INVESTIGATION INTO THE FUNCTIONS OF THE POLLEN SPECIFIC GENES PiVAMP721 AND PiSCP1 IN POLLEN TUBE GROWTH

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INVESTIGATION INTO THE FUNCTIONS OF THE POLLEN SPECIFIC GENES PiVAMP721 AND PiSCP1 IN POLLEN TUBE GROWTH

Abstract

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PiVAMP721 is a member of the plant unique VAMP7 proteins, which is expressed specifically in pollen. The VAMP7 (longin) proteins are involved in endosomal trafficking across species. PiVAMP721 was colocalized with an endocytic marker, FM4-64 and it's over-expression was found to be inhibitory to pollen tube growth. PiVAMP721/C, a truncated constitutively active form of this protein, generated bulging pollen tube tips at a frequency 3 fold higher than the than wild type protein, and lead to increased growth inhibition (mean pollen tube length 26% shorter than tubes expressing the wild type protein). In contrast expression of the truncated N-terminal "longin" domain, PiVAMP721/N, in pollen tubes did not generate a discernable phenotype. Interestingly PiVAMP721/N was found to be able to partially rescue inhibition caused by expressing PiVAMP721/C, suggesting that PiVAMP721/N has a negative regulatory function and that the two regions of this protein are able to interact and function "normal" even when not translationally fused.

Although the small novel protein, PiSCP1, has two homologs in *Arabidopsis*, neither has a known function. We showed that PiSCP1 is a peroxisomal protein by colocalization

with a peroxisome marker. Overexpression of PiSCP1 did not generate a significant phenotype. However, the protein encoded by this gene interacted with CDPKs in yeast two hybrid assays and mutations of two potential sites of serine phosphorylation lead to proteins that inhibited pollen tube growth. These results suggest that phosphorylation of either of these two residues is important to the overall function of PiSCP1. The conserved N-terminal "A" and C-terminal "C" domains both retained the ability to localizes to peroxisomes and may still retain the ability to interact with CDPK(s). The divergent central B domain was found to be largely cytosolic and not to interact with CDPK(s). Here, we further showed that PiCDPK2 is also a peroxisomal protein. PiCDPK2 was shown to colocalize with PiSCP1 and interact with this protein in a yeast two hybrid assay, suggesting that PiCDPK2 is likely the CDPK isoform that interacts with PiSCP1 *in vivo*. Neither PiSCP1 nor PiCDPK2 have classic peroxisome targeting signals, and the potential targeting/sorting signals of these two proteins are discussed.

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CHAPTER 1

The contribution of endocytosis and peroxisomes to pollen tube growth

Abstract

Pollen tubes are a model system to study tip growth. Growing pollen tubes can be structurally and functionally divided into apical, subapical and distal regions. The apical region is the "clear zone" where transport vesicles are concentrated, while organelles are concentrated in the subapical region; the distal region is where the male germ unit and a large vacuole are located. Special actin organization, ion gradients and exo/endocytosis are necessary for maintaining this zonation of pollen tubes. The phospholipid signaling system which intersects the above three systems may provide communication between interdependent pathways. The zonation must be dynamically maintained, and endocytic recycling, proteolytic degradation, phosphorylation/dephosphorylation are among the mechanisms by which this is achieved.

Endocytosis has only recently been widely accepted in plants, available genomic data suggest that plants possess the whole set of molecular machinery for endocytosis including receptor kinases, Rab GTPases, SNAREs and clathrins among others. Endocytosis in plants probably follows a plasma membrane, trans-Golgi network (TGN), prevacuolar compartment (PVC) to vacuole route with TGN and PVC corresponding to the early and late endosomes. Membrane fusion events involving endocytic vesicles are mediated by SNARE proteins. R.III VAMP7 family longin proteins are the R-SNARE proteins responsible for membrane fusion in endocytosis. Plants have an unusually large family of VAMP7 proteins relative to animals.

Peroxisomes are ubiquitous organelles in eukaryotes and contain proteins originating from both the ER and mitochondria. Peroxisomes are recognized to have important functions in signaling beside the traditional lipid metabolism function. Peroxin proteins are responsible for the peroxisome biogenesis, division and protein import. The potential roles of peroxisomes in pollen tube growth include energy reserve mobilization, developmental signaling and membrane production. Mutations in some peroxisomal proteins show male sterile phenotype with defects in pollen tube growth and pollen tube reception.

Introduction

Pollen tubes are an excellent tip growth model to study a broad spectrum of fundamental plant events including signal transduction, exo/endocytosis and pollen-pistil communication (Feijo et al., 2004). Pollen must first initiate germination, then a pollen tube is extended and grows toward the ovule, this growth process is where most research is focused, partly because it is easy to induce *in vitro*; once the pollen tube grows near the ovule, another set of signal transduction events different from its guidance during the extension phase must take place for the pollen tube to enter the micropyle, at the final stage, pollen tubes arrest growth and burst to release the sperm cells (Franklin-Tong, 1999). Great advances have been made recently in our understanding of pollen tube growth, partly attributed to the following factors. First the comparison with other polar growth models such as root hairs, neurite outgrowth, and fungal hyphae (Samaj et al., 2006). Each model offers unique advantages and valuable insights can be gained through comparisons, for example, signal transduction pathways in pollen tubes can be postulated

based on work on mammalian neurites. Second is the availability of genomic data, particularly that of *Arabidopsis*, through it we know that essential factors for polar growth in other systems are present in pollen (Sanderfoot et al., 2000; Shiu and Bleecker, 2001, Yang, 2002). The third contributing factor is the availability of molecular probes, such as GFP fusion proteins and vital fluorescent markers such as FM dyes (Camacho and Malhó, 2003). The wide use of advanced microscopic techniques such as confocal microscopy is also an important contributing factor. This chapter will first discuss some of these recent advances particularly in the light of structural and functional compartmentalization of growing pollen tubes and then focus on the roles of endocytosis and peroxisome in pollen tube growth.

Zonation and maintenance of pollen tube

The most prominent feature of an extending pollen tube under a light microscope is its clear zone at the tip and the so-called reverse-fountain-like vigorous cytoplasmic steaming right behind it. Ultrastructural analysis of pollen tubes of various species has shown the clear zone is virtually devoid of any organelle except transport vesicles, which are invisible under a light microscope (Lennon and Lord, 2000). In the subapical region, organelles such as mitochondria, ER, and Golgi bodies can be observed moving along cytoplasmic streaming (Lennon and Lord, 2000; Lovy-Wheeler et al., 2007). More distal from the tip, the vegetative nuclei and two sperm cells are located. Similar observations were made both *in vitro* using TEM and *in vivo* using GFP marker proteins targeted to specific organelles (Lennon and Lord, 2000; Parton et al., 2001; de Graaf et al., 2005). From these observations, pollen tubes can be structurally and functionally divided into

the apical region which is the clear zone, the subapical region in which organelles are concentrated and the distal region which include most of the shank with the male germ unit and a large vacuole (Lennon and Lord, 2000; Cheung and Wu, 2007).

