The Roles Vacuolar Sorting Receptor (VSR) and Secretory Carrier Membrane Protein (SCAMP) in Pollen Germination

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Statement

All experiment works reported in this thesis were performed by the author, unless specially stated otherwise in the text.

WANG, Hao

Abstract

Abstract of thesis entitled:

The Roles of Vacuolar Sorting Receptor (VSR) and Secretory Carrier Membrane Protein (SCAMP) in Pollen Germination

Submitted by WANG, Hao

For the degree of Doctor of Philosophy

at the Chinese University of Hong Kong in (September 2010)

The plant endomembrane system contains several membrane-bound organelles including the endoplasmic reticulum (ER), Golgi, trans-Golgi network (TGN) or early endosome (EE), prevacuolar compartment (PVC) or late endosome (LE). In tip-confined rapid growing pollen tube, delivery of cell membrane and wall constructional materials to fast expanding tip region requires spatial and temporal coordination of secretory vesicle trafficking and endocytosis. Thus, polarized and rapid growing pollen tube is regarded as an excellent single cell model system to study molecular mechanisms of protein trafficking, vesicular secretion and endocytosis in plant cells. In this thesis research, I have made the following accomplishments in studying how pollen regulate and co-ordinate exocytosis and endocytosis processes to achieve rapid and polarized growth. Firstly, I have tested and optimized a pollen grain transient expression procedure for pollen grains of three commonly used plant species including lily, tobacco and *Arabidopsis* for studying protein subcellular localization and organelle dynamics. Secondly, using newly

cloned lily vacuolar sorting receptor (VSR) and secretory carrier membrane protein (SCAMP) cDNA (termed *LIVSR* and *LISCAMP* respectively) as well as specific antibodies against VSR and SCAMP1 as tools, I have demonstrated that in growing lily pollen tubes i) transiently expressed GFP-VSR/GFP-LIVSR located throughout the pollen tubes except the apical clear zone region whereas GFP-LISCAMP was mainly concentrated in the tip region; ii) VSRs are localized to multivesicular body (MVB) and vacuole, while SCAMPs are localized to apical endocytic vesicles, TGN and vacuole; and iii) microinjection of VSR or SCAMP antibodies and *LIVSR* or *LISCAMP* siRNAs significantly reduced the growth rate of the lily pollen tubes. Taken together, these results demonstrate that both VSRs and SCAMPs are essential for pollen tube growth, likely working together coordinately in the secretory and endocytic pathways respectively.

摘要

植物細胞的內膜系統包含了幾種膜結構的細胞器，它們分別是內質網，高爾基體，反式高爾基網狀系統或初級內膜體，液泡前體或次級內膜體。在頂尖快速生長的花粉管在伸長過程中，細胞需要在時間與空間上同時協調胞內囊泡分泌和細胞內吞來實現細胞膜與細胞壁迅速擴張所需物質的運輸。所以，快速，極性生長的花粉管是做爲研究蛋白質運輸，囊泡分泌和細胞內吞良好的單細胞模式系統。在本論文的研究中，我通過研究花粉管如何協調與調控細胞外吐和細胞內吞過程來最終達到快速，極性的生長得出以下成果。第一，我測試並優化了三種廣泛用於研究蛋白質定位和細胞器動力學研究的植物：百合，煙草和擬南芥花粉暫態表達和體外萌發體系。第二，以新克隆百合的 VSR 和 SCAMP 的 cDNA （分別命名爲 LlVSR 和 LlSCAMP）以及 VSR 和 SCAMP1 的特異性抗體爲工具，我證明在花粉管的生長過程中：i）暫態表達 VSR 和 LlVSR 融合綠色螢光蛋白發現該蛋白定位在除了頂端透明區域的整個花粉管中，而表達 LlSCAMP 綠色融合蛋白則發現該蛋白主要集中定位在花粉管頂尖區域。 ii）VSR 主要定位在液泡前體和液泡，而 SCAMPs 定位在細胞內吞的內吞囊泡，反式高爾基網狀系統和液泡。 iii）顯微注射 VSR 和 SCAMP 的特異性抗體和 *LlVSR* 或 *LlSCAMP* 的小干預 RNA 可以顯著減緩百合花粉管的生長。總體來說，以上結果證明 VSR 和 SCAMP 對花粉管的生長至關重要，它們分別作用於細胞的分泌與內吞途徑來共同調節花粉管的生長。

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List of Abbreviations

BFA	Brefeldin A
CCVs	Clathrin coated vesicles
CM fraction	Cell membrane fraction
CS fraction	Cell soluble fraction
CT	Cytoplasmic tail
ER	Endoplasmic reticulum
GFP	Green fluorescent protein
immunoEM	immunocytochemical Electron Microscopy
LV	Lytic vacuole
MVBs	Multivesicular bodies
PI 3-kinases	Phosphatidylinositol 3-kinases
PSV	Protein storage vacuole
PVCs	Prevacuolar compartments
PME	Pectin methylation enzyme
SCAMP	Secretory carrier membrane protein
TGN	*trans*-Golgi network
TIP	Tonoplast intrinsic protein
TMD	Transmembrane domain
VSDs	Vacuolar sorting determinants
VSR	Vacuolar sorting receptor
Wort	Wortmannin

CHAPTER 1

General Introduction

1.1 The Plant Secretory Pathway and Vacuolar Sorting Receptor (VSR)

Eukaryotic cells share a common organization of organelles within their endomembrane systems, where each is a membrane-bound compartment which defines a separate environment for specific functions, and different organelles communicate with each other via transport vesicles. In general, a unique type of vesicle is required for each step in traffic, and transmembrane receptor proteins that are specific for one vesicle type recruit cargo that will be transported from one organelle to another in the step mediated by that vesicle (Pryer *et al.*, 1992; Paris *et al.*, 1996; Gurkan *et al.*, 2007; Paul and Frigerio, 2007; Robinson *et al.*, 2008). A general principle that applies across eukaryotic species defines vesicle specificity: the cytoplasmic coat proteins that cause a vesicle to bud from its organelle source interact with the specific receptor proteins and cause them to partition with their cargo into the budding vesicle (Robinson and Depta, 1988; Robinson, 1996). Thus, in general terms, a sorting receptor is specific for one vesicle type that traffics in one specific step between two endomembrane organelles (Figure 1).

In plant cells, the search for vacuolar sorting receptors (VSRs) lead to the identification of an 80 kD protein called BP80 (Kirsch *et al.*, 1994; Paris and Neuhaus, 2002). BP80, the first identified vacuolar sorting receptor, is a type I membrane protein and a member of a highly conserved family of proteins in plants termed vacuolar sorting receptors (VSRs). BP80/VSRs localize primarily to the PVC

(Sanderfoot *et al.*, 1998; Li *et al.*, 2002; Tse *et al.*, 2004). The Arabidopsis genome contains seven VSR proteins (defined as AtVSR1–7) with high conservation at the amino acid level, in particular in their transmembrane domain (TMD) and cytosolic tail (CT) regions. The expression of AtVSRs was detected in most Arabidopsis tissue types, including root, leaf, stem, flower, pollen, and seed (Miao *et al.*, 2006). Confocal immunofluorescence studies demonstrated that all GFP fusions with AtVSRs TMD and CT region exhibited typical punctate signals that mostly colocalized with endogenous VSR proteins in transgenic BY-2 cell lines (Miao *et al.*, 2006). However, based on microarray expression profile analysis (www.genevestigator.com/gv/index.jsp), the seven AtVSRs are not equally expressed in these tissues and thus might suggest distinct functions in various tissues.

1.2 The Plant Endocytosis and Secretory Carrier Membrane Protein (SCAMP)

Endocytosis is an essential process in all eukaryotic cells that involves in the internalization of molecules from the plasma membrane and extracellular environment, plasma membrane recycling, including uptake and the degradation of signal molecules (Mellman, 1996). Recent research has indicated that secretory pathways in both animal and plant cells are integrated closely with their endocytic networks (Figure 1).

Endocytosis plays an important role in polarity and tip growth (Cheung and Wu,

2008; Yang, 2008; Zonia and Munnik, 2008; Wang *et al.*, 2010). For example, the endocytic protein Sla2p/End4, which links the endocytic machinery with the actin cytoskeleton, is crucial in establishing zones of polarized growth in yeast (Wesp *et al.*, 1997; Iwaki *et al.*, 2004). Tip-growing plant cells, such as root hairs and pollen tubes, have persistent polarized growth that depends on both secretory and endocytic pathways. It is believed that tip-growing cells need balanced exocytosis and endocytosis to regulate the amount of plasma membrane at the apices of these cells, and to maintain their highly polarized pattern of growth. Importantly, endocytosis is concentrated in the growing tips of these highly polarized cells (Camacho and Malho, 2003; Cheung and Wu, 2007; Lee and Yang, 2008; Yang, 2008; Wang *et al.*, 2010).

Secretory carrier membrane proteins (SCAMPs) were initially identified as secretory vesicle components in mammalian exocrine glands and later found to be ubiquitous proteins in eukaryotes (Fernandez-Chacon and Sudhof, 2000). In NRK cells, SCAMPs are found in both the TGN and the endosomal recycling compartment, and they appear to be concentrated within the motile population of early and recycling endosomes (Castle and Castle, 2005). Plant SCAMP homologs have been found in rice (*Oryza sativa*), *Arabidopsis*, and pea (*Pisum sativum*) and are thought to be present in many other plant species. Recent studies of rice SCAMP1 protein reveal that SCAMP-labeled tubular-vesicular structures resemble the *trans*-Golgi network in plant cells (Lam *et al.*, 2007a; Lam *et al.*, 2007b). However, little was known about the function of plant SCAMPs.

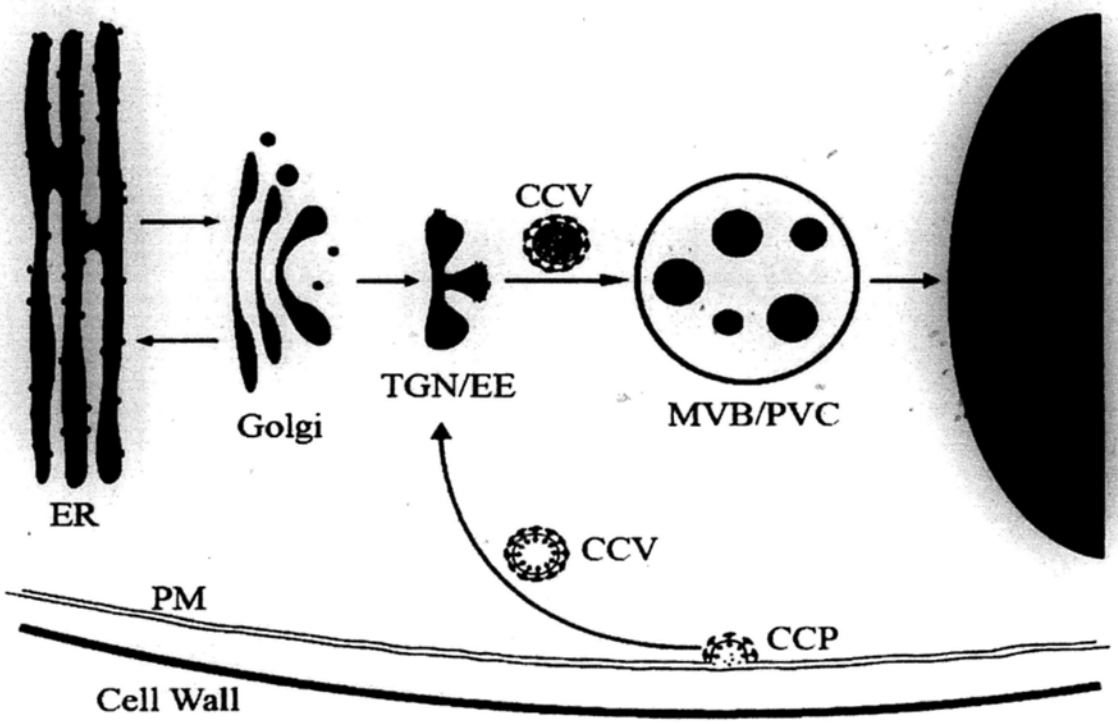

Figure 1. The plant secretory and endocytic pathways.
The proteins sorting to vacuoles begin from ER and proteins are transported to Golgi and finally reach the vacuoles via prevacuolar compartments (PVCs). The endocytosis begins at the plasma membrane (PM) forming clathrin coated pit (CCP), pass through trans-Golgi network (TGN) and reach the vacuole via clathrin-coated vesicles (CCVs).

1.3 Pollen Tube: A Model System for Studying Protein Trafficking and Signal Transduction

Pollen, the male gametophyte of flowering plant, plays an important role in sexual reproduction. Once landing on the sigma for pollination, the pollen grain begins to dehydrate and produces a long pollen tube to deliver two sperm cells towards ovule where the double-fertilization occurs. Pollen tubes elongate rapidly within specific pistil tissues, targeting ovules that are often located at distances thousands of times that of the diameter of the grain away from the sigma (Hepler *et al.*, 2001; Krichevsky *et al.*, 2007). Pollen tube growth is characterized as one of the fastest and polarized growth cell type like the budding yeast and neuron synapse in the world. In addition, pollen tubes from different species show a broad range of growth efficiency. For instance, lily pollen tubes grow at an outstanding average speed of 200-300 nm/sec in vitro. Growth rates ranging from about 25-30 to 80-100 nm/sec have been reported for in vitro-grown tobacco pollen tubes. In vivo, tobacco pollen tubes may reach 4.5 cm within the pistil in approximately 30 h to reach the ovules. The *Arabidopsis* pistil is approximately 2.5 mm long; pollen tubes reach to the most distal ovules about 10-15 h after pollination. In vitro, *Arabidopsis* pollen tubes may attain length as long as 800 μm with in the fist 6 h growth (Hepler *et al.*, 2001; Krichevsky *et al.*, 2007).

Pollen tube growth is restricted to the tube apex. Newly synthesized membrane and cell wall components were packed, sorted and transported via Golgi derived

secretion vesicles to pollen tube apical region. At there, numerous small secretion vesicles whose diameters are usually 100-200 nm will dock and fuse with the plasma membrane to meet the requirement of rapid cell surface-expansion (Figure 2). Meanwhile, active apical endocytosis also occurs to recycle the excessive transporting vesicles and cell wall or membrane material for another around trafficking (Taylor and Hepler, 1997; Hepler *et al.*, 2001; Micheli, 2001; Krichevsky *et al.*, 2007; Yang, 2008). During this process, the apical cell wall of growing pollen tube should be not only strong enough to withstand the internal turgor pressure but also plastic enough to integrate all the membrane and cell wall materials to support directional polarized tip growth (Hepler *et al.*, 2001; Krichevsky *et al.*, 2007; Cheung and Wu, 2008; Yang, 2008; Zonia and Munnik, 2008).

