACCCUGAAGUUCAU-3′; anti-sense, 5′-GAACUUCAGGGUCAGCUUGC CG-3′).

Microinjection procedures

Lily pollen grains were suspended in germination medium and spread out on 1% low melting agarose in a 9-cm-diameter Petri dish. About 45 min after germination, pollen tubes around 150 μm in length were selected for injection, as described by Lin and Yang (1997), using a Nikon inverted microscope (TE2000-U; http://www.nikon.com). About 2 nl of affinity-purified antibody (0.2 mg ml^{-1}) or *LIVSR* siRNA (50 nM) was gently loaded into the pollen cytoplasm, as estimated by Wolniak and Larsen (1995). Five min after injection, micropipette tips were slowly removed from the pollen tube, followed by time-lapse recording of fluorescent signals in the growing pollen tube.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Amino acid sequences comparison of LIVSR and LISCAMP.

Figure S2. Dynamics and distribution of GFP-HDEL in germinating tobaccco pollen tubes.

Video Clip S1. Dynamics of GFP-LIVSR in a growing lily pollen tube.

Video Clip S2. Dynamics of GFP-LISCAMP in a growing lily pollen tube.

Video Clip S3. Dynamics of GFP-LISCAMP and RFP-LISCAMP co-expressing in a growing lily pollen tube.

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