The apical region is dedicated to exocytosis through which cell wall building factors and enzymes are secreted (Taylor and Hepler, 1997; Hepler et al., 2001). Accompanying this exocytosis, an appreciable amount of endocytosis must take place as the amount of membrane expansion as a result of exocytosis far exceeds the amount needed for tube growth (Derksen et al., 1995). Aside from membrane recycling, endocytosis is perhaps more important in recycling and deactivating various protein factors; in pollen tube growth *in vivo*, endocytosis may also be involved in the uptake of both guidance cues and nutrients from female tissue. Another aspect of the apical region is the fact that prominent actin cables found in the shank of the tubes are absent and instead short fragmented actin filaments and actin patches can be observed (Cheung and Wu, 2007).

In the subapical region, high concentrations of fast-moving organelles are observed, powered by actin-dependent cytoplasmic streaming. Studies employing EM and *in vivo* marker proteins have clearly established that organelles such as ER, Golgi, MT are present in this region (Lennon and Lord, 2000; Parton et al., 2001). Actin cables are also a prominent feature of this region, a variety of techniques including phalloidin staining, immunodetection with different antibodies such as anti-actin, and GFP tagged marker proteins such as GFP-actin depolymerizing factor (ADF), have revealed a subapical mesh or fringe of action cables (Gibbon et al., 1999; Geitmann et al., 2000; Vidali et al., 2001; Lovey-Wheeler et al., 2005). Structurally speaking, actin cables in this

region are essential for proper organelle positioning, and disruption of actin polymerization inevitably causes the invasion of various organelles into the apex. The mechanism that controls this actin positioning, however, is not well understood. Recent advances in phosphoinositides (PI) signaling provide evidence for cross-talk between PI and actin. For example, reduction of intracellular PA level caused rearrangement of microfilaments, dispersal of apical accumulation of secretory vesicles and movement of organelles into the apical region (Monteiro et al., 2005). Also worth noting is that the subapical actin mesh is very sensitive to various actin depolymerization agents, in fact, much lower concentrations of these agents leads to breakdown of this structure than is required for disruption of shank actin cable or inhibition of germination (Gibbon et al., 1999).

The distal region is a somewhat overlooked region in the pollen, although the ultimate purpose of pollen tube growth is to deliver the sperm to the ovules. Apparently, not much takes place in this region except that these structures are being “dragged” along. Microtubules have been shown to be important for this dragging which is intriguing as no important function has been assigned to microtubules although they are abundant in pollen tubes and also form a fringe like actin (Joos et al., 1994; Lovey-Wheeler et al., 2005). Although fragmented tubular vacuoles are present throughout the subapical region, one large vacuole usually dominates the distal region. The vacuole has long been treated as a passive organelle where unwanted materials are sequestered. Recent evidence suggests that it is used to sequester stylar S-RNases that mediate gametophytic self-incompatibility in compatible pollinations, while releasing them in an incompatible

reaction, this finding clearly highlights its active role in pollen tube growth (Goldraij et al., 2006).

Three well established mechanisms are necessary for the above mentioned zonation. First, exo/endocytosis and their associated signaling components are essential for the apical region, actin dynamics and signaling factors associated with it, for example Rop GTPase is required for proper positioning and moving of organelles and various vesicles; overlaying the above structural segregations, a number of gradients are found, with ion gradients being the most prominent.

Although exocytosis has long been proposed in pollen tubes, endocytosis has only been well accepted recently. Genes for various components of the endocytic machinery have been identified in *Arabidopsis*; various membrane impermeable vital dyes have been shown enter pollen tubes through endocytosis; finally, charged nanogold particles are internalized by tobacco pollen tubes providing definite evidence that endocytosis does take place in pollen (Moscatelli et al., 2007). In fact, more than one mode of endocytosis has been proposed. Beside a conventional plasma membrane-endosome-vacuole route, a quick recycling route has to be in operation as various membrane factors such as have been shown to recycle in this manner (Russinova et al., 2004; Takano et al., 2005; Geldner et al., 2007). Recent microscopic evidence demonstrated that vesicles and endosomes show dramatically different modes of motion supporting this notion (Voigt et al., 2005; Wang et al., 2006).

Actin is a special case of differential distribution in that it is largely absent in the form of cables from apical region, forms a mesh or fringe right behind the apical region, while in the shank it forms long cables. This form of actin organization is essential for

proper organelle positioning as discussed above. Disruption of actin organization or depolymerization of actin inevitably results in the blurring of zonation especially in the apical and subapical region. After such treatments, invasion of organelles such as the ER, vacuole and mitochondria into the apical region has been well documented by TEM and confocal microscope (Lovey-Wheeler et al., 2005).

Although organelle movement is dependent upon the actinomysin system, the mechanism(s) by which the apical vesicles move is still unclear, on one hand this movement is dependent upon actin polymerization, on other hand, prominent actin cables are virtually absent from this region. One attractive hypothesis is that apical vesicles are propelled by actin polymerization or an actin comet tail. This mode of movement is generated by actin polymerization and depolymerization alone, neither motor proteins nor long actin tracks are required. Voigt et al. showed the movement of root hair endosomes to be insensitive to myosin inhibitor, 2,3-butanedione monoxime, but sensitive to actin depolymerization agents, and proposed that the actin comet tail may propel endosome movement in root hair cells (Voigt et al., 2005). Although long prominent actin cables are absent in tip region, short fragmented actin filaments and actin patches are present and an actin gel structure has been proposed to form in the tip region (Holdaway-Clarke and Hepler, 2003). However, the movement of the root hair endosome is highly variable and different from pollen tube apical secretory vesicles (Voigt et al., 2005), it would be interesting to investigate whether or not pollen apical endo/exocytosis is dependent upon myosin motors.