The complexity of tip-growth is evident and the significance of periodicity is intriguing. These essential rheological properties appear to be controlled by pectins, which constitute the principal component of the apical cell wall (Micheli, 2001; Bosch *et al.*, 2005). Current evidence indicates that these pectins are synthesized and methylesterified in the Golgi and thereafter secreted into the wall in a highly methylesterified state via exocytosis pathway. Pectins are subsequently deesterified by the enzyme pectin methylesterase (PME) in a process that exposes acidic residues. These carboxyls can be cross-linked by calcium, which structurally rigidifies the cell wall. Alternatively, it has been proposed that the localized reduction in pH, due to the deesterification process, could promote cell wall extension or growth by stimulating the activity of several cell wall-loosening hydrolases, such as polygalacturonases and

pectate lyases. Both exocytosis and endocytosis should be tightly coordinated and regulated spastically and temporally to support fast pollen tube growth (Micheli, 2001; Bosch *et al.*, 2005).

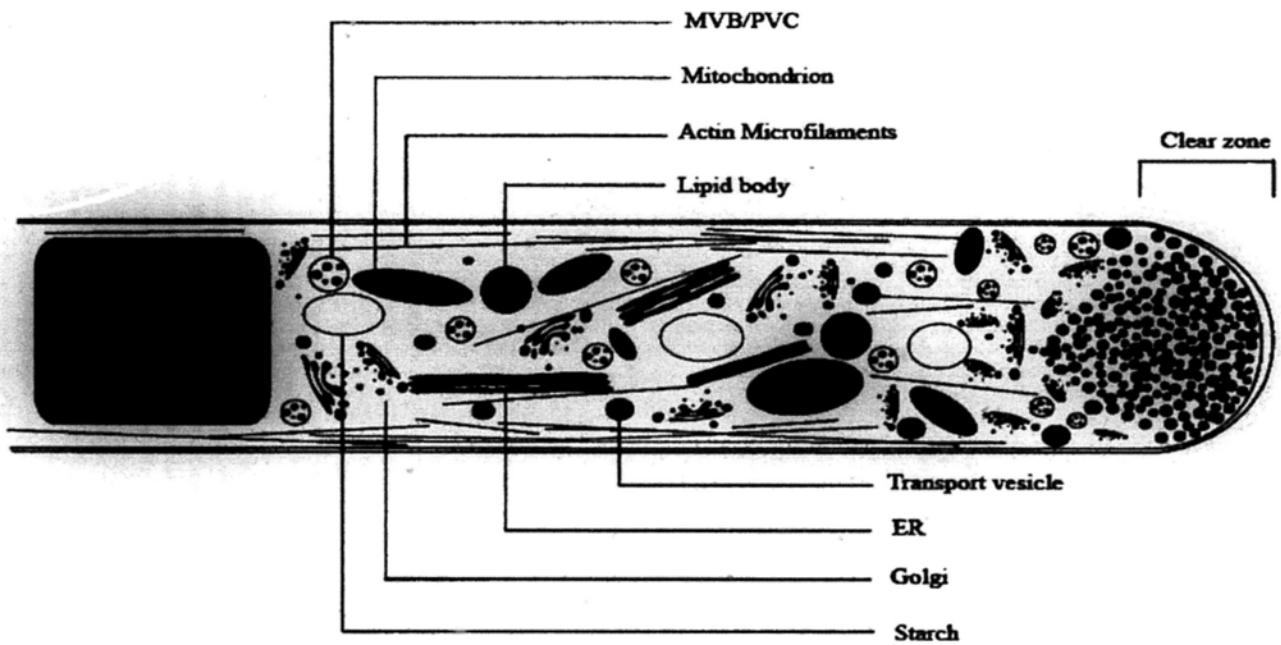

Figure 2. Structure of a pollen tube.
Pollen tube is composed of many organelles which play essential roles in the polarized and rapid growth, these organelles include: mitochondria, actin microfilaments, large vacuoles, endoplasmic reticulum (ER), Golgi, Starch granules, lipid bodies, transport vesicles and clear zone at the apex of pollen tube with enriched Golgi-derived secretory vesicles to meet the rapid pollen plasma membrane and cell wall expansion.

The dynamic organization of the actin cytoskeleton plays an essential role in tip growth. Both pharmacological and genetic perturbation of actin assembly ultimately results in inhibition of polar growth pollen tubes (Samaj et al., 2004). The presence of filamentous actin within the growing tips of pollen tubes remains controversial. The presence of actin filaments has been unambiguously established within the clear zone using several independent approaches such as immunolocalization with actin antibodies, actin visualization with fluorescently labeled phalloidin and GFP recombinant technology (Gibbon et al., 1999; Fu et al., 2001; Lovy-Wheeler et al., 2005). Genetic manipulation of the function of small GTPases including ROP1, ROP2, ROP4, RAC10 and RAB11B resulted in actin aberrations in pollen tubes (Fu et al., 2001; Molendijk et al., 2001; Fu et al., 2002). Recently, the ROP1 target proteins RIC3 and RIC4 (ROP-interactive CRIB motif containing protein 3 and 4) (Wu et al., 2001) were shown to be involved in two counteracting pathways that regulate actin cytoskeleton dynamics in pollen tubes (Gu et al., 2005): in which the RIC4 pathway promotes F-actin assembly while the RIC3 pathway activates calcium signalling leading to F-actin disassembly (Gu et al., 2005). Actin in tip-growing cells may have distinct functions, including delivery of secretory vesicles to the tip, maintenance of clear zone structure and/or supporting of endocytic vesicle uptake (Hepler et al., 2001; Samaj et al., 2004).

The apical plasma membrane of tip-growing pollen tubes is defined as a domain with specific localization of Rho/RAC GTPases, functioning in the organization of actin cytoskeleton, the generation of reactive oxygen species (ROS) and

calcium-dependent signaling. ROP1 is associated with the apical plasma membrane of pollen tubes in different species (Fu *et al.*, 2001; Molendijk *et al.*, 2001; Fu *et al.*, 2002; Gu *et al.*, 2005). In addition, Rho activators such as ROP-GEFs are localized at the apical membrane, where ROP-GEF1 can interact with ROP1 (Gu *et al.*, 2005). So far, apical plasma membrane localization is thought to be the most important factors for the proper function of Rho/RAC proteins in tip growth. However, little is known about the events that lead to the distribution of Rho/RAC proteins to the apex and their maintenance at this location, as well as the mechanism of their retrieval or recycling from the apex. It has recently been proposed that some Rho GTPases and Rho-GEFs interact functionally with receptor-like kinases (RLKs) in pollen tubes (Gu *et al.*, 2005). In light of this hypothesis, one would expect these proteins to be recycled alongside the RLKs via endocytosis. Indeed, in addition to the apical plasma membrane, several ROPs are found to localize in intracellular spots (Jones *et al.*, 2002), indicating their transportation to the apex, or recycling and/or turnover of ROPs via endocytosis in growing pollen tubes.

1.4 Project Objectives

Two striking characters of plant pollen tube are rapid and polarized growth. They make pollen tube as an ideal and model system to study signal transduction, protein dynamics, membrane trafficking, exocytosis/endocytosis and cell wall construction. In order to better study the protein trafficking the secretory pathway and endocytosis process in growing pollen tube, I have chosen two distinct maker proteins for the biosynthetic and endocytic pathways respectively to investigate how pollen tube regulate and coordinate secretion and endocytic processes to maintain its rapid growth rate and polarity.

Specific objectives of this thesis research include the following:

1. To establish a fast transient expression system to study the dynamics and subcellular localization of florescent fusion proteins in growing lily, tobacco and Arabidopsis germinating pollen tubes.
2. To study the dynamics, subcellular localization and function of VSR and SCAMP in lily pollen tube growth.
3. To study the functional roles of VSR and SCAMP proteins in lily pollen tube growth.

CHARPTER 2

Transient Expression and Analysis of Fluorescent Reporter Proteins in Plant Pollens

2.1 Introduction

Pollen, the male gametophyte of flowering plant, plays an important role in sexual reproduction. Upon landing on the sigma for pollination, the pollen grain produces a long pollen tube to deliver sperm to the ovule for fertilization. Pollen tube growth is an extremely polarized and highly dynamic cellular process, making it an ideal single-cell model system for studying molecular mechanisms of signal transduction, protein trafficking, vesicular secretion and endocytosis in plant cells (Taylor and Hepler, 1997; Hepler et al., 2001; Samaj et al., 2006; Krichevsky et al., 2007). Several approaches have been used to transformed genes into pollen grains or germinating pollen tubes in these studies, including micro-injection of plasmid DNA into pollen cells (Raghavan, 1997) and transgenic plants-derived pollens. However, since it takes at least several months to generate transgenic plants including *Arabidopsis*, tobacco, maize and rice via *Agrobacterium*-mediated transformation (Nishimura et al., 2006; Sparkes et al., 2006; Zhang et al., 2006; Ishida et al., 2007) while the microinjection of pollen tubes is a technically challenging and low-efficiency approach, transient expression of pollen grains has thus recently became an alternative and more attractive system for studying protein localization and organelle dynamics in germinating pollens (Zhang et al., ; Twell et al., 1989; de Graaf et al., 2005; Helling et al., 2006; Xiang et al., 2007; Cheung et al., 2008; Lee et al., 2008; Vidali et al., 2009).

Transient expression of fluorescent fusion proteins has been useful tool for

quickly illustrating organelle dynamic and protein localization in various plant cell types and tissues using various methods of DNA delivery, including electroporation- or PEG-mediated transient expression of protoplasts derived from suspension cultured cells (Lippincott-Schwartz et al., 2001; Sheen, 2001; Miao and Jiang, 2007; Yoo et al., 2007) and *Agrobacterium* infiltration of tobacco (Sparkes et al., 2006). However, all these methods cannot be simply applied to pollen grains because of the pollen cell wall and coat, that function in protecting pollen from various environmental conditions whereas the coat surface molecules provide contact and initiate the signaling necessary for successful adhesion to stigma and pollen tube germination (Taylor and Hepler, 1997). Removal of pollen coat and cell wall will result in poor pollen germination. Therefore, transient expression of fluorescent reporter proteins in growing pollen tubes via biolistics has been wildly used for studying protein dynamics, membrane trafficking, cytoskeleton organization, signal transduction and exocytosis or endocytosis in pollen tubes of different plant species (Zhang et al., ; Twell, 1991; Kost et al., 1998; Fu et al., 2001; Cole et al., 2005; de Graaf et al., 2005; Okada et al., 2005; Xiang et al., 2007; Cheung et al., 2008; Hwang et al., 2008; Lee et al., 2008; Vidali et al., 2009).

Here, I have tested and optimized a pollen grain transient expression procedure and *in vitro* species-specific pollen tube germination media/conditions for pollens of three commonly used plant species: lily, tobacco and *Arabidopsis* (Table 2). The application of this protocol consists of four essential stages: 1) coating gold microcarrier with plasmid DNA, 2) preparation of pollen grains for transient

expression, 3) transformation of DNA into pollen gains via particle bombardment, and 4) *in vitro* of pollen germination for dynamic analysis of fluorescent-tagged proteins in germinating pollen tubes. Using this protocol, more than 95% of the lily and tobacco pollen grains as well as 80% of the *Arabidopsis* pollen grains are capable of germinating into pollen tubes respectively whereas about 10% of the germinated lily and tobacco pollen tubes and 5-6% of the bombarded *Arabidopsis* pollen tubes show good fluorescent signals several hours after the bombardment. As a proof of principle, I used this protocol to test known protein markers of prevacuolar compartment (PVC) and apical endocytosis (Wang et al., 2010) for their subcellular localization, dynamics as well as their response to drug treatments in germinating pollen tubes, as compared to those previously obtained from transgenic tobacco BY-2 and *Arabidopsis* suspension cells (Jiang and Rogers, 1998; Tse et al., 2004; Mayo et al., 2006; Miao and Jiang, 2007; Lam et al., 2007a; Lam et al., 2007b; Lam et al., 2008; Miao et al., 2008). Identical results were obtained when the same fluorescent reporter proteins were transiently expressed either in germinating pollen tubes or in tobacco BY-2 and *Arabidopsis* protoplast (Miao and Jiang, 2007; Miao et al., 2008). This protocol is also compatible with pharmaceutical treatments (such as wortmannin treatment, see ANTICIPATED RESULTS).

Since it takes several months to generate transgenic plants to obtain the needed transgenic pollen grains for dynamics studies in germinating pollen tubes (Hicks et al., 2004; de Graaf et al., 2005), this transient expression approach will therefore represent an attractive alternative approach for studying large amount of proteins in

germinating pollen tubes quickly. In addition, since germinating pollen tubes are highly dynamics in endocytosis and exocytosis (Yang, 2008) as well as an excellent single-cell system (Cheung and Wu, 2008), this protocol thus introduces a very fast and reliable transient expression system for studying protein trafficking, protein localization, organelle dynamics, and protein-protein interaction in the rapid tip-focused growing pollen tubes of various plant species including the three plant models of lily, tobacco and Arabidopsis.

2.2 Experiment Design

2.2.1 Selection of Pollen Specific Promoter and Expression Vector

To increase the transient expression efficiency of the fluorescent reporter proteins in germinating pollen tubes, pollen or pollen tube specific promoters *ZM13* (Hamilton et al., 1992; Hamilton et al., 1998; Hamilton, 1998) or *Lat52* (Twell et al., 1991; Muschietti et al., 1994) rather than the constitutive cauliflower mosaic virus (CaMV 35S) promoter (Miao and Jiang, 2007), are used in this protocol. *ZM13* promoter and *Lat52* is derived from maize and tomato respectively and have been successfully used to control the expression of various proteins in pollen grains or pollen tubes of monocot and dicot (Wang et al., 2010; Twell, 1991; Muschietti et al., 1994; Hamilton et al., 1998; Hamilton, 1998). The expression vectors for transient expression should

have a high expression level and small in size (about 3-6 kb). In this protocol, I usually use constructs derived from the pBI221 under the control of the *ZM13* promoter for lily pollens or the *Lat52* promoter for tobacco or *Arabidopsis* pollens and the nopaline synthase (*NOS*) terminator (Clontech).

2.2.2 DNA quality

DNA used for transient expression in pollen grains or pollen tubes demands high purity and high quality. Conventional DNA preparation method was usually used, in which bacteria are lysed by lysozyme-Triton solution, followed by plasmid DNA purification via phenol/chloroform extraction. Generally, purified DNA shall have an OD_{260}/OD_{280} value close to 1.8. In addition, plasmid DNA with higher quality will further improve the transformation and expression efficiency. Therefore, both CsCl-gradient centrifugation methods (Bosch et al., 2005) and maxi-DNA (Qiagen) preparation kit were usually used with higher expression efficiency in this protocol.

2.2.3 Pollen tube germination ratio

The pollen tube germination ratio is one of the key factors that significantly affect the efficiency of transient expression. Factors such as germination medium, temperature and plant species determine the germination rate of pollen grains. In this protocol, three types of pollen grains from lily, tobacco and Arabidopsis were tested for

transient expression, because pollen grains from different plant species require species-specific *in vitro* germination medium and conditions. Table 2 summarizes the optimized germination media and conditions for these three types of pollen grains tested in this protocol, with an *in vitro* pollen germination rate of 95% for lily and tobacco or 80% for Arabidopsis.