Actin dynamics are fine tuned by a number of factors, such as ADF/cofilin, profilin, gelsolin and GTPases (Ren and Xiang, 2007). Rop/Rac GTPases are well-

established molecular switches in controlling actin dynamics and 11 Rop/Rac genes are present in *Arabidopsis*. They function as molecular switches in the sense that they are active in GTP-bound form and inactivate in GDP-bound form. GTPase cycles between these 2 forms and their functions are modulated by a number of regulatory factors such as guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP dissociation inhibitors (GDIs). For example, constitutively active AtRop1 results in depolarized growth in pollen tubes, actin cables in these pollen tubes show a spiral-like organization, GDI and GEF interacting with AtRop1 have also been identified (Fu et al., 2001; Gu et al., 2006) .

The Rop/Rac GTPases of *Arabidopsis* belong to a distinct subfamily of Rho class of GTPase. Several Rop GTPases was localized to the apical plasma membrane of pollen tubes (Zheng and Yang, 2000; Yang, 2002). Over-expression studies with wild type and mutant forms of Rop have shown they are critical for pollen tube growth and may be involved in polarity establishment. The over-expression of a dominant negative form of *Arabidopsis* Rop1 GTPase caused strong inhibition of pollen tube growth in tobacco while retaining normal pollen tube morphology. The over-expression of constitutively active mutants resulted in a loss of polarity, tip swelling and rearrangement of the actin filaments into transverse arrays (Kost and Shimmer, 1998). A number of potential downstream effectors of Rop, termed RICs (Rop-interactive CRIB motif-containing proteins), have also been identified. Preliminary over-expression studies with RICs suggested they are targets of Rop GTPase in pollen tube growth (Wu et al., 2001).

It has long been known that a steep tip focused Ca^{2+} gradient is essential for pollen tube growth. Pollen tubes exhibit a characteristic tip-focused gradient in the

extreme apex of the growing pollen tube where the cytosolic calcium concentration may approach 10 mM (Messerli et al., 2000). Only growing pollen tubes exhibit this gradient and it is lost when pollen tube growth stops. Further, pollen tubes exhibit a reorientation response toward a localized increase in Ca^{2+}concentration (Malho et al., 1994). The gradient drops off sharply and reaches a basal level of 150-200 nM within approximately 20 µm from the apex. Ratiometric imaging of this cytosolic calcium reveals the dynamic nature of this calcium gradient, which pulses coincident with the growth phase of pollen tube (Holdaway-Clarke and Hepler, 2003). Strong evidence also suggests different types of calcium channels and pumps must operate in concert to produce and maintain this sharp gradient. Recent cloning of a cyclic nucleotide-gated calcium channel from *Arabidopsis* provided direct evidence for the operation of channels in pollen tubes (Frietsch et al., 2007). However, signaling pathways up- and downstream of calcium dynamics are still not well understood. Potential candidates may include lipid signaling, Rop GTPase, calmodulin, cAMP and CDPK (Moutinho et al., 2001; Rato et al., 2004; Yoon et al., 2006). Of these, CDPKs which are a family of plant unique calmodulin-independent calcium dependent protein kinases are particularly intriguing. There are 34 CDPK genes identified in *Arabidopsis* and they are localized to diverse locations such as plasma membrane, ER, cytosol, peroxisome and nucleus (Dammann et al., 2003; Hrabak et al., 2003; Chehab et al., 2004). CDPKs consist of four domains, a regulatory calmodulin-like domain, an auto-inhibitory domain, a kinase domain, and a variable N-terminal domain usually contains lipid modification sites which may be responsible for their subcellular localization (Dammann et al., 2000; Harper and Harmon, 2005). They are responsible for the majority of calcium-stimulated protein phosphorylation in plants

and their diverse localizations support the notion they participate in a variety of signaling events. A number of CDPKs have been functionally characterized including PiCDPK1 and PiCDPK2 in our laboratory, which are involved in pollen tube growth (Yoon et al., 2006). Apart from the well established Ca^{2+} gradient, a number of other ion gradients and molecular/functional gradients are also present (Holdaway-Clarke and Hepler, 2003). For example, there is evidence for the presence of an H^{+} gradient in pollen tube producing a slightly acidic tip followed by a so-called "alkaline band" (Feijo et al, 1999). This phenomenon may be important in maintain the plasticity of the cell wall at the tip (Denes et al., 2000).

Besides ion gradients, localization or activity of virtually all signaling molecules is polarized. In most cases, these molecules are not uniformly distributed. For example, Rop1, PIP2, and DAG are localized to the tip membrane (Fu et al., 2001; Helling et al., 2006), Pet PLC1 and tobacco Rab11 are focused at the tip region (Dowd et al., 2006; De Graaf et al., 2005), Nt PLC3 is focused at the subapical membrane (Helling et al., 2006). In some cases, the distribution is uniform but the activity forms a gradient, for example, calmodulin is found to be nearly evenly distributed in pollen tube but only active at certain region as its activity is linked to the Ca^{2+} gradient (Rato et al., 2004). The disruption of proper localization of many of these molecules results in the perturbation of pollen tube growth.

Clearly, a number of interdependent parallel signaling pathways must operate in concert to ensure proper pollen tube growth. Signaling components in those pathways are not arranged in a linear hierarchy but rather in a network where perturbation of one factor could result in a number of changes elsewhere which may amplify or compensate for the

primary effect. Supporting this notion, it is generally observed that exo/endocytosis, Ca^{2+} gradient, actin dynamics and pollen tube polar growth are tightly coupled, perturbation of one aspect generally leads to the perturbation of all aspects of pollen tube growth. Also consistent with the idea that signaling pathways are well balanced, most perturbations of factors involved in pollen tube growth result in growth inhibition.

Pollen tube extension is among the fastest growth processes in plants, it is clear that the zonation discussed above is transient in nature and must be dynamically maintained. Fluorescent maker proteins provide vivid pictures of this dynamic zonation and in real-time. The pulse and wave-like fluctuations of ion gradients clearly provide a mechanism for this dynamic transition and integration (Holdaway-Clarke and Hepler, 2003). The dynamic maintenance of molecule gradients is discussed below.

As pollen tubes extend, a formerly pinpoint growth focus along with polarity-establishing factors must become diffused as a result of a retrograde membrane flow caused by exocytosis. For pollen tubes to maintain polarized growth, they must constantly re-establish a focus to grow, and continuously neutralize the extended focus. Localization of focus-establishing factors to the very tip clearly provides a mechanism for establishing polarity. Some factors can be passively deactivated, this is true for factors which activity is position-dependent such as calmodulin and CDPK. When they are no longer in an activating context such as high Ca^{2+} concentration, they are inactive. Most factors, however, have to be actively deactivated. Possible mechanisms include enzymatic deactivation, for example by degradation of bound GTP to GDP, or by phosphorylation or dephosphorylation; another possible route for membrane associated

molecules is through endocytosis, followed by either recycling or proteolytic degradation (Helling et al., 2006; Klahre et al., 2006; Sheng et al., 2006; Zi et al., 2007).

Endocytosis and SNARE proteins

Endocytosis and exocytosis are essential cellular processes, which cells depend on for intra/inter cellular material and signal exchange. This is particularly true for cells specialized in polar growth, such as neurons, pollen and root hairs. They depend upon exocytosis to directionally deliver materials for plasma membrane and cell wall expansion. Endocytosis accompanies this exocytic process to ensure regulated polarized growth (Battey et al., 1999; Samaj et al., 2006). Endocytosis in plants has been implicated in diverse events such as polar growth in pollen tubes and root hairs (Samaj et al., 2006), shoot and root gravitropism (Friml et al., 2002; Silady et al., 2004), auxin transport (Geldner et al., 2001), stomatal closure (Blatt, 2000), cell plate formation (Dettmer et al., 2006; Chow et al., 2008), stress responses (Mazel et al. 2004) and pathogen defense (Robatzek et al. 2006). Mounting evidence suggests there is an intimate relationship between endocytosis and signaling (Di Fiore and De Camilli, 2001; González-Gaitán and Stenmark, 2004). Endocytosis provides a convenient way for the regulated internalization and degradation of receptors and enzymes, such as the cases for endo-1,4-ß-D-Glucanase KORRIGAN1 (KOR1) involved in cellulose synthesis (Robert et al., 2005) and the flagellin receptor FLS2 (Robatzek et al., 2006).

The investigation of endocytosis lags behind in plants when compared to yeast and mammals due to several reasons. First, even the existence of endocytosis in plant has long been questioned as the presence of a rigid cell wall and high turgor pressure has

thought to make endocytosis energetically unfavorable (Saxton and Breidenbach, 1998). In addition, the presence of a cell wall makes the use of endocytic tracers and protein markers, successful approaches used in yeast and mammalian systems, difficult in plants. Compounding the difficulties is the inherent complexity of the plant endomembrane system (Vitale and Galili, 2001). Numerous experimental data now supports the existence of endocytosis in plants, these include the uptake of endocytic marker dyes (Ueda et al., 2001; Cholewa and Peterson, 2001), as well as localization and functional studies employing filipin-labeled plant sterols (Grebe et al., 2003) and green fluorescent protein (GFP)-tagged marker proteins (Ueda et al., 2001). The availability of genomic data provides additional support as plant genomes possess members of all the necessary gene families to perform endocytosis (Sanderfoot et al., 2000; Shiu and Bleecker, 2001; Yang 2002).

The technical difficulties previously mentioned were circumvented or solved by techniques such as the use of protoplasts in studying endocytosis, the use of membrane-impermeable styryle dyes such as, FM 1-43 and FM 4-64 as endocytic tracers (Parton et al., 2001; Ueda et al., 2001), the establishment of Rab GTPases Ara6, Ara7 and Rha1 compartments (Ueda et al., 2001, 2004; Sohn et al., 2003), and the use of the fungal toxin BFA and wortmannin as inhibitors of endocytosis (Nebenfuhr et al., 2002). Among these, FM dyes are particularly notable. FM dyes are a series of cationic styryl dyes that typically have a lipophilic tail at one end and a highly hydrophilic charged head group at the other. These molecules only fluoresce when associated with a membrane and are widely used as endocytic tracers. Externally applied FM dyes gradually start to fluoresce as the bind to the plasma membrane and are internalized via rapidly moving endosomes,

eventually reach the vacuole. It has been shown that this internalization is dependent on the vesicle transport machinery (Ueda et al., 2001; Emans et al., 2002).

Although the investigation of vesicle traffic and fusion in plant systems lags behind animal and yeast systems, the availability of genomic data has led to the identification of homologs to essentially all of the genes shown to be involved in endo/exocytosis in other systems. One function of endocytosis in mammalian systems is to deactivate receptor kinases. Although such a function has not been well established in plants, receptor-like kinases (RLKs), once thought to be absent in plants, have been found to be abundant in plant genomes. In fact, there are more than 600 RLKs identified in the *Arabidopsis* genome, making them the largest family of putative kinases (Shiu and Bleecker, 2001; 2003). Although most RLKs are not functionally characterized, the presence of such a great number suggests that they may function in the perception of a wide range of signals or stimuli. Recently LePRK1 and LePRK2 receptor kinases were found to be involved in pollen tube growth by interacting with PRONE-domain containing kinase partner protein, a new family of Rop-GEF proteins (Kaothien et al., 2005; Berken et al., 2005). This indicates that RLKs may transduce extracellular signals through Rop GTPase pathways. Rab GTPases and SNARE proteins are important components of vesicle budding, docking and fusion machinery. In total 93 small GTPases have been identified in the *Arabidopsis* genome, with 57 Rab GTPases and 11 Rho GTPases, which are known to be involved in membrane trafficking (Yang, 2002; Vernoud et al., 2003). Rab11 from tobacco has been shown to localize to the apical region and important in pollen tube polar growth (De Graaf et al., 2005). Analysis of the

Arabidopsis genome has revealed 54 SNARE and clathrin genes (Sanderfoot et al., 2000; Yang, 2002; Uemura et al., 2005).

It is believed that there are at least 4 types of endocytosis in operation in mammalian systems, namely, clathrin coated receptor mediated endocytosis, lipid raft-mediated endocytosis, fluid phase endocytosis (pinocytosis) and phagocytosis, all of which also exist in plants (Schultze and Kondorosi, 1998; Samaj et al., 2004). The best studied type of endocytosis in mammals is clathrin-mediated receptor-ligand uptake in which receptor-ligand complexes are specifically internalized to desensitize receptor-mediated responses. These ligand-receptor complexes cluster into specific protein coated pits at the plasma membrane. Clathrin is one of the major scaffolding proteins of coated pits. The pits then bud off into vesicles, fuse into early endosomes, and from early endosomes, either be recycled back to plasma membrane or go further to late endosomes and finally the lysosome. There are specific molecular markers such as SNAREs, Rab GTPase, which characterize each step of the endocytic pathway (Marinissen and Gutkind, 2001, Gonzalez-Gaitan and Stenmark, 2003). Recently, GFP-tagged *Arabidopsis* flagellin perception LRR-RLK flagellin sensitive 2 (FLS2) was found to exhibit ligand-induced internalization and accumulate in endocytic vesicles, which made FLS2 the first documented ligand-mediated endocytosed RLK in plants (Robatzek et al. 2006). The receptor for brassinosteroids, BR1, and boron transporter BOR1, are also regulated in a similar fashion (Russinova et al., 2004; Geldner et al., 2007; Takano et al., 2005).