2.3 Material and Methods

2.3.1 Reagents

- Lily (*Lilium longiflorum*), Tobacco (*Nicotiana tabacum*) or *Arabidopsis thaliana* (Columbia 0) pollen grains,
- Sucrose (Fluka, cat. no. 84097)
- H_3BO_3 (USB, cat. no. 10043-35-3)
- $CaCl_2$ (Ajex chemical, cat. no. 960)
- KNO_3 (Sigma-Aldrich, cat. no. P-8291)
- $MgSO_4$ (Sigma-Aldrich, cat. no. M2643)
- Agar (Sigma-Aldrich, cat. no. A-7921)
- 1.0 μm gold microcarrier (Bio-Rad, cat. no. 165-2263)
- Spermidine (Sigma-Aldrich, cat. no. S-2626)
- $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, cat. no. M1880)
- $CaCl_2 \cdot 2H_2O$ (Ajax Chemicals, cat. no. 127)

- Ethanol (Merck, cat. no.1.00983.2511)

2.3.2 Reagent Setup

●Pollen grains preparation:

Lily (*Lilium longiflorum*) plants are grown in the green house of the Chinese University of Hong Kong according to standard conditions. Mature pollen grains are collected from anthers and after air dried for one day at room temperature, the pollen grains are then stored at 4 °C before use. Tobacco (*Nicotiana tabacum*) plants are grown in green house at 22 °C under a light cycle of 12 hr light and 12hr darkness (Fu et al., 2001). *Arabidopsis thaliana* plants are grown in growth chambers under standard condition (Detlef Weigel, 2002). Fresh pollen grains were collected from these individual plants and used for transient expression via particle bombardment.

'CRITICAL' In order to maintain a high germination rate, the storage time for lily pollen grains should not be longer than 7 days at 4 °C. Longer storage of the pollen grains at 4 °C will significantly reduce the pollen germination rate. To obtain high germination ratio for tobacco and Arabidopsis pollen grains, freshly collected samples for biolistics from these two plant species are highly recommended.

- Pollen tube germination buffer:

To germinate pollen grains into pollen tubes *in vitro*, pollen grains are suspended in the species-specific germination medium as listed in Table 2.

'A CRITICAL' The pollen germination medium should be freshly prepared just

prior to use or sterilized via autoclave or filtration for long time storage because the medium contains sucrose.

- Spermidine solution:

0.1 M spermidine working solution can be prepared by diluting 1 M spermidine stock solution with sterilized double-distilled H_2O.

▲ CRITICAL 1 M spermidine stock solution should first be prepared to avoid degradation. Sterilize the solution by filtration through a 0.22 mm syringe filter and store at -80°C. For working solution, dilute 1 M spermidine to 0.1 M with sterilized double-distilled H_2O, aliquot 100 ml each and store at -20 °C.

- Gold microcarrier solution:

To prepare 60 μg $liter^{-1}$ microcarrier solution, 1.0 μm gold particles were first washed with absolute ethanol and vortexed continuously for 3 min. The gold particles were spin down at max speed for 1min with a bench top centrifuge, followed by discarding of the supernatant and washing with distilled water twice. Finally, the particles were re-suspended in 50% (wt/vol) glycerol and stored at -20 °C.

2.3.3 Equipment

- PSD-1000/He particle delivery system (Bio-Rad, cat. no. 165-2257)
- Confocal microscopy (Leica, TCS SP5)
- Macrocarriers (Bio-Rad, cat. no.165-2335)
- Rupture disk (Bio-Rad, cat. no.165-2329)
- Stopping screen (Bio-Rad, cat. no.165-2336)

- Shaker set at 85 r.p.m. at 27.5 °C
- 20 ml syringes
- 0.22 m syringe filter
- Petri dishes (85 mm x 15 mm)
- 50 ml conical tubes
- Bench top centrifuge
- Water bath set at 22.5 °C
- Confocal dish (coverglass bottom dish) (Life Scientific, cat. no. SPL-100350)
- Funnel Buchner (Nalgene, cat. no.4280-0700) and 500 ml filtering flask (Nalgene, cat. no. DS4101-0500)
- Microscope slide and cover slide
- 70 mm filter paper (Whatman, cat. no. 1001070)

Vacuum pump (VacuGene XL Blotting Pump 220 VAC, Amersham Biosciences, cat. no. 80-1265-15) In order to get an even distribution of the pollen grains on the filter paper, the pump should work gently and slowly.

2.3.4 Equipment Setup

- PSD-1000/He particle delivery system

The settings of the PSD-1000/He particle delivery system are as follow: 1100 psi, 29-mm Hg vacuum, 1-cm gap distance and 9-cm particle flight distance.

2.3.5 Procedure

Coat gold particle with plasmid DNA 30 min

1| Vortex gold mircocarriers solution extensively for 3 min. Pipette out 1.5 mg (25 µl) gold particles into a new 1.5-ml eppendorf tube.

'? TROUBLESHOOTING'

2| Firstly, vortex mixing the gold particles with 10 µl of 0.1 M spermidine. Secondly, add 5 µl of 1µg/µl plasmid DNA and keep vortexing for another 1 min. Thirdly, slowly add 25 µl of 2.5 M $CaCl_2$ solution and at the meantime keeping constant mixing for 3 min.

▲ The order for the addition of solutions should be followed as described, because spermidine will first give gold particles positive electronic charges so that the negatively charged plasmid DNA can bind to the gold microcarriers subsequently, finally $CaCl_2$ can precipitate the DNA on the microcarriers.

'? TROUBLESHOOTING'

3| Spin down the gold mircocarriers using a bench top centrifuge at max speed for 5 sec and remove supernatant. Wash with 200 µl of absolute ethanol and spin down at max speed for 5 sec.

4| Re-suspend the gold particles in 18 µl of absolute ethanol and aliquot 6 µl particles suspension onto three macrocarriers and let them air dry.

'▲ CRITICAL' It is essential to completely re-suspend the gold particles by

vortexing or pipetting up and down with the pipette tips. Make sure that the gold particles are evenly distributed in the center of macrocarriers. Preventing the gold particles from forming aggregations is crucial for achieving high transformation efficiency.

'? TROUBLESHOOTING'

Preparation of pollen grains for transient expression 10 min

5| Harvest anthers from 2 lily flowers, 15-20 tobacco flowers or 40-50 Arabidopsis flowers and transfer them into 20 ml species-specific pollen germination medium in a 50-ml Falcon tube. Vortex vigorously 1 min to release the pollen grains into the medium and remove the anthers with forceps.

'? TROUBLESHOOTING'

6| Connect the vacuum pump with the filtering flask with robber pipes and put the funnel Buchner in the filtering flask tightly. To prepare lily and tobacco pollen grains for bombardment, pre-wet a 70 mm filter paper with pollen germination medium, turn on the vacuum pump and adjust the vacuum to 40 mbar. Directly vacuum filtrate the 20 ml pollen grain suspension onto the pre-wetted filter paper so that the pollen grains are collected and eventually distributed onto the filter paper. To prepare Arabidopsis pollen grains for bombardment, because of its less abundance as compared to lily or tobacco pollen grains, it is necessary to spread the Arabidopsis pollens on a smaller area of the filter paper. Firstly use a pencil to draw a square of 20 mm×20 mm in the center of the 70 mm filter paper. Secondly pre-wet the filter paper with germination medium, turn on the vacuum pump and adjust the vacuum to

40 mbar. Thirdly transfer the suspended Arabidopsis pollen grains with a pipetman from the Falcon tube onto the marked 20 mm×20 mm square. In order to have an even distribution of the Arabidopsis pollen grains on the marked area of the filter paper, individual droplets of pollen grains should be added one by one to different positions of the marked area and allow each droplet to be completely stick on the filter prior to applying the next one. The filter paper with the pollen grains facing up is then transferred onto an 85mm-Petri dish containing 2% agar.

'▲ CRITICAL' To avoid losing of pollen grains from the filter paper during vacuum, make sure that there is no air bubble between the pre-wetted filter paper and funnel Buchner.

Transfer DNA into pollen grains via particle bombardment 30 min

7| The settings of the PSD-1000/He particle delivery system are as follow: 1100 psi, 29-mm Hg vacuum, 1-cm gap distance between the rupture disk and macrocarrier and 9-cm particle flight distance between macrocarrier and pollen grain samples.

8| Bombard the pollen grains on the filter paper for three times at three different positions in order to increase the transformation efficiency. Immediately after the bombardment, identify the bombarded areas that are visible on the filter paper because of the brown color of gold particles. Then, use a knife to cut out the filter paper about 2 mm wider along the bombarded areas and use 5 ml of pollen germination buffer to wash the bombarded pollen grains into a 50-ml conical tube (for lily and tobacco pollen germination), or use 250-300 µl of Arabidopsis pollen germination medium to wash the bombarded Arabidopsis pollen grains into a 1.5 ml

eppendorf tube for germination.

'! CAUTION' Wear safety glasses when operating the PSD-1000/He system because potentially dangerous conditions like high pressure gas and high speed particles associated with the system.

Pollen tube germination *in vitro* 2-10 h

9| Germinate the bombarded pollen grains into pollen tubes in the species-specific germination medium and conditions (Table 2) for 2-10 hours prior to observation for fluorescent signals.

'! CAUTION' The expression efficiency and fluorescent signal intensity are gene and pollen species-dependent.

Step 1-2, 30 min

Step 3-6, 10 min

Step 7-8, 30 min

Step 9, 2-10 hr

TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1. Troubleshooting table.

STEP	PROBLEM	POSSIBLE REASON	SOLUTION
5	Low pollen tube germination rate	Un-fresh pollen grains or long-period storage of pollen grains	Use freshly collected pollen grains.
2	No expression	Un-suitable promoter	Use pollen specific promoter such as ZM13 or Lat52 promoter instead of the CaMV 35S promoter.
5		Low germination rate of pollen grains	Use fresh grains or pollen grains stored at 4 °C less than 7 days for lily. Use freshly collected pollen grains of tobacco or Arabidopsis.
2		Poor DNA quality	Use higher quality plasmid DNA prepared from commercially available max plasmid extraction kit or CsCl-gradient centrifugation methods.
1, 2	Low expression efficiency and level	Low efficiency of DNA coating onto gold microcarrier	(i) Vortex the gold particle solution extensively before use to prevent aggregation of gold particles. (ii) During the procedure of microcarrier coating, maker sure to follow the proper order of mixing solutions: firstly microcarrier, secondly spermindine, thirdly plasmid DNA and finally $CaCl_2$. Keep vortexing throughout the whole procedure.
4		Aggregation of gold particles on macrocarriers	Before loading gold particles onto the macrocarriers, completely re-suspend the gold particles by vortexing or pipetting up and down with the pipette tips to prevent aggregations. Make sure that the gold particles are evenly distributed in the center of the macrocarriers.

2.4 Anticipated Results

This protocol describes a rapid transient expression method for introducing fluorescent reporter proteins into pollen grains of lily, tobacco and Arabidopsis to study protein localization and organelle dynamics in germinating pollen tubes, which is one of the fascinating single cell model systems in polar growth studies (Taylor and Hepler, 1997; Hepler et al., 2001; Samaj et al., 2006; Krichevsky et al., 2007). Transient expression of fluorescent fusion proteins can be achieved by transferring DNA constructs into the pollen grains via particle bombardment. These constructs are usually under the control of the pollen specific promoter *ZM13* (Hamilton et al., 1992; Hamilton et al., 1998; Hamilton, 1998) for lily pollen grain or *Lat52* (Twell, 1991; Muschietti et al., 1994) for tobacco and Arabidopsis pollen grains in pBI221-derived expression vectors (Jiang and Rogers, 1998). Subsequently, subcellular localization, dynamic study and drug treatments can be carried out on germinating pollen tubes. More than 95% of the tested lily and tobacco pollen grains and 80% of Arabidopsis pollen grains can germinate (Figure 3a and Figure 4. a, c and e) whereas about 10% of the lily and tobacco pollen tubes expressing a cytosolic GFP construct showed good fluorescent signals 1-2 hrs after transformation (Figure. 3a/b). Owing to relatively lower pollen tube germination ratio and smaller size of pollen grains, only about 5-6% of the *Arabidopsis* pollen tubes expressing a cytosolic GFP construct showed good fluorescent signals at about 8-10 hrs after transformation (Figure 3a/b and Figure 4e).

As a proof of principle, I transferred and tested GFP and a GFP fusion protein with a vacuolar sorting receptor (VSR) BP-80 (GFP-BP-80) that is known to locate to prevacuolar compartments (PVCs) of the plant secretory pathway in both tobacco BY-2 and *Arabidopsis* suspension culture cells (Jiang and Rogers, 1998; Tse et al., 2004; Miao et al., 2006; Miao and Jiang, 2007; Miao et al., 2008), and the lily secretory carrier membrane protein (LlSCAMP) that is know to involve in the endocytosis during pollen tube growth (Wang et al., 2010). When the soluble GFP was transiently expressed in the lily pollen tubes, the fluorescent signal was found in the whole germinating pollen tube in a diffused pattern as expected for cytosolic proteins (Figure 3c). The expressed GFP-BP-80 fusion proteins exhibit the typical PVC punctate patterns throughout most of the germinating pollen tube except in the inverted cone region at the pollen tube apex (Figure 3d, as indicated by an asterisk). Interestingly, when an uptake study was carried out using the endocytic pathway marker FM4-64 (Lam et al., 2007a) in germinating pollen tube expressing the GFP-BP-80, the internalized FM4-64 was found mainly in the inverted cone region lacking the GFP-BP-80 signals (Figure 3e), a result indicating the distinct difference between the GFP-BP-80-marked secretory pathway and the FM4-64-marked endocytic pathway in germinating pollen tubes. In addition, the punctate GFP-BP-80 signals may represent the PVCs because wortmannin treatment (+Wort) also cause them to form ring-like structures in germinating pollen tubes (Figure 3f), a unique feature of PVCs in both tobacco BY-2 and *Arabidopsis* culture cells (Tse et al., 2004; Miao et al., 2006; Miao and Jiang, 2007; Miao et al., 2008). Furthermore, the

GFP-BP-80 proteins are highly dynamics and extremely active, moving along with the cytoplasmic streaming in pollen tube which exhibit vigorous "reverse fountain" motion. In contrast, the expressed RFP-LlSCAMP proteins are highly concentrated on the tip region of a growing pollen tube (Figure 3h), consistent with the pattern of the endocytic marker FM4-64 dye where active and vigorous endocytosis occurs. When co-expressed together in the same lily pollen tube, GFP-LlVSR and RFP-LlSCAMP show distinct pattern of distribution in which GFP-LlVSR (green) is missing from the tip region with rich RFP-LISCAMP (red) signals (Figure 3i).