Available genomic data suggest that plants possess clathrin and also putative homologs of most of the proteins necessary for clathrin-dependent endocytosis such as budding (epsin), scission (dynamin) and uncoating (auxilin, synaptojanin) (Holstein,

2002). The vast number of RLKs present in plant genome further support the notion that clathrin-mediated endocytosis exists in plants (Shiu and Bleecker, 2001). The final destination in plant endocytosis pathway is the vacuole. The nature of early endosomes and late endosomes in the plant endocytic pathway remain controversial, the general view is that trans-Golgi network (TGN) or partially coated reticulum (PCR) in plants corresponds to the "early endosomes" of animals (Müller et al., 2007; Lam et al., 2007b). This means that the plant TGN functions in both secretory and endocytic pathways which is rather unusual as the TGN in animal systems is an independent entity from early endosome and does not have an endocytic function. In mammalian cells, the position of Golgi stacks is usually close to the cell center and TGN is tightly associated with the trans-side of the Golgi stacks (Kupfer et al., 1982). While in plants Golgi stacks and associated TGN are considered to be a unit and the units or clusters of these units are scattered over the entire cell. TGN can be found either close to or loosely associated the *trans* side of Golgi stacks. Under EM, the TGN is described as a cluster of electron translucent round and tubular vesicles reaching up to 100 to 150 nm in size, these vesicles show an electron-dense clathrin coat at their surface and are often interconnected and sometimes called the PCR (Dettmer et al., 2006; Hause et al., 2006; Preuss et al., 2006; Lam et al., 2007a).

A number of molecular markers have been described for the TGN such as *Arabidopsis* SNARE protein SYP41 (Uemura et al., 2004) and the vacuolar membrane V-ATPase subunit VHA-a1 (Dettmer et al., 2006), an endocytosis-mediating protein secretory carrier membrane protein1 (SCAMP1) (Lam et al., 2007a). A small Rab-GTPase RabA4b in *Arabidopsis* root hairs, which is a homolog of the mammalian Rab11

family, is localized in the clear zone, this RabA4b compartment was shown to have a TGN-like localization using immuno-gold electron microscopy (Preuss et al., 2004, 2006). Another isoform, RAB11B in tobacco pollen tubes, was also localized in the clear zone and colocalizes with the endocytic marker, FM4-64 (de Graaf et al., 2005). It has been proposed that in rapidly secreting cells such as pollen and root hairs, the TGN is vesicular and labeled by Rab4 in root hairs and Rab11 in pollen tubes. These TGN vesicles accumulate within the clear zone at the tip to serve both secretory and endocytic functions (Samaj et al., 2006).

The prevacuolar compartment (PVC) or multivesicular body (MVB) in plants is believed to correspond to late endosomes (Tse et al., 2004). In animal cells, MVBs are defined as late endosomes, and have dual roles in the late endocytic and the secretory pathways. Plant PVC/MVBs are spherical electron translucent structures reaching sizes up to 200 to 500 nm. It is clear now that the plant PVC functions in both endocytosis and secretory pathways. In fact, the TGN to PVC to vacuole pathway is an endocytic pathway as well as a secretory pathway (Lam et al., 2007b). Molecular markers described for PVC are vacuolar sorting receptors (VSRs) which mediate lytic vacuole targeting such as BP80 (Li et al, 2002), the *Arabidopsis* syntaxin SYP21 (Uemura et al., 2004) and Rab GTPases such as Ara6/RabF1, Ara7/RabF2B and Rha1/RabF2A (Ueda et al., 2001; Sohn et al., 2003). There has been some controversy about the localization of endosomal Rab GTPases in plants as their localizations are not identical, but partial colocalization of their specific compartments has been obtained (Ueda et al., 2004) and immunogold labeling show all three Rabs are associated with PVC/MVB (Haas et al., 2007). It is worth noting that these Rab GTPase compartments show as FM4-64 labeled punctuate structures

distributed along the whole length of root hairs (Voigt et al., 2005) but their morphology in pollen tubes remain to be established.

Endocytosis is eventually accomplished by membrane fusion events in which the contents of a compartment is mixed with that of another compartment. In his seminal 1993 SNARE hypothesis, Rothman proposed that a class of SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors) proteins is sufficient to guide the specificity of and mediate membrane fusion events (Söllner et al., 1993a, 1993b). SNAREs are usually small proteins ranging from 150-300 amino acids with a membrane-anchored C terminus and an N terminal cytosolic SNARE motif which functions in membrane fusion. The α-helical coiled-coil 'SNARE motifs' are composed of a repeated hydrophobic heptad register, which enables them to potentially form complexes with each other. SNAREs are necessary and sufficient to mediate specific membrane fusion events at least *in vitro* (Weber et al., 1998). The SNARE hypothesis is largely validated by the classical synaptic Ca^{2+} regulated exocytosis in which a heterotrimeric *trans*-SNARE complex or SNAREpin is formed between SNAP25 (2 SNARE motifs), syntaxin 1 and synaptobrevin/VAMP (Weber et al., 1998). A number of heterotrimeric or heterotetrameric SNAREpins were later identified in other systems, crystal structures of some of these molecules have also been resolved (Gonzalez et al., 2001; Sutton et al. 1998; Tochio et al. 2001). Membrane fusion is mediated through the formation of a very stable four-helix bundle *trans*-SNAREpin complex by three Q (t) SNAREs and a R (v) SNARE. The *trans*-SNAREpin complex then becomes *cis* during which vesicle contents are mixed. The SNARE complex then dissembles and is recycled for another round of membrane fusion events (Bonifacino and Glick, 2004). Other factors,

such as NSF, SNAP, SM proteins, Rab GTPases and tether proteins are also important in this process (Malsam et al., 2008). Traditionally, SNAREs are known as v- or t-SNAREs, depending on their localization to either the transport vesicle (v) or the target (t) membrane (Fasshauer et al., 1998). Q- and R- SNAREs are classified based on a conserved arginine or glutamine polar residue at the position of the so-called 'zero' ionic layer of the four-helix bundle. The two light chains of a Q- SNARE can come from one protein, such as SNAP25, or from two proteins, such as syntaxin 7 and syntaxin 8. Q-SNAREs can be subdivided into Qa- (syntaxin), Qb- (25-kDa synaptosomal-associated protein (SNAP-25) N-terminal SNARE motif) or Qc- (SNAP-25 C-terminal SNARE motif) SNAREs (Scales et al., 2000, Bock et al., 2001). The R-SNAREs can be also further divided into short VAMPs (vesicle-associated membrane proteins) or 'brevins' and long VAMPs or 'longins', on the basis of whether they contain a short variable domain or a conserved longin domain of 120–140 amino acids at their N terminus (Filippini et al., 2001).

Longins can be further functionally and structurally divided into R.I (Sec22-like), R.II (Ykt6-like) and R.III (VAMP7-like) groups while brevins forms the R.IV group. Comparisons between available data suggest that R.I group functions in the ER, R.II in the ER to Golgi and intra-Golgi trafficking, R.III in endosome trafficking (Kloepper et al., 2007). From this classification, the R-SNAREs mediating membrane fusion in endocytosis should be VAMP7 family proteins. The well-studied human VAMP7 or Ti-VAMP largely validates this hypothesis, although it does mediate some exocytic processes originating from the lysosome (Advani et al., 1999; Pryor et al., 2004, 2008). Preliminary characterization of Arabidopsis VAMP7s showed them to be localized to the

PM, endosomes and vacuole, also consistent with this rule (Uemura et al., 2004). Although plants have to use VAMP7 for exocytosis since they do not have the R.IV brevin type SNAREs which are used by animal and yeast cells for secretion (Kloepper et al., 2008). The presence of a relatively large family of VAMP7s in Arabidopsis also leads support to the notion that VAMP7 may mediate diverse membrane fusion events (Sanderfoot et al., 2000). However, characterization of the function of individual VAMP7 isoforms in their proper physiological context has not been done (Uemura et al., 2004).

Peroxisomes and their role in pollen tube growth

Peroxisomes are small organelles virtually ubiquitous to eukaryotes. This inconspicuous single-membrane bound organelle is versatile and participates in different metabolic pathways. Peroxisomes are believed to contain proteins of eukaryotic origin and alaph-proteobacteria origin which may be transferred from the ER and mitochondria (Gabaldón et al., 2006). The minimal ancestral peroxisome or "universal" peroxisome is believed to contain a core set of peroxin (Pex) proteins and enzymes that function in fatty acid metabolism (Tabak et al., 2006). Although plant peroxisomes do have the signature function of β-oxidation and scavenging the resulting hydrogen peroxide, they also have the classic plant-specific function in photorespiration and some unique features. Plant peroxisomes are able to differentiate in specific cell types and each type contains a unique set of enzymes corresponding to the specific requirements of that cell type (Nishimura et al., 1996). Glyoxyosomes in endosperm and cotyledons use fatty acid β-oxidation and glyoxylate cycle enzymes during germination and seedling establishment. Leaf peroxisomes contain enzymes of the photorespiratory glycolate pathway to process

phosphoglycolate produced during C3 plants photosynthesis dark reaction. Peroxisomes in root nodules participate in ureide biosynthesis and peroxisomes in senescent tissue are sometimes referred to as gerontosome (Hayahsi and Nishimura, 2006). In addition to metabolic functions, evidence suggests that plant peroxisomes also participate in stress responses and plant pathogen defense (Titorenko and Rachubinski, 2004; Baker et al., 2006). Plant peroxisomes are involved in the biosynthesis of signaling molecules such as indole acetic acid (IAA), jasmonic acid (JA) and salicylic acid. This is accomplished though the multifunctional ß-oxidation pathway in peroxisomes as it is able to accommodate a variety of substrate including aromatic or cyclopentanone rings (Reumann et al., 2004).

Peroxisomes can vary dramatically in size and number according to environmental, metabolic or developmental requirements. This phenomenon was first observed in yeast cells grown on oleic acid as the sole carbon source and screening for mutants defective in peroxisome biogenesis led to the identification of a group of proteins late named peroxins (Pex) (Veenhuis et al., 1987, Erdmann et al., 1989, 1991). Pex proteins were found to be pivotal in peroxisome membrane assembly, their division, proliferation and maturation, and finally peroxisomal membrane protein insertion and matrix protein import (Brown and Baker, 2003; Platta and Erdmann, 2007). There have been 33 peroxin genes identified in yeast and 23 in *Arabidopsis* (Mullen and Trelease, 2006). Pex proteins are believed to be of ER origin and the core Pex proteins show similarity in sequence, subcellular localization and function to ERAD system proteins (Gabaldón et al., 2006). For example, Per1 and Per6 are AAA cassette containing proteins with homology to Cdc48, Pex2 and Pex10 are ubiquitin ligase (RING-finger)

domain containing proteins with homology to ERAD ubiquitin ligase Hrd1 and Pex4 are E2 ubiquitin conjugating enzyme domain containing protein with homology to ERAD Ubc1 (Gabaldón et al., 2006, Platta and Erdmann, 2007).

Although controversial historically, it is now generally accepted that peroxisomes have at least two ways to originate, one *de novo* pathway from the ER and one budding pathway from pre-existing peroxisomes (Mullen and Trelease, 2006). Multiple lines of evidence has confirmed that there is extensive traffic between the ER and peroxisomes. The budding pathway has long been established as the dominant peroxisome biogenesis model and the ER pathway has only been recently reemerged as a viable alternative. In the ER pathway, a subset of peroxisomal membrane proteins are first sorted to a subdomain of the ER, the so-called "peroxisomal ER", and then bud off to form a peroxisome (Titorenko and Rachubinski, 2001; Titorenko and Mullen, 2006; Mullen and Trelease, 2006). An elegant real time observation of *de novo* peroxisome formation conducted by Hoepfner et al. provided convincing evidence for the ER pathway as well as explaining the source of peroxisome membrane (Hoepfner et al., 2005; Kunau, 2005; van der Zand et al., 2006). The ER pathway indicates there must be extensive traffic between the ER and peroxisomes to the extent that peroxisomes should legitimately be considered to be part of the endomembrane system (Titorenko and Mullen, 2006). This model also suggests there may be several intermediate stages between the ER and mature peroxisomes although the exact nature of these compartments remains to be established (Titorenko and Mullen, 2006). Molecules involved in vesicle budding and trafficking such as dynamin-related proteins, and Arf and Rab GTPases were also found to be involved in these steps (Lay et al., 2006; Hashimoto et al., 2008).