With optimized specie-specific germination media and condition for high germination rate of pollen grains (Table 2), this protocol also applies successfully to both tobacco and Arabidopsis pollen grains. More than 95% of the tobacco pollen grains and 80% of the Arabidopsis pollen grains germinated under these media/conditions (Figure 4a/c). When an endoplasmic reticulum (ER) reporter GFP-HDEL (His-Asp-Glu-Leu, an ER retention signal) was transiently expressed in germinating tobacco pollen tube under the control of the Lat52 pollen specific promoter, typical ER network pattern was exhibited (Figure 4b). An overview of a 3-D projection of Arabidopsis pollens grains transformed with the cytosolic GFP was shown in Figure 4. e. Furthermore, when the GFP-AtSCAMP4 was transiently expressed in germinating Arabidopsis pollen tube, similar to RFP-LISCAMP expressing in lily pollen tube (Figure 4d), the signals were mainly found in the tip region (Figure 4d). Taken together, all these results from germinating pollen tubes of lily, tobacco and Arabidopsis are consistent with those obtained from other plant

cells (Wang et al., 2010; Tse et al., 2004; de Graaf et al., 2005; Miao et al., 2006; Miao and Jiang, 2007; Lam et al., 2007a; Lam et al., 2007b; Miao et al., 2008).

TABLE 2. Species-specific germination media and culture conditions.

SPECIES	MEDIUM COMPOSITION	CONDITIONS & TIME	REFERENCES
Lily (*Lilium longiflorum*) pollen grains	1.3 mM Boric Acid 2.9 mM KNO_3 9.9 mM $CaCl_2$ 10% (wt/vol) sucrose pH 5.8	27.5 °C 85 rpm/min Shaker 1.5-2.5 hr	(Wang et al., 2010; Parton et al., 2001; Lovy-Wheeler et al., 2006; Xiang et al., 2007; Wang et al., 2008)
Tobacco (*Nicotiana tabacum*) pollen grains	0.01% Boric Acid 1mM $CaCl_2$ 1mM $Ca(NO_3)_2 \cdot 4H_2O$ 1mM $MgSO_4 \cdot 7H_2O$ 10% (wt/vol) sucrose pH 6.5	27.5 °C 85 rpm/min Shaker 2-3 hr	(Kost et al., 1998; Fu et al., 2001; de Graaf et al., 2005; Hala et al., 2008; Lee et al., 2008)
Arabidopsis thaliana pollen grains	0.01% Boric Acid 5mM KCl 1mM $MgSO_4$ 5mM $CaCl_2$ 10% (wt/vol) sucrose pH 7.5	22.5 °C Water bath 8-10 hr	(Fan et al., 2001; Molendijk et al., 2001; Cole et al., 2005; Boavida and McCormick, 2007; Hala et al., 2008; Hwang et al., 2008)

Lily pollen tubes

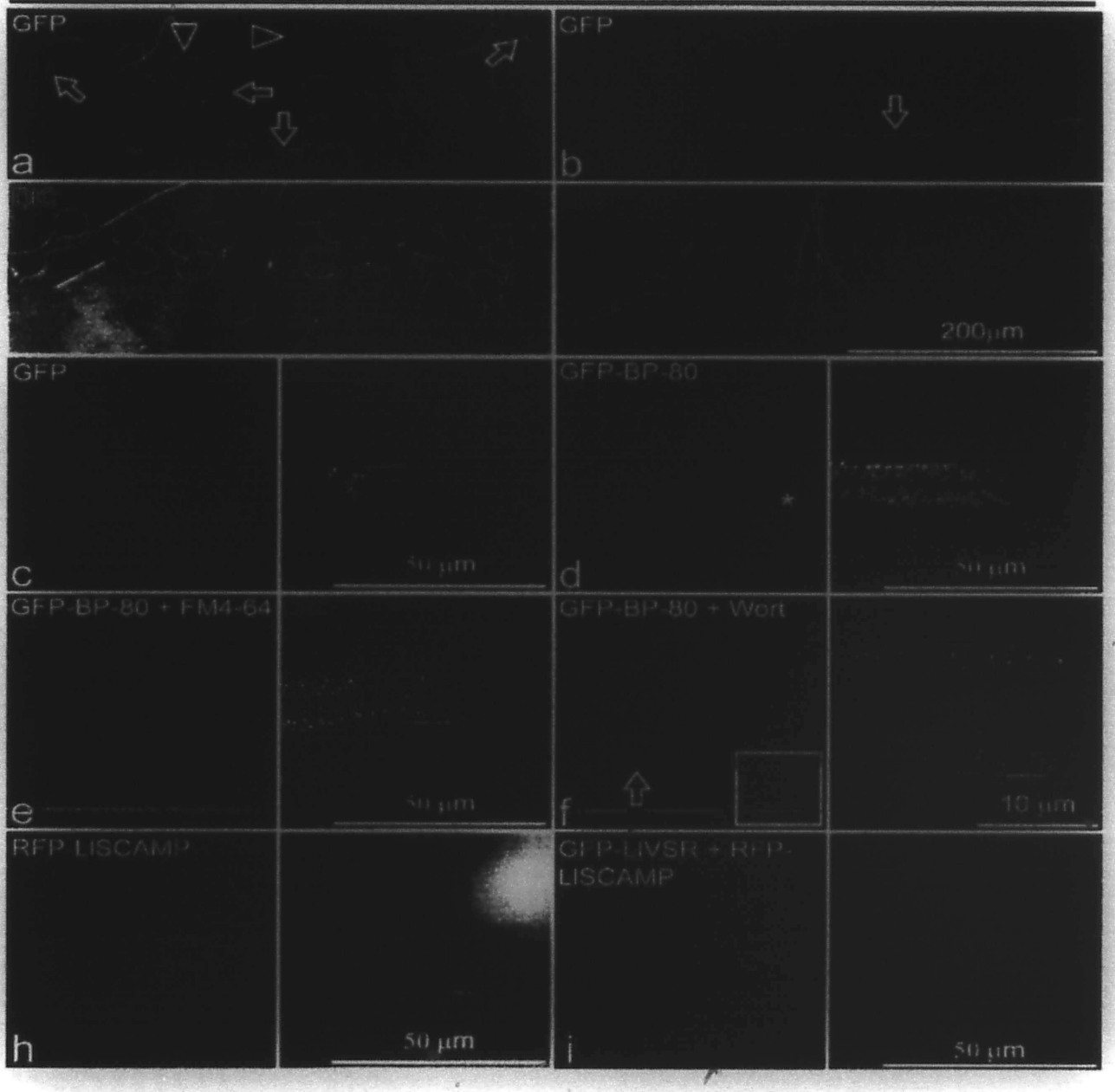

Figure 3. Transient expression of fluorescent reporter proteins in growing lily pollen tubes.

(a) & (b) Transient expression of cytosolic GFP in germinating lily pollen tubes with an efficiency of about 10%. Arrows indicate examples of germinating pollen tubes with fluorescent signals, whereas the signals from pollen grains (arrowheads) are due to autofluorescence of the pollen coats. (c) Cytosolic localization of the expressed GFP in germinating lily pollen tube. (d) Localization and dynamics of the transiently expressed GFP-BP-80, a reporter for prevacuolar compartment (PVC), in germinating lily pollen tube. Asterisk indicates the tube apex lacking the GFP signals. (e) Germinating lily pollen tube expressing the GFP-BP-80 was incubated with the endocytic marker FM4-64 dye (red) for 10 min before image collection. (f) Lily pollen tube expressing the GFP-BP-80 was treated with wortmannin (Wort) at 8.25 μM for 30 min before image collection, showing the wortmannin-induced enlarged PVC (arrow). (h) Localization of RFP-LlSCAMP, a reporter for endocytosis, in germinating lily pollen tube. (i) Co-expression of LlVSR and RFP-LlSCAMP in germinating lily pollen tube.

BP-80, the transmembrane domain and cytoplasmic tail of the pea binding protein 80 kDa; LlSCAMP, *Lilium longiflorum* secretory carrier membrane protein; LlVSR, *Lilium longiflorum* vacuolar sorting receptor; DIC, differential interference contrast; GFP, green fluorescent protein; *NOS*, nopaline synthase terminator; Wort, wortmannin.

Tobacco
pollen tubes
GFP-HDEL
b
25 µm
Arabidopsis pollen tubes
GFP-AtSCAMP4
d
12.5 µm
GFP
e
DIC
400 µm

Figure 4. Transient expression of fluorescent reporter proteins in growing tobacco and Arabidopsis pollen tubes.

(a) The germination rate of tobacco pollen grains was about 95% (as determined by the growth of pollen tube after 2.5 hrs incubation in the tobacco germination medium). (b) Expression and subcellular localization of an ER fluorescent reporter GFP-HDEL in germinating tobacco pollen tube, showing typical ER network pattern. (c) The germination rate of Arabidopsis pollen grains was more than 80% (as determined by the growth of pollen tube after 8 hrs incubation in the Arabidopsis germination medium at 22.5 ^{0}C). (d) Expression and subcellular localization of a GFP fusion with the Arabidopsis SCAMP4 (GFP-AtSCAMP4) in germinating Arabidopsis pollen tube, the signals were mainly found in the inverted cone region of the pollen tip and plasma membrane with some punctuate signals in the sub-apical region. (e) An overview of a 3-D image of green florescent signals from germinating Arabidopsis pollen tubes and pollen grains. Arabidopsis pollen grains (Columbia 0) were transformed with the cytosolic GFP construct and germinated for 10 hours in optimized Arabidopsis pollen grain germination condition before confocal imaging.

AtSCAMP4, Arabidopsis secretory carrier membrane protein4; HDEL, His-Asp-Glu-Leu, an ER retention signal; PVC, prevacuolar compartment; DIC, differential interference contrast; GFP, green fluorescent protein.

CHAPTER 3

Vacuolar Sorting Receptors (VSRs) and Secretory Carrier Membrane Proteins (SCAMPs) are Essential for Pollen Tube Growth

3.1 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, and 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 (Shimada et al., 2003; 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, I have chosen to investigate the location and functionality of two different proteins which 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 (TMD) that are ubiquitously found in many eukaryotes including nematodes, insects, fish, amphibia, mammals and in both monocot and dicot plants, but not 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 SCAMP1 (OsSCAMP1) and its YFP fusions were expressed in tobacco BY-2 cells, the fluorescent proteins were found to localize to the plasma membrane (PM), to an early endosome (EE) - identified as the *trans*-Golgi network (TGN), and also to secretory vesicles (Lam *et al.*, 2007a; Lam *et al.*, 2007b; Toyooka *et al.*, 2009b). Interestingly, SCAMPs also accumulate in the PM of the developing cell plate during cytokinesis (Lam *et al.*, 2008; Toyooka *et al.*, 2009a). 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 (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, 2005). This is a type I integral membrane protein with a single transmembrane domain (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, colocalizes with endogenous VSR proteins at a multivesiculate, prevacuolar compartment (PVC) (Jiang and Rogers, 1998; Tse et al., 2004; Tse et al., 2006). This demonstrates that the TMD and CT are sufficient for VSR targeting. Indeed, using a similar reporter system, all seven *Arabidopsis* VSRs (AtVSR1-7) were found to colocalize with endogenous VSRs to the PVC in both tobacco BY-2 and Arabidopsis cells (Miao et al., 2006; Miao et al., 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.

I show here that the dynamics and subcellular localizations of VSR and SCAMP in lily 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 except at 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 *LlVSR* siRNAs severely inhibited the growth rate of the lily pollen tubes, pointing to essential roles for VSR and SCAMP in pollen tube growth.

3.2 Materials and Methods

3.2.1 Plant Materials, Pollen tube germination and Chemicals

Lily (*Lilium longiflorum*) 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, 1.3mM H_3BO_3, 2.9mM KNO_3, 9.9mM $CaCl_2$, pH 5.8 at 27.5°C for 45 min. Stock solutions of wortmannin (2.5mM in DMSO; Sigma-Aldrich, St. Louis, MO, U.S.A), BFA (1mM in DMSO; Sigma-Aldrich, St. Louis, MO, U.S.A) and Latrunculin B (1mM in DMSO; Sigma-Aldrich, St. Louis, MO, U.S.A) were aliquoted and stored at -20°C.

3.2.2 Cloning of LlVSR and LlSCAMP 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. cDNA was synthesized from each RNA sample using the Invitrogen SuperScript III first-strand synthesis system for RT-PCR using the oligo(dT) primer according to the kit's instructions. A Clontech SMART RACE cDNA Amplification Kit 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*, rice *SCAMPs*, two primers (forward primer, 5'-ATGAGAACTGAGAGTGCTTTG-3'; reverse primer, 5'-GGCCACG CGTCGACTAGTACTTTTTTTTTTTTTTTT-3) were used to get the 3' end of *LlSCAMP*. Based on the known 3' end gene sequence another two primers (forward primer was provided and used according to the kit's instructions; reverse primer, 5'-GTGCTGCCTCGCGCTTCAACTCTGCAGC-3') were used to get the 5' end of *LlSCAMP*. The *LlSCAMP* gene was then amplified from lily germinating pollen tube cDNA with two primers with restriction enzyme XbaI/NcoI sites (forward primer, 5'-GGGTCTAGAATGGCGGGCCGCTACGACAGC-3'; reverse primer, 5'-GGGC CATGGTAATGTTGCCCTCAAGGCACCAC-3') and then *LlSCAMP* gene was subcloned into *ZM13pro-GFP* transient expression vector with the same restriction sites. A similar strategy was also used to clone the *LlVSR* gene from germinating lily pollen tubes based on the alignment of Arabidopsis *VSRs*, rice *VSRs*, *BP-80* and pumpkin *PV70*. *LlVSR* gene spacer with TMD and CT region was then amplified with two primers with restriction enzyme XbaI/NcoI sites (forward primer, 5'-GGGTCTAGAACATGCATCAGTAAAAAACC-3'; reverse primer, 5'-GGG CCATGGTCATATATCGCCATGCGATACG-3') and then *LlSCAMP* gene was subcloned into *ZM13pro-GFP* transient expression vector with the same restriction sites.

3.2.3 Particle Bombardment of Pollen

Ten anthers were harvested from 2 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 were vacuum filtrated onto a prewetted filter paper. The filter paper covered with the pollen grains was then immediately transferred surface-up onto 2% agar in a 85mm-Petri dish. For 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 for 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 hours before observation of fluorescent signals.

3.2.4 Electron Microscopy 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 frozen immediately in a high–pressure freezing apparatus (EMP2; Leica). Immunogold EM labeling was carried out using VSR, BP-80 CT and SCAMP1 antibodies at 40 μg/mL 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 Technologies) operating at 80 kV.

3.2.5 Protein Extraction, Protein Gel and Immunoblotting

Harvested lily pollen grains were germinated in pollen germination medium for 2 h. Germinated lily pollens were grinded into 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 leupeptin) and western blot analysis with various antibodies at 4 mg/mL as previously described (Tse et al., 2004; Lam et al., 2007a).