It has long been known that pollen has oil bodies and peroxisomes, but the relationship between these organelles is unclear and whether pollen peroxisomes are specialized is still under investigation (Zhang et al., 1994; Piffanelli et al., 1998). Theoretically speaking, potential roles of peroxisomes in pollen tube growth may include, reserve mobilization by β-oxidation for energy, cell signaling - as peroxisomes have been shown to produce signaling molecules such as GABA, JA, IAA and NO (Prado et al., 2004, Baker et al., 2006) and structural functions in membrane production. Despite the fact that a *bon fide* NOS has not been identified in plants, pollen peroxisomes have been shown to produce NO (Prado et al., 2004). The glyoxysome enzymes such as isocitrate lyase and malate synthase, which are generally considered glyoxysomal signature enzymes have been shown to be expressed in pollen tubes in some species (Zhang et al., 1994). Oil reserves in pollen may either be respired to provide energy or converted to membranes or both (Piffanelli et al., 1998). Even if the glyoxylate cycle is not active in pollen tube peroxisomes, citrate can still be exported for respiration (Baker et al., 2006). Whatever their specific function, peroxisomes are important in pollen tube growth as a number of peroxisome mutants including *cts* and *amp2/amc* mutants are male sterile (Footitt et al., 2007; Boisson-Dernier et al., 2008).

Genetic screens have yielded a few photorespiration mutants due to its obvious phenotype, other peroxisome mutations have proven difficult to isolate, a few peroxisomal mutants did emerge recently which have defective male gametophytes, with processes such as anther dehiscence, pollen germination and pollen tube growth and reception being affected (Hayashi and Nishimura, 2006). *cts* mutants have reduced fertilization due to impaired pollen tube growth which results in unfertilized ovules and

aborted embryos. *In vitro* pollen tube growth experiments have shown that *cts* pollen tubes have comparable growth rate to wild type in normal growth media, but germination and growth were reduced if exogenous carbon source was absent in growth media. *CTS* was found to encode an ATP-binding cassette transporter required for substrate import into peroxisomes for β-oxidation. CTS was proposed to be necessary in fulfilling energy requirements during pollen tube growth since pollen represents a symplastically isolated sink (Footitt et al., 2007). In *amp2/amc,* reduced male transmission efficiency was found to be due to pollen tube overgrowth and disruption of sperm discharge at the final stage of male-female gametophyte reception (Mano et al., 2006; Boisson-Dernier et al., 2008). Pollen tube reception is impaired only when both parents bore the *amc* mutation. *amc/amp2* encode for Pex 13 which is essential for peroxisomal protein import and *AMC* mutation disrupts PTS1/2-dependent protein import. These reports suggest that functional peroxisomes in either the male or female gametophyte are essential for proper pollen-tube reception and subsequent sperm cell discharge.

Exciting advances have recently been made regarding pollen tube growth, however notable gaps remain. No clear pathway has emerged in RLK signaling even though hundreds of RLKs are present, equally puzzling few reports are available for a role of tyrosine phosphorylation in polar growth. Recent identification of a family of plant specific GEFs highlights the inadequacy of relying solely on extrapolation from mammalian systems (Berken et al., 2005). Although endocytosis is well established in plants, we lack detailed information of the specific components of the endocytic molecular machinery such as SNAREs in pollen tubes. The roles played by less conspicuous intracellular compartments, for example peroxisomes, are virtually unknown.

This research is focused on two *Petunia inflata* proteins involved in pollen tube growth, PiVAMP721, a SNARE protein, which we hypothesize to be involved in endocytosis and PiSCP1, a novel protein identified as a calcium dependent protein kinase substrate, which we have determined is targeted to peroxisomes.

The purpose of the research present in this dissertation was to identify molecules important in pollen tube growth and through functional characterization attempt to integrate them into the emerging polar growth signaling network. A flowering plant, Petunia, is used as the model system in this study. Although not a crop plant itself, as a member of the Solanaceae, Petunia is closely related to a considerable number of species of agronomic importance. The genus Petunia has been well-studied, an established transformation system, a comprehensive genomic BAC library and assorted cDNA libraries are available. Petunia is well suited for pollen studies as it is easy to keep plants flowering year round and harvest large amounts of pollen. It is an excellent model system that will generate results applicable to other members of the Solanaceae and beyond.

This first chapter has been written in the format of a review to summarize our current understanding of pollen tube growth. The subsequent chapters of this dissertation are written in formats for publication in suitable journals. Chapter 2 has been split into two parts, chapter two A, a SNARE protein, PiVAMP7 is identified and characterized. The function of its longin domain and SNARE domain of this protein is explored, this section is a single publication unit. Chapter two B, which investigates the effect of potential phosphorylation on PiVAMP721 is rather preliminary and the results presented are not anticipated to be published with those of chapter two A. In chapter three, the

function of two peroxisomal proteins, PiSCP1 and PiCDPK2 is investigated. Conclusions and future directions are discussed in chapter four.

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CHAPTER 2A

Identification and characterization of the role of an R-SNARE protein PiVAMP721 in pollen tube growth in *Petunia inflata*

Abstract

Pollen tubes are a particularly useful model with which to study exocytosis and endocytosis as the pollen tube apex is dedicated to these processes. Membrane fusion is a key step during exocytosis and endocytosis. VAMP7 proteins are SNAREs (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptors) shown to mediate cognate membrane fusion. *Arabidopsis* VAMP7s localize to the plasma and endosome membranes. Here we show that PiVAMP721 of *Petunia inflata* plays a key role in pollen tube growth. Phylogenetic analysis suggested that AtVAMP725 is the closest *Arabidopsis* homologue of PiVAMP721. PiVAMP721 is expressed specifically in pollen as demonstrated by RT-PCR. Over-expression of full-length PiVAMP721 fused N terminally to YFP (yellow fluorescence protein) inhibits pollen tube growth; expression of N terminus alone had no effect, whereas the expression of the C terminus alone strongly inhibited tube growth. At low expression levels PiVAMP721 colocalized with the endocytic marker FM4-64 to the cone of transport vesicles at the tube tip. Interestingly high levels of over-expression, which were associated with growth inhibition, induced the formation of novel membrane sacs at the tube tip to which both endo- and exocytic markers localized, perhaps implicating PiVAMP721 in "fast recycling" of endosomes. We hypothesize that PiVAMP721 is involved in an endocytic pathway important to pollen tube growth to balance membrane dynamics and that its N terminus has a negative regulatory function.

INTRODUCTION

Endocytosis and exocytosis are essential cellular processes through which cells exchange intra/inter cellular material and in addition regulate the abundance of plasma membrane receptors. Exocytosis is a particularly important process for cells specialized for polar growth, such as neurons, pollen and root hairs, as this process is the vector through which materials for growth are delivered in a directional manner. Endocytosis accompanies this exocytic process to ensure regulated polarized growth. As the molecular mechanisms responsible for endocytosis and exocytosis are beginning to be unravelled, an increasing volume of evidence suggests that endocytosis and exocytosis have a closer relationship than previously thought. It is clearly informative to view endo/exocytosis as a complete cyclic process where vesicles are continuously formed, released, transported, docked, fused and recycled rather than as two independent processes (for recent reviews, see Bonifacino and Glick 2004; Samaj *et al.* 2005).