3.2.6 Design and Synthesis of LlVSR and LlSCAMP siRNAs

LIVSR siRNA was designed based on its *LIVSR* gene sequence. Double-strand 21 nt length siRNA was designed with 3' overhang TT dinucleotides and synthesized by the Sigma Company. 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, 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 were scrambled and thereby lacking any significant sequence homology to other genes. To make sure the designed siRNA

specifically targeted the *LlVSR* gene, I compared the potential target sites in the plant genome database and eliminated from consideration any target sequences with of homology genes or other coding sequences in the database. Additional one more *GFP* siRNA was included as another negative control which is commercially available from Invitrogen and was proved to be effective in down-regulating *GFP* expression (Caplen et al., 2001). The sequences of siRNAs are as follows: *LlVSR* siRNA1 (sense, 5'-UAGGCAAUACAGAGGUAAATT-3'; anti-sense, 5'-UUUACC UCUGUAUUGCCUATT-3'); *LlVSR* siRNA2 (sense, 5'-GUACCGAAUCAGGA GUUACTT-3'; anti-sense, 5'-GUAACUCCUGAUUCGGUACTT-3'); *LlVSR* scramble siRNA (sense, 5'-GACGAAGGUACGAAUAUAATT-3'; anti-sense, 5'-GUAACUCCUGAUUCGGUACTT-3'); *LlSCAMP* siRNA1 (sense, 5'-GGGCU GGAAUUGUAUAGATT-3'; anti-sense, 5'-UCUAUAACAAUUCCAGCCCTT-3'); *LlSCAMP* siRNA2 (sense, 5'- CCUGGAGCUUAUGUGUUAUTT-3'; anti-sense, 5'-AUAACACAUAAGCUCCAGGTT-3'); *LlSCAMP* scramble siRNA (sense, 5'-GAGCAUGGAUGUGUAUGUATT-3'; anti-sense, 5'-CTCGUACCUACTCAU ACAUTT-3') and *GFP* siRNA (sense, 5'-GCAAGCUGACCCUGAAGUUCAU-3' ; anti-sense, 5'- GAACUUCAGGGUCAGCUUGCCG-3').

3.2.7 Microinjection Procedures

Lily pollen grains were suspended in germination medium and spread out on 1% low melting agarose in a 9-cm Petri dish. About 45 min after germination, pollen tubes

around 150 μm were selected for injection as described (Lin and Yang, 1997) using a Nikon inverted microscope (TE2000-U, Japan). About 2 nL of affinity-purified antibody (0.2 mg/mL) or *LIVSR* siRNA (50 nM) was gently loaded into the pollen cytoplasm, as estimated (Wolniak and Larsen, 1995). Five minutes after injection, micropipette tips were slowly removed from the pollen tube, followed by time-lapse recording of fluorescent signals in growing pollen tube.

3.3 Results

3.3.1 Dynamics and Distribution of VSR in Germinating Lily Pollen Tubes

I 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. Since GFP-VSR fusion reporters with the TMD and CT of VSRs were found to colocalize with the endogenous VSR proteins (Jiang and Rogers, 1998; Tse et al., 2004; Miao et al., 2006; Miao et al., 2008), I employed a similar approach for studying VSRs in pollen tubes. I therefore cloned a full-length lily (*Lilium longiflorum*) VSR cDNA (termed *LIVSR* in this study) via 5' and 3' RACE using cDNA derived from mRNA isolated from germinating lily pollen, which is similar to VSRs of pea and Arabidopsis (Figure 5). I also generated a similar GFP-LIVSR construct under the

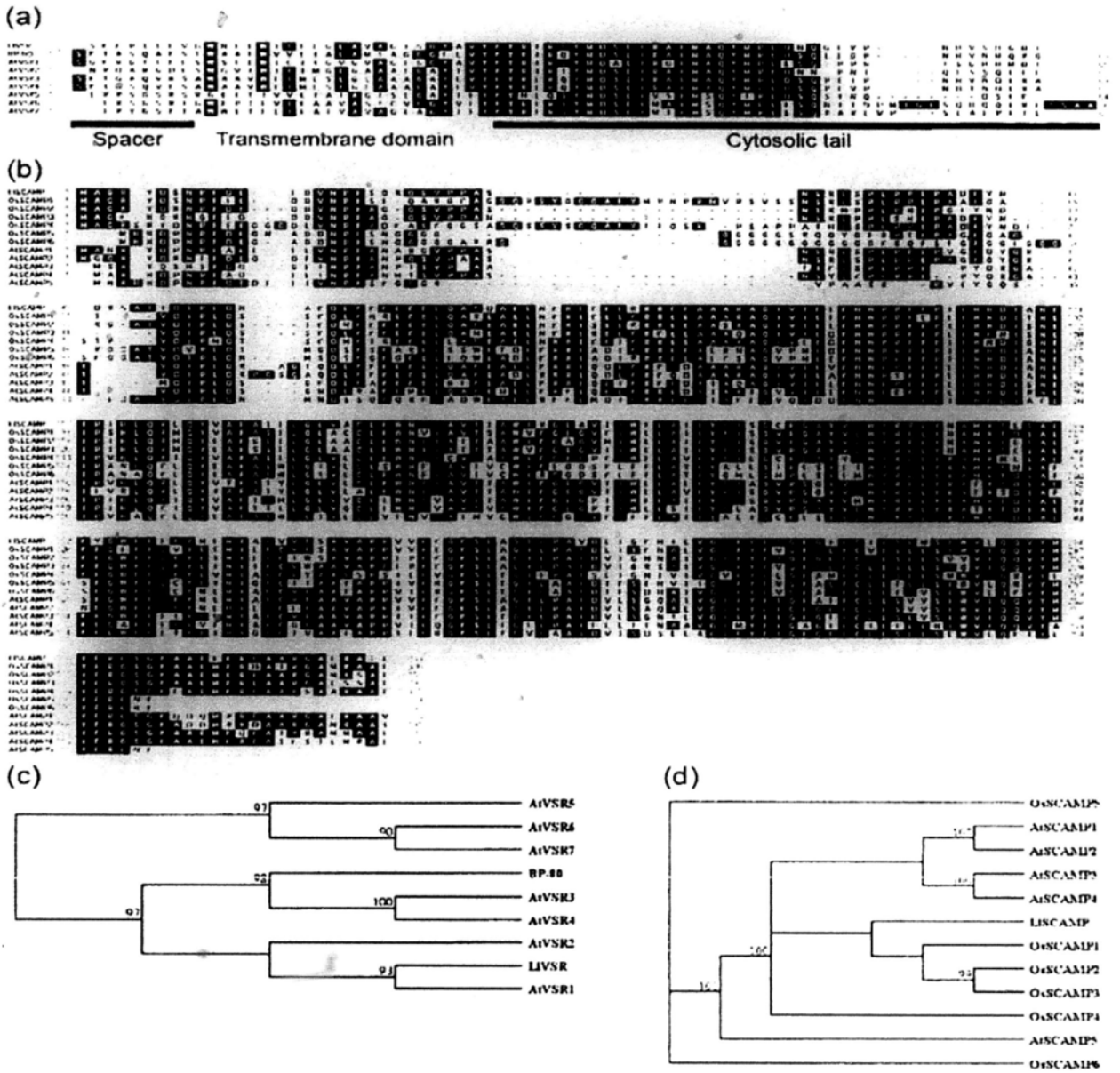

Figure 5. Amino acid sequences comparison of LIVSR and LISCAMP.
(A) Comparison of the LIVSR with BP-80 and the other seven Arabidopsis VSR1-7 in their spacer-TMC-CT regions at the amino acide level.
(B) Comparison of the LISCAMP with the six rice SCAMPs (OsSCAMP1-6) and the five Arabidopsis SCAMP (AtSCAMP1-5) at the amino acid level.
(C) Phylogenetic analysis of VSRs as indicated.
(D) Phylogenetic analysis of SCAMPs as indicated.

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, I also modified the original PVC marker GFP-BP-80 that colocalized 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 from the apical inverted-cone region of the clear zone (Figure 6b and 6c). In contrast, control cytosolic GFP signals were found to be evenly distributed in the growing pollen tube (Figure 6a). Interestingly, the clear zone at the tip matched perfectly with the distribution of the internalized endocytic marker FM4-64 (Figure 6d). The clear zone consistently lacked the GFP-LIVSR signal during pollen tube growth (Figure 6e). In contrast to the diffuse cytosolic GFP signal (Figure 6a), signals from GFP-VSR fusions (Figure 6b to 6e) 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 6e). 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 7). In addition, the GFP-tagged ER marker was excluded only from the very tip of the apex, but GFP-LIVSR was missing from the much larger v-sharp region of the apex (Figure 6). 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.

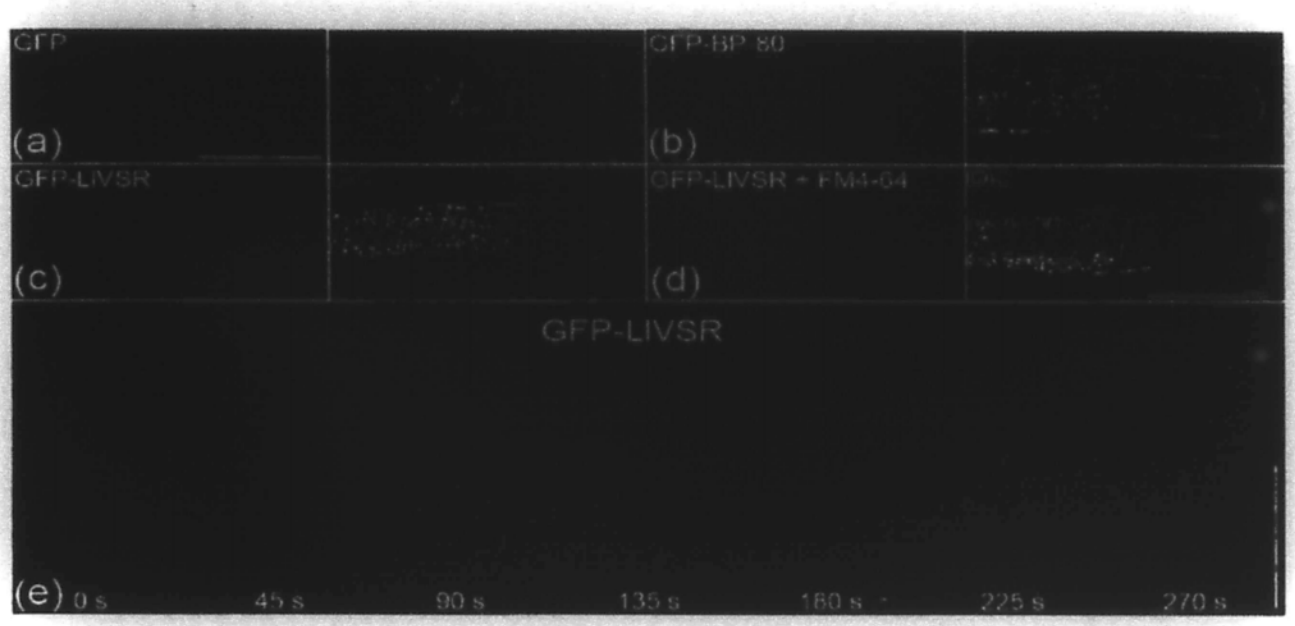

Figure 6. Dynamics of GFP-VSR in germinating lily pollen tubes.
Germinating pollen were transfected with various GFP fusions as indicated via bombardment for transient expression, followed by confocal imaging. Representative confocal images are pollen tubes expressing free GFP protein (A), GFP-BP-80 (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. s, seconds. Scale bar = 25 μm.

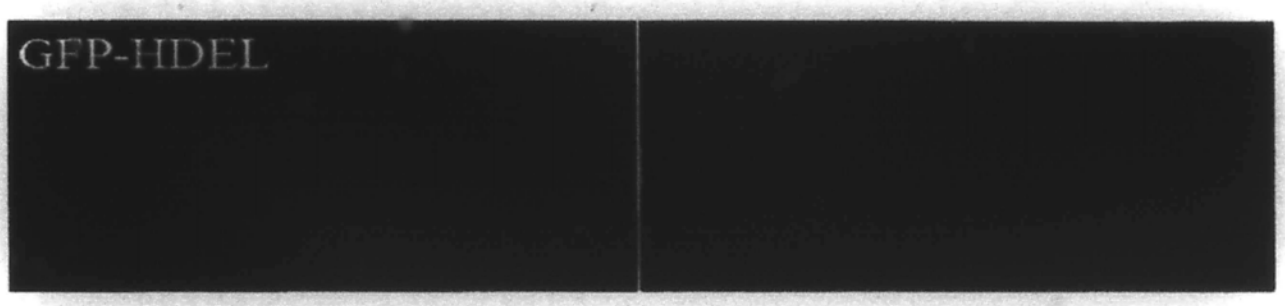

Figure 7. Dynamic and distribution of GFP-HDEL in germinating tobacco pollen tubes.
Germinated tobacco pollen tubes were transiently expressed with GFP-HDEL. DIC, differential interference contrast. Scale bar = 50 μm.

3.3.2 Dynamics and Distribution of SCAMP in Germinating Lily Pollen Tubes

Since both YFP-SCAMP1 and SCAMP1-YFP fusion constructs were found to colocalize with the endogenous SCAMP1 proteins to both PM and TGN in tobacco BY-2 cells (Lam et al., 2007a; Lam et al., 2007b), we decided to use a similar reporter system for studying SCAMP in pollen tubes. I 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 (Figure 5). I also cloned and used the rice SCAMP6 as another control in this study. I 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 8a and 8b). 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 8a to 8c). The tip-localized GFP-SCAMP signals were also obvious in time-lapse confocal images collected from a growing pollen tube expressing GFP-LISCAMP (Figure 8c), where the GFP signals remained concentrated at the elongating tip region (Figure 8d). In addition, the GFP-SCAMP-labeled apical inverted-cone zone region

continually streams backward like the tail of a comet.

To decide whether there is a direct relationship between VSR and SCAMP in pollen, I co-expressed GFP-LlVSR together with RFP-LlSCAMP and followed their dynamics in the same growing pollen tube. As shown in Figure 9, GFP-LlVSR and RFP-LlSCAMP showed clearly distinct distributions in the growing pollen tube: RFP-LlSCAMP was predominantly present in the apical region whereas GFP-LlVSR is missing at this location (Figure 9a). Such a distinct but coordinated dynamic distribution between GFP-LlVSR and RFP-LlSCAMP remained constant as the pollen tube continuously elongated (Figure 9b). 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.

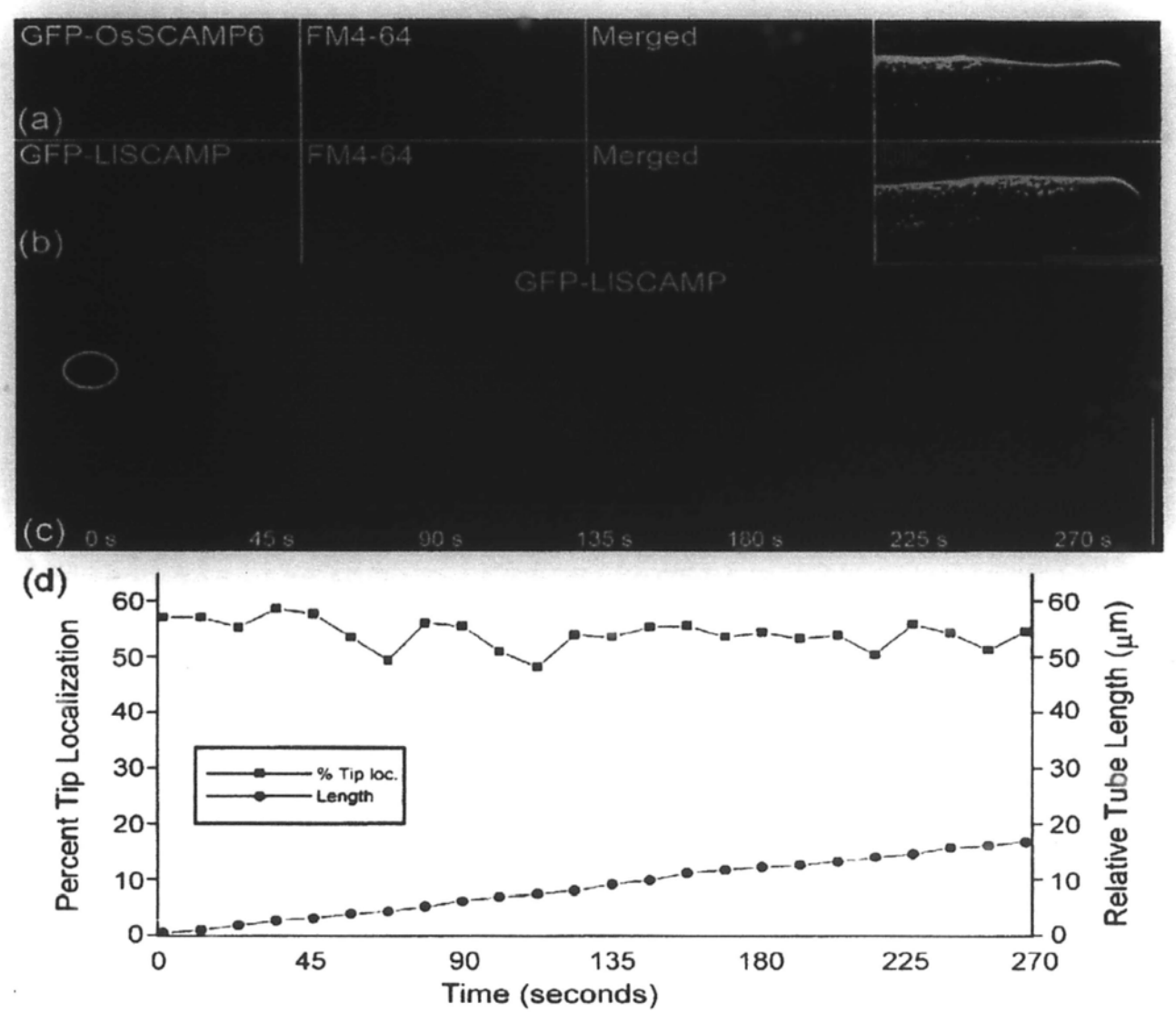

Figure 8. Dynamics of GFP-SCAMP in germinating lily pollen tubes.
(A) and (B) Colocalization GFP-OsSCAMP6 or GFP-LISCAMP with FM4-64 in germinated lily pollen tubes expressing the reporters. Scale bar = 25 μm.
(C) A time-lapse conofcal images of pollen tube expressing GFP-LISCAMP. s, seconds; Scale bar = 25 μm.
(D) The relationship between the percentage of tip-localized GFP-LISCAMP and the relative length of the growing pollen tube.

Figure 9. Dynamics of GFP-LIVSR and RFP-LISCAMP upon their co-expression in growing lily pollen tube.

(A) Relationship between GFP-LIVSR and RFP-LISCAMP upon their co-expression in growing lily pollen tube.

(B) Time-lapse confocal images of pollen tube co-expression GFP-LIVSR and RFP-LISCAMP. s, seconds; Scale bar = 25 μm.

3.3.3 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, I applied three drugs, wortmannin, brefeldin A (BFA) and latrunculin B (Lat B). As mentioned above, wortmannin typically induces the vacuolation of GFP-tagged PVCs. Lat B at low concentrations (2.5 nM) is known to specifically de-polymerize short actin microfilament bundles present in the clear zone region, while 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; Chen et al., 2007). Moreover, Lat B at low concentrations does not stop cytoplasmic streaming although further pollen tube growth is inhibited.

Wortmannin (8.25 μM) treatment of bombarded lily pollen tubes for 15 min induced the cytoplasmic GFP-LIVSR punctae to form ring-like structures (Figure 10a), with on the average about 10-12 such ring-like structures per whole growing pollen tube. 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 10b). Nevertheless, the GFP-LIVSR punctae remained motile moving towards the apex and returning through the tube centre (data not shown). On the other hand, LatB-induced disruption of the inverted cone-region at

the tip prevented the endocytic uptake of FM4-64 (Figure 10c). This inhibition was reversible after washing out the drug for 10 to 15 min, leading to the reconstitution of the apical inverted cone region (Figure 10d). These results indicate that a) the dynamics of GFP-LlVSR trafficking is independent of the short actin microfilament bundles at the apex of the pollen tube, and b) 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.

I 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 already causes changes at the tip of the lily pollen tube, as monitored by FM4-64 uptake (Figure 11a). The FM4-64 labeled inverted cone region became less organized and a large sub-apical aggregate was formed upon BFA treatment (Parton et al., 2001; Parton et al., 2003). However, treatment with BFA at 10 µg/ml 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 11b). Similar results were obtained when the same BFA treatments were carried out in pollen tubes expressing GFP-LISCAMP (Figure 11c, d and f). Here, GFP-LISCAMP positive BFA-induced aggregation was obvious in the tip region with aggregates present throughout the whole tube within 10 min

after BFA treatment (Figure 11c). The sub-apical localized BFA-induced aggregate was gradually disrupted and changed into diffuse pattern in less than 10 min (Figure 11f), and eventually changed into a cytosolic pattern lacking tip-localization within 30 min of BFA treatment (Figure 11d). Nevertheless, the cytosolic BFA-induced aggregates marked by GFP-LlSCAMP 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-LlSCAMP was also disrupted. However, the punctate GFP signals representing endosomal compartments remained highly dynamic although the growth of the pollen tube was stopped (Figure 11e). These results again demonstrate that the dynamics and maintenance of apical-localized GFP-LlSCAMP is dependent on the short actin microfilament bundles.

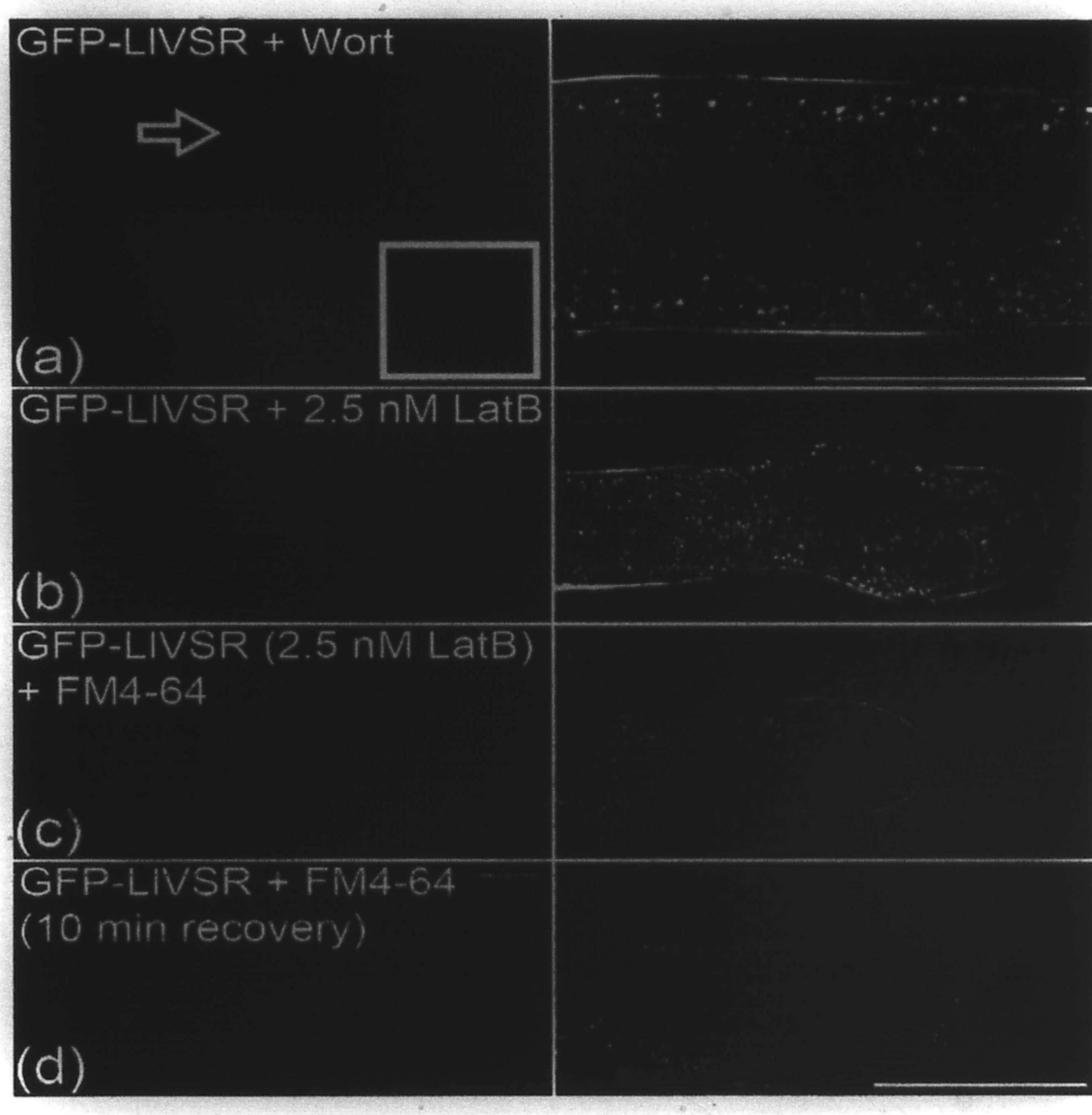

Figure 10. Dynamics of GFP-LIVSR in response to drug treatments.
Upon transformation of GFP-LIVSR via bombardment, the transformed lily pollens were germinated and (A) treated with 8.25 μM Wortmannin for 15-min before confocal imaging; or (B) treated with 2.5 nM Latrunculin B (LatB) for 15 min before confocal imaging; (C) further uptake with FM4-64 after treatment with 2.5 nM LatB before confocal imaging; and (D) washing off the LatB and allow 10-min recovery before FM4-64 uptake and confocal imaging.
DIC, differential interference contrast. Scale bar = 10 μm in (A), 25 μm in (B), (C) and (D).

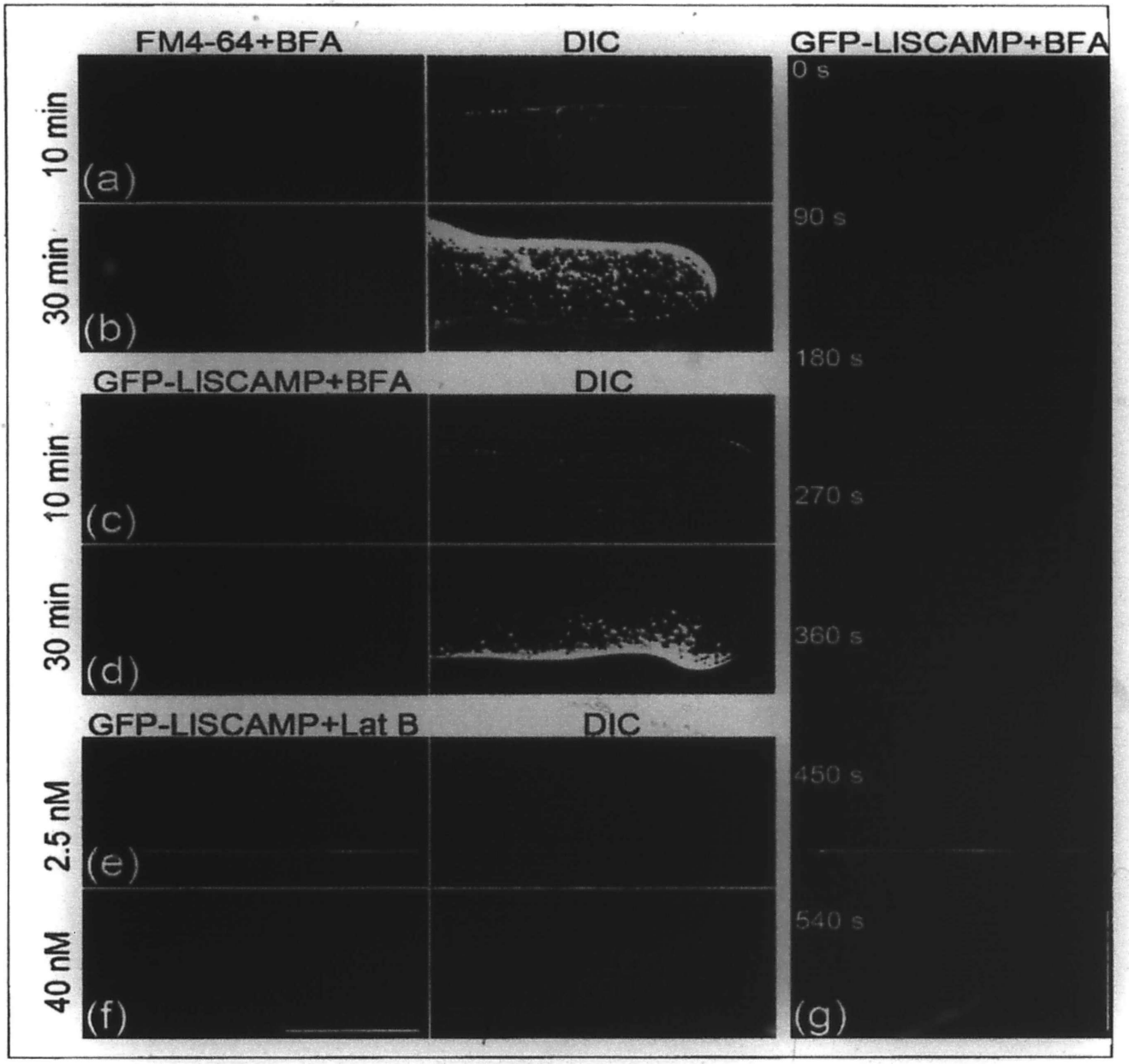

Figure 11. Dynamics of GFP-LISCAMP in response to drug treatments.
(A) and (B) Germinated lily pollen tubes were subjected to FM4-64 dye uptake, followed by 10-min BFA treatment at 10 μg/ml, followed by anther 10- or 30-min incubation as indicated prior to conofcal imaging.
(C) and (D) Germinated lily pollen tubes expressing the GFP-LISCAMP were treated with BFA at 10 μg/ml for 10 and 30 min prior to confocal imaging.
(E) and (F) Germinated lily pollen tubes expressing the GFP-LISCAMP were treated with 2.5 nM (E) or 40 nM (F) LatB for 15 min before confocal imaging.
(G) Germinated lily pollen tubes expressing the GFP-LISCAMP were treated with BFA at 10 μg/ml for 30 min prior to time-lapse confocal imaging at indicated times from 0 to 540 seconds. s, seconds; Scale bar = 25 μm.

3.3.4 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, I 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 12, both VSRat-1 and BP-80 CT antibodies detected a major protein band at about 80 kDa in the membrane fraction with a very weak protein band at around 50 kDa, which 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.

I performed immunogold EM on ultra-thin 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 putative PVC/MVB (Figure 13a, b and c) and small vacuole-like structures (Figure 13d and e).

Similarly, SCAMP1 antibodies also labeled the TGN (Figure 14d) and vacuole-like structures (Figure 14e). In addition, SCAMP1 antibodies also specifically labeled small vesicles that were highly enriched in the apical region of the tube (Figure 14a-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 immunogold EM labeling was deemed to be highly specific (data not shown).

A statistical analysis on the immunogold EM VSR- and SCAMP1-labelled sections for the distribution of gold particles in various organelles was also carried out. As shown in Table 3, gold particles on VSR-labeled sections were mainly found over PVC/MVB and small vacuoles with an average of 3.08 and 6.36 gold particles per organelle respectively, with little background labelling 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 4).

Taken together, these results indicate that in germinating lily pollen tubes VSRs localize to 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 given in previous studies (Tse et al., 2004; Lam et

al., 2007a) and the dynamic live cell distribution of LlVSR and LlSCAMP in this study. However, the additional localizations SCAMPs to the apical vesicles seem to be a pollen-specific feature with possible functional implications (see Discussion).

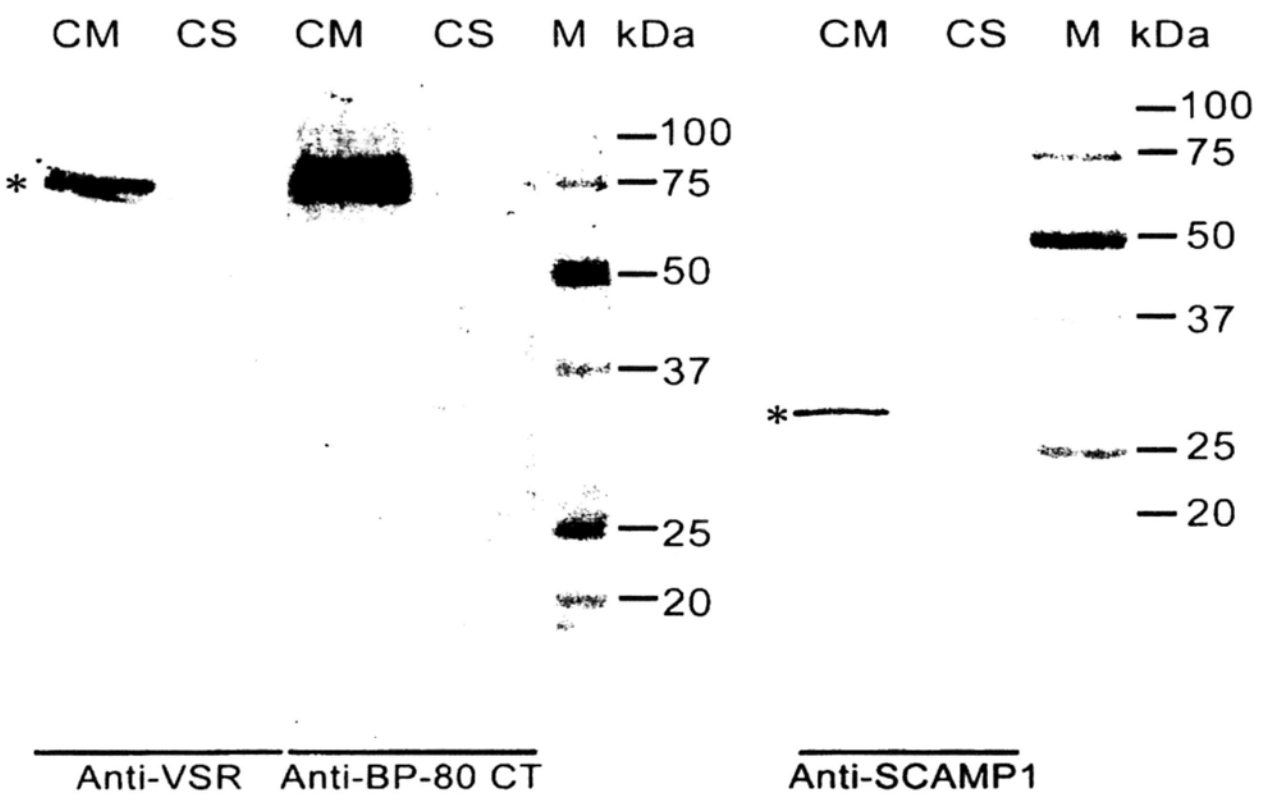

Figure 12. Western blot analysis of VSR and SCAMP1 proteins in germinating lily pollen.

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. Asterisk indicates positions of the target proteins. M, molecular weight marker in kDa.

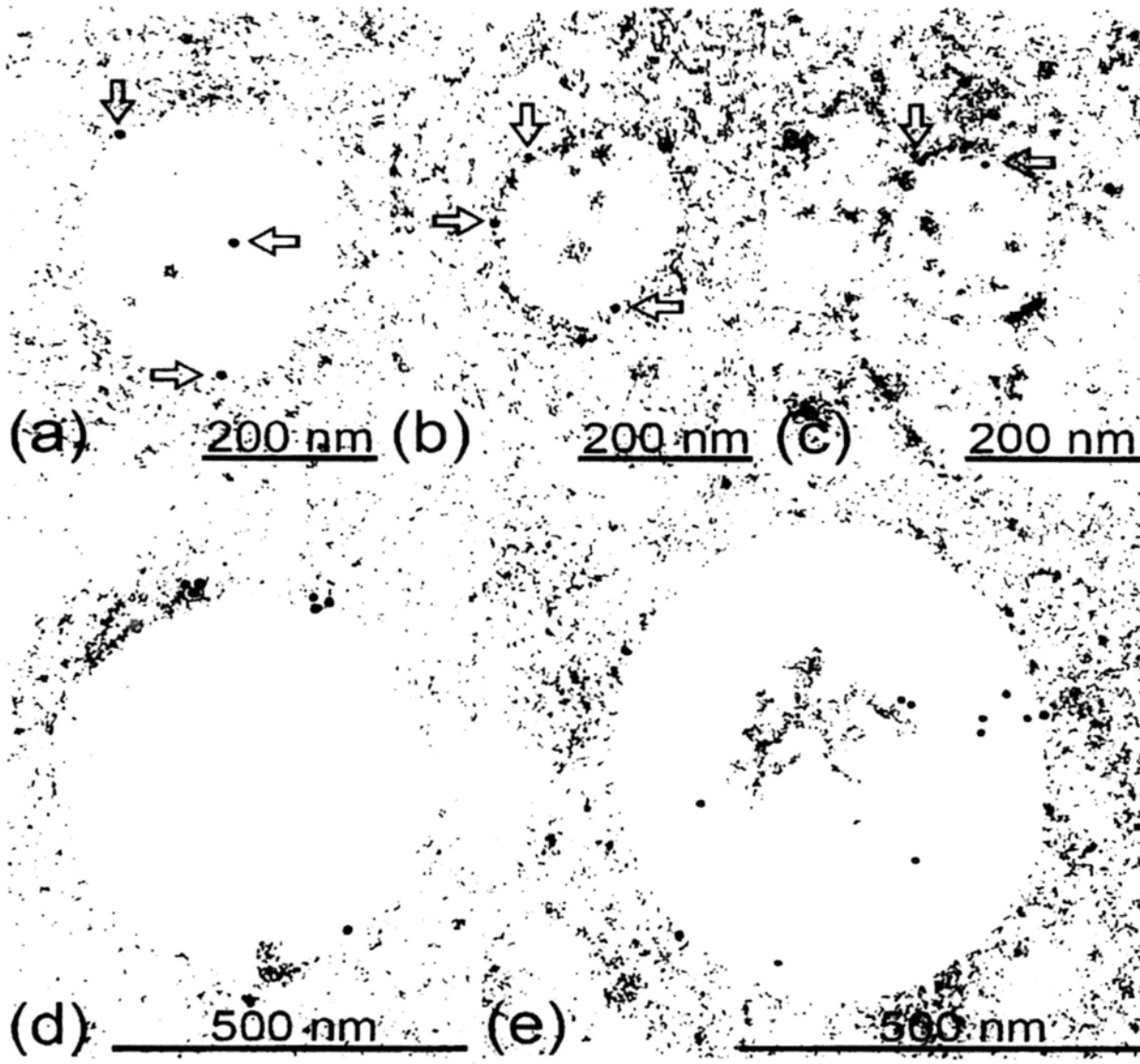

Figure 13. Immunogold EM localization of VSR in lily pollen tubes.
Ultrathin sections prepared from high-pressure freezing/freeze-substitute pollen samples were labeled with VSRat-1 antibodies. Arrows indicate examples of gold particles on putative PVC/MVB (A, B and C), and vacuole-like structure (D and E). Scale bar = 500 nm in (D) and (E) while in (A), (B) and (C) is 200 nm.

Table 3. Distribution of Gold Particles (GP) for VSR antibodies in immunogold EM labeling of germinating lily pollen tubes.

Organelle	GP No.	Organelle No.	GP per Organelle
MVB/PVC	77	25	3.08**
Small vacuole	159	25	6.36**
Mitochondria	4	25	0.16**

Significant differences between two organelles was analyzed using one-side paired *t* test ($P < 0.01$ [**]). Data was collected and analyzed from seven independent labeling experiments. GP, Gold particle.

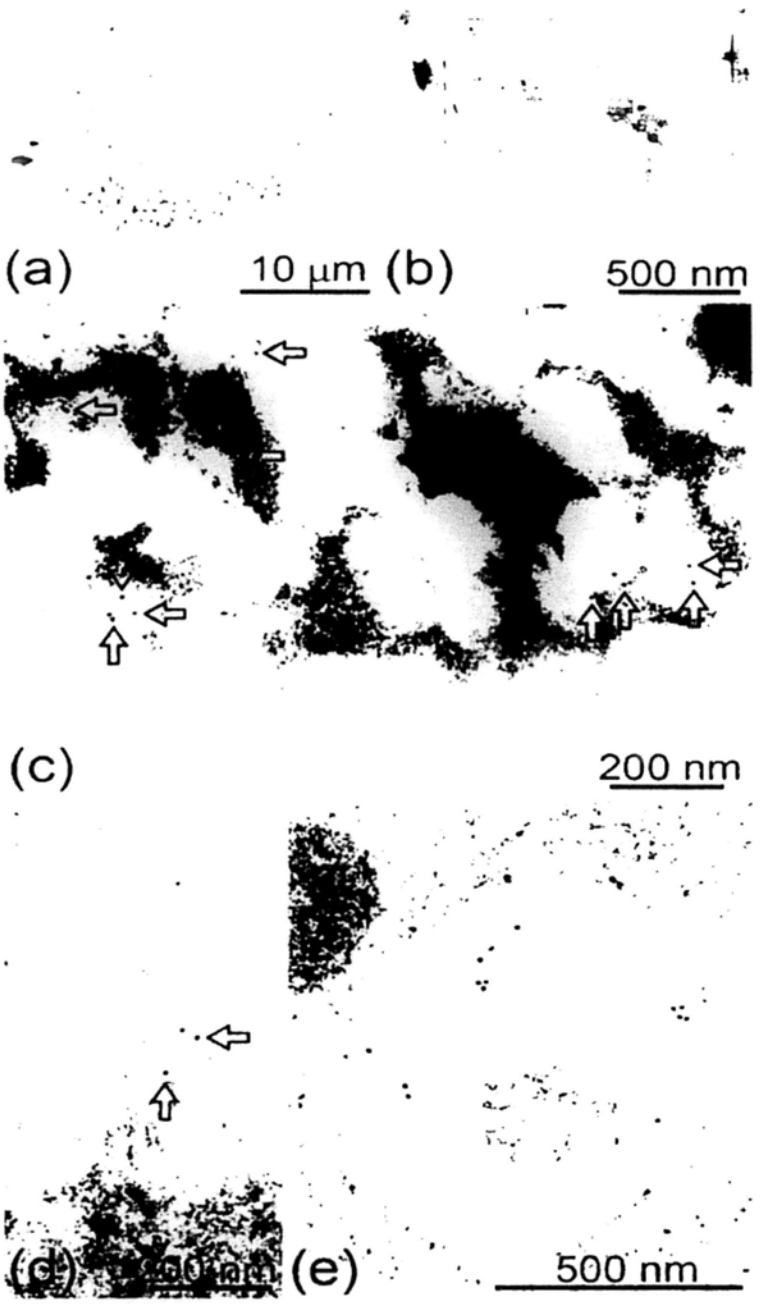

Figure 14. Immunogold EM localization of SCAMP1 in lily pollen tubes.
Ultra-thin sections prepared from high-pressure freezing/freeze-substitute pollen tube tip were labeled with SCAMP1 antibodies. (A) Overview of the ultrathin section across the tip of a lily pollen tube used for labeling. (B) The enlarged apical region of the pollen tube from (A) as indicated and (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 (TGN) (D) and vacuole-like structure (E).
Scale bar = 10 μm in (A); 500 nm in (B), (E); and 200 nm in (C), (D).

Table 4. Distribution of Gold Particles (GP) for SCAMP1 antibodies in immunogold EM labeling of germinating lily pollen tubes.

Organelle	GP No.	Organelle No.	GP per Organelle
TGN	87	25	3.48**
Small vacuole	167	25	6.68**
Tip-endocytic vesicles	24	25	0.96**
Mitochondria	3	25	0.12**

Significant differences between two organelles was analyzed using one-side paired *t* test ($P < 0.01$ [**]). Data was collected and analyzed from seven independent labeling experiments. GP, Gold particle.

3.3.5 Microinjection of VSR and SCAMP Antibodies Strongly Inhibit Pollen Tube Growth

To investigate further the functional roles of VSRs and SCAMPs in pollen tube growth, I performed microinjection experiments with antibodies, a technique which has been previously shown to be effective in addressing the function of proteins during pollen tube growth (Lin and Yang, 1997). I therefore microinjected VSR and SCAMP antibodies into growing pollen tubes cultured on low-melting agar pollen germination medium, followed by observation of the subsequent tube growth. As shown in Figure 15a and 15b, microinjection of either BP-80 CT or SCAMP1 antibodies into pollen tubes significantly reduced the growth rate of the pollen tubes within 10 min after the completion of the microinjection as compared to normal lily pollen tubes (Figure 15a). These negative effects were specific for these two antibodies, since when identical microinjection experiments were carried out using buffer or GFP or α-TIP antibodies, the injected pollen tubes retained normal growth rates (Figure 15a). These observations were supported by a statistical analysis of data collated from multiple replicate experiments (Figure 15a). 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.

3.3.6. Microinjection of siRNA of LIVSR and LISCAMP Strongly Inhibit Pollen Tube Growth

Direct delivery of synthesized siRNA into cells to inhibit gene expression is the fastest and most direct way to down-regulate a gene of interest (Elbashir *et al.*, 2001a; Elbashir *et al.*, 2001b; 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 became a reliable tool to study the functions of specific genes in mammalian cell (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 I have just demonstrated through control experiments, growing pollen tubes can be successfully microinjected without necessarily perturbing their function. Thus, to provide further support that LlVSR and LlSCAMP are essential for lily pollen tube growth, I injected *LlVSR* siRNAs and *LlSCAMP* siRNAs into growing lily pollen tubes to specifically knock-down the expression of the *LlVSR* and *LlSCAMP* genes. As shown in Figure 15b, the injection of either the two designed *LlVSR* siRNA1 and *LlVSR* siRNA2 or *LlSCAMP* siRNA1 and *LlSCAMP* siRNA2 dramatically reduced the growth rate of the pollen tubes, as did microinjection of the general protein synthesis inhibitor cycloheximide (Figure 15b). This inhibition was specific since injection of the control scramble siRNA and *GFP* siRNA sample did not slow down pollen tube growth (Figure 15b). These observations were supported by a statistical analysis of data collated from multiple replicate experiments (Figure 15b).

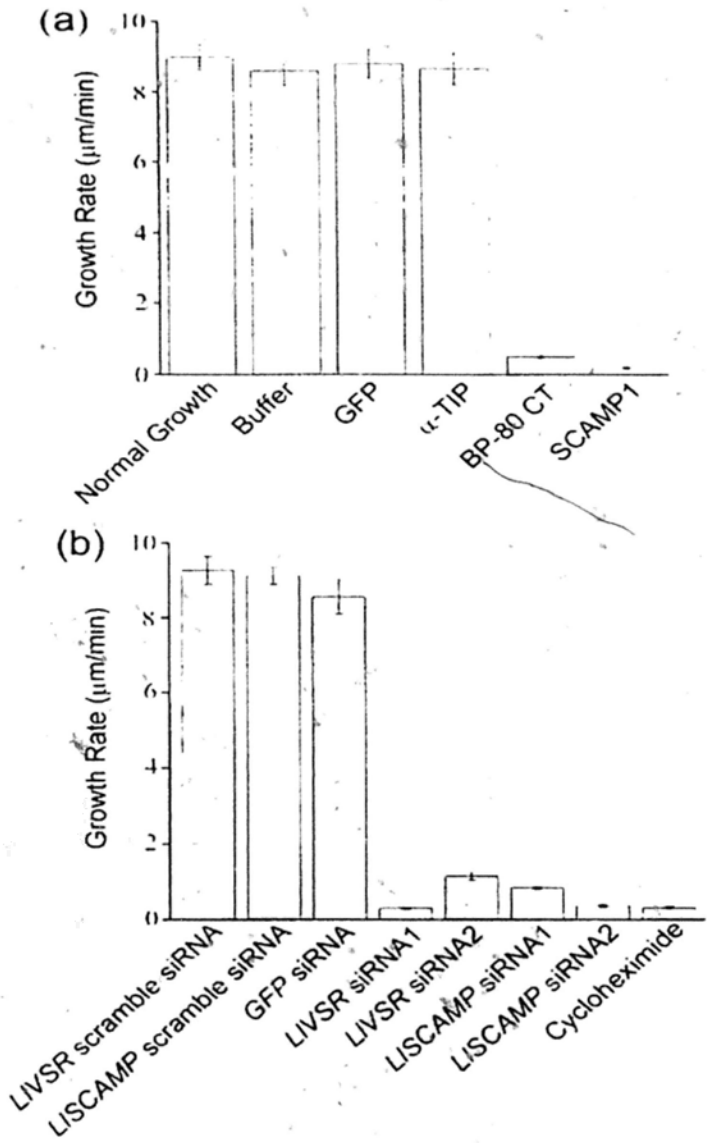

Figure 15. Effects of antibody and siRNA injection on lily pollen tube growth. Growing lily pollen tubes were microinjected with various antibodies (BP-80 CT, SCAMP1, GFP and alpha-TIP) together with control buffer as indicated and various siRNAs (*LIVSR* siRNA, *LISCAMP* siRNA, Scramble siRNA and *GFP* siRNA) together with 100 nM cycloheximide as another control. After 5-min recovery, micropipette tips were slowly removed from the pollen tube and pollen tube length was measured and 10 min later after injection.

(A) Shows the statistical analysis of growth rate vs. antibody injection using data from at least ten independent experiments and expressed as mean values ± SE.

(B) Shows the statistical analysis of growth rate vs. siRNAs or 100 nM cycloheximide injection using data from at least ten independent experiments and expressed as mean values ± SE.

3.4 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, I 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 (Tse et al., 2004; Lam et al., 2007a).

3.4.1 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* down-regulation 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 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 to the pollen of other plant species such as Arabidopsis or tobacco. In fact, the first example for microinjection was performed in using RopIPs 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, I microinjected two affinity-purified polyclonal antibodies into growing lily pollen tubes: one generated against a synthetic peptide corresponding to the cytoplasmic tail (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 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 15a). A similar result was obtained when *LIVSR* siRNAs and *LISCAMP* siRNAs were used in identical microinjection experiments (Figure 15b). These results came as a surprise in particular for VSR, because it would indicate that this receptor must recycle 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 cell 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 since they did not significantly perturb the "reverse fountain" cytoplasmic steaming 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 pollen tube? The BP-80 CT antibodies recognize the cytoplasmic tail 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 which 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 (AP) for the formation of clathrin coated vesicle (CCV) (Jiang and Rogers, 1998; Sanderfoot et al., 1998), 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; Lam et al., 2008) would be prevented. Following this line of argumentation, I consider that it is more than likely that both VSR-mediated protein trafficking and SCAMP-mediated endocytosis are essential for the pollen tube growth.

Western blot analysis showed that both VSRat-1 and BP-80 CT antibodies detected a major protein band about 80 kDa, while OsSCAMP1 antibodies detected a major protein band about 30 kDa in the membrane protein fractions of germinating lily pollen (Figure 12). I would maintain that these antibodies detected their corresponding lily homologs because the molecular weight based on the newly cloned LIVSR and LISCAMP are also predicted to be 80 kDa and 30 kDa respectively (Figure 5). 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, both LIVSR was cloned using cDNA derived from mRNA isolated from growing lily pollen tubes.

3.4.2 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; Parton et al., 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 amount of plasma membrane 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: where 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 colocalizing with the internalized endocytic marker FM4-64 (Figures 6, 8 & 9). Since 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 vs. 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). Since GFP-SCAMP (Figures 8 & 9) 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 that caused GFP-LlSCAMP to form aggregates in the sub-apical region of a growing pollen tube (Figure 11), a perturbation typically representing the accumulation of endocytic vesicles in the inverted cone region (Cheung and Wu, 2008).

3.4.3 Functional Implications of the Distinct Distributions and Dynamics of VSRs vs. SCAMPs in Pollen Tube Growth

Pollen tube growth 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 to other plant cells, the secretion and endocytosis in the growing pollen tube are site-directed and 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 around 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 process are closely related, but occur in two distinct regions of the pollen tube. Whereas endocytosis occurs at 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 missing from the tip. Since GFP-tagged VSR and SCAMP are reliable markers representing protein trafficking in the plant secretory and endocytic pathways respectively in plant cells (Jiang and Rogers, 2003; Lam et al., 2007a; 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? 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). I 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, I am 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.

Chapter 4

Conclusion and Perspectives

In summary, using a combination of cell biology, molecular biology and biochemistry approaches, I have made the following contributions in my thesis research:

i) I have developed and optimized a pollen grain transient expression system using three wildly studied pollen grains of lily, tobacco and Arabidopsis pollen via particle bombardment. Florescent-tagged proteins were introduced into pollen grains via biolistic transformation. Subsequent protein subcellular localization, kinetic and protein association studies largely depend on the in vitro germination ability of pollen grains. Therefore, I optimized pollen grain germination medium and culture condition for lily, tobacco and Arabidopsis pollens. I found that nearly 95% lily and tobacco pollen grains can germinate and approximately 10% of germinated pollen tubes showed good florescent signals. For Arabidopsis, about 85% pollen grains are able to germinate and 5-6% of germinated pollens had good florescent signals.

Significance: This transient expression protocol provides a quick and convenient tool for studying protein dynamics and subcellular localization in growing pollen tubes. The detailed procedures, optimized in vitro germination media and conditions for pollen grains in this protocol are of especially useful and helpful for un-experienced researchers who want to choose pollen tubes as the model system to study protein trafficking, signal transduction and polarized growth. Moreover, this optimized biolistic pollen

transformation method can also be used as an alternative method to generate transgenic plants.

ii) I have cloned *VSR* and *SCAMP* cDNA from lily pollen (termed *LIVSR* and *LISCAMP* respectively). Since pollen tube growth is an extremely polarized and highly dynamic cellular process with rich membrane trafficking, vesicular secretion and endocytosis, here I study the dynamics and functional roles of VSR and SCAMP in pollen tube growth using lily (*Lilium longiflorum*) pollen as a model. Using florescent fusion protein with newly cloned LIVSR and LISCAMP, I here demonstrate that VSRs are localized to PM, multivesicular body (MVB) and vacuole, while SCAMPs are localized to PM, TGN and vacuole in growing lily pollen tubes. In growing pollen tubes transiently expressing GFP-VSR or GFP-SCAMP reporters, GFP-LIVSR located throughout the pollen tubes except the apical clear zone region but GFP-LISCAMP was mainly concentrated in the tip region.

iii) In order to study the possible roles of VSR and SCAMP in germinating pollen tubes, I used VSR and SCAMP1 specific antibodies as well as siRNAs for *LIVSR* and *LISCAMP* as tools. Microinjection of VSR or SCAMP antibodies and *LIVSR* or *LISCAMP* siRNAs significantly reduced the growth rate of the lily pollen tubes. Taken together, both VSR and SCAMP are required for pollen tube growth, likely working together in regulating protein trafficking in the secretory and endocytic pathways needed for supporting pollen tube elongation.

Significance: In the past years, pollen tube become a very good model system in plant cells to study protein dynamics, membrane trafficking, cytoskeleton organization, signal transduction and exocytosis or endocytosis. This research is the first attempt to study the roles of VSR and SCAMP proteins in pollen tube growth, which further strengthen our knowledge in understanding the molecular mechanisms of how the fast and polarized pollen tube growth be maintained and how pollen tube regulates its growth. Moreover, this study provides the first evidence that, among all the VSR and SCAMP family members, the pollen-specific VSR and SCAMP proteins are required for pollen tube growth, which is also consistent with microarray data analysis.

Based on the results obtained from this thesis research, I have proposed a working model of trafficking networks regulating pollen tube growth that is integrated with vesicle trafficking and the hydrodynamic cycle (Figure 16). Other experimental data in pollen tubes and other plant cells show that cell hydrodynamics can activate and deactivate many key structural and signalling elements, including membrane trafficking, exocytosis and endocytosis, cell elongation and growth, ion flux, phospholipid and inositol polyphosphate signalling, and deposition and assembly of the apical pectin cell wall. Cell hydrodynamics has the potential to regulate other key elements including actin, cytoskeletal organization and small GTPases. Future work will be directed to experimental investigation of these potential links.

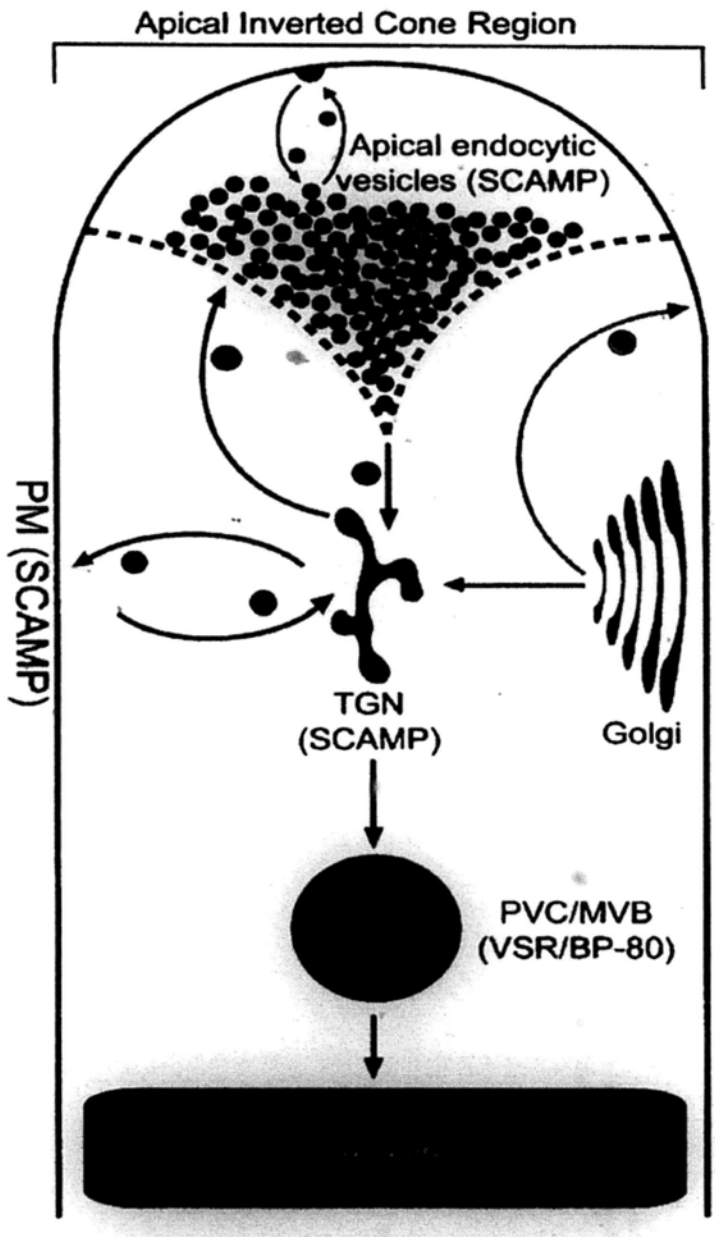

Figure 16. Possible roles of VSR and SCAMP in growing pollen tube.
Shown is a working model on the localization, dynamics and possible functional roles of VSR and SCAMP proteins in germinating pollen tubes. SCAMP is highly enriched in the apical region of the pollen tube which is missing the VSR. VSR/BP-80 are involved in the ER-Golgi-TGN-PVC/MVB-vacuole transport pathway. Similarly, SCAMP could reach the PM from either Golgi or TGN and internalize from the PM via endocytosis colocalizing with the internalized endocytic marker FM4-64. The SCAMP-positive small vesicles enriched in the apical region are believed to be derived directly from the Golgi apparatus or via TGN and endocytic vesicles from PM. Both VSR and SCAMP were found to reach the vacuole lumen in immunogold EM, presumably for degradation.

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