The polar growth nature of pollen tube growth makes it an excellent model with which to study endocytosis and exocytosis (Samaj et al., 2006). In this particular polar growth model, the pollen tube apex is a “clear zone” is void of organelles other than transport vesicles and is dedicated to endocytosis and exocytosis, these vesicles have been observed to be moving rapidly by active “reverse fountain” cytoplasmic streaming (Taylor and Hepler, 1997; Lovy-Wheeler et al., 2007). Pollen tubes are extended by the exocytic deposition of cell wall building materials, such as pectin esters. Concomitant vigorous endocytosis is required for the uptake of nutrients, recycling of membrane and maintaining of a proper tube shape (Samaj et al., 2004). However, the identity and

regulation of the specific molecules responsible for membrane fusion in pollen tube growth such as SNAREs remain largely unknown (Samaj et al., 2005, 2006).

SNARE (soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor) proteins are membrane associated proteins proposed to mediate membrane fusion. Endocytosis and exocytosis is eventually accomplished by membrane fusion leading to the contents of one membrane bound compartment being mixed with that of another or the environment. According to the SNARE hypothesis (Söllner et al. 1993a, 1993b), SNARE proteins are sufficient to guide the specificity of, and mediate, membrane fusion event. SNAREs are usually small proteins ranging from 150-300 amino acids with a membrane-anchored C terminus and an N terminal cytosolic SNARE motif, which functions in membrane fusion. The α-helical coiled-coil 'SNARE motifs' are composed of a repeated hydrophobic heptad register, which enables them to potentially form complexes with each other. SNAREs are necessary and sufficient to mediate specific membrane fusion at least *in vitro* (Weber et al., 1998). The SNARE hypothesis is largely validated by the classical synaptic Ca^{2+} regulated exocytosis in which a heterotrimeric *trans*-SNARE complex or SNAREpin is formed between SNAP25 (2 SNARE motifs), syntaxin 1 and synaptobrevin/VAMP (Weber et al., 1998). A number of SNAREpins were later identified in other systems and the crystal structures of some of these molecules are also been resolved (Gonzalez et al., 2001; Sutton et al., 1998; Tochio et al., 2001). The general model of membrane fusion which has emerged from these SNAREpins suggests that membrane fusion is mediated through the formation of a very stable four-helix bundle SNAREpin complex of Q (t) SNAREs and an R (v) SNARE. Q- and R- SNARE are classified on the basis of a conserved arginine or glutamine residue at

the position of the so-called ‘zero’ ionic layer of the four-helix bundle (Hong 2005). The R-SNAREs can be subdivided into short VAMPs (vesicle-associated membrane proteins) or ‘brevins’ and long VAMPs or ‘longins’, on the basis of whether they contain a short variable domain or a conserved longin domain of 120–140 amino acids at their N terminus (Figure 1, Filippini et al., 2001).

The investigation of vesicle trafficking and fusion in plant systems lags behind animal and yeast systems. However, the availability of genomic data has led to the identification of homologues of essentially all genes shown to be involved in endo/exocytosis in other systems. In total 93 small GTPases have been identified in the *Arabidopsis* genome, with 57 Rab GTPases and 11 Rho GTPases, classes which are known to be involved in membrane trafficking (Yang, 2002; Vernoud et al., 2003). SNARE and clathrin genes have also been identified in the *Arabidopsis* genome (Sanderfoot et al., 2000; Holstein, 2002). This conservation highlights the universal importance of many aspects of endo/exocytosis, on the other hand, some notable differences have also emerged. Plants have some unique features, such as the presence of storage and lytic vacuoles, the plant unique Rop GTPase family, calcium-dependent protein kinase (CDPK) family and a large VAMP7 family which lacks members of the brevin subfamily, but has a greatly expanded longin subfamily relative to animals (Sanderfoot et al., 2000; Vitale and Galili, 2001; Yang, 2002).

Vamp7 are a subclass of R-SNARE longins, sequence analysis and comparisons across available genomic data suggest they are involved in endocytic trafficking (Kloepper et al., 2007). The only VAMP7 of human, Ti-VAMP, has been implicated in the polar growth of neurites. Neurites grow longer when the TiVAMP C terminal domain

is over-expressed while neurite outgrowth is inhibited by over-expression of the N terminal domain (Martinez-Arca et al., 2000). Subsequent studies have showed TiVAMP is also involved in endocytosis (Alberts et al., 2003). A large family of VAMP7s has been identified in the *Arabidopsis* genome, and expression studies show that they largely localize to the PVC (prevacuolar compartment), TGN (Trans-Golgi network) and plasma membrane as one would predict (Uemura et al., 2004). However, tissue specific functional characterization of the members of this gene family is lacking. As plants lack brevin R-SNAREs and VAMP7s are their biggest group of R-SNAREs, understanding the individual function each VAMP7 in the cell types and physiological contexts in which they are expressed *in vivo* is important to our understanding of the plant endocytic network.

Here we report the identification of a pollen specific VAMP7 from *Petunia inflata* and provide evidence that it is important to pollen tube growth in this species. We show that GFP-tagged PiVAMP721 localizes to the transport vesicles of the apical clear zone of pollen tubes and colocalized with an endocytic marker, FM4-64. We also provide data that suggest that its SNARE domain-containing C terminus can promote membrane fusion while its N terminal longin domain negatively regulates this function.

Material and Methods

Plant material

Petunia inflata and *Petunia hybrida* were grown under green house conditions with a supplemental light regime of 16 hr light and 8 hr dark.

Yeast 2-hybrid library screening

We used the modified system developed for yeast two hybrid library screening (James et al, 1996). The yeast strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$-GAL_{TATA}-HIS3, MEL1, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ) was used in the screen. AH109 was sequentially transformed first with pGBD/ΔN-PiCDPK2 bait construct, and then with a *Petunia inflata* pollen cDNA library in pGAD424 by the lithium acetate method (Gietz et al., 1995). The transformed cells were plated onto synthetic drop-out (SD) medium lacking leucine, tryptophan and histidine. After 7 days, cells were replicated onto SD medium lacking leucine, tryptophan, histidine and adenine, and positive transformants subjected to a colony lift assay as described in yeast protocol handbook (Clontech, Palo Alto. CA). Yeast plasmid DNA was extracted from positive clones and transformed into competent XL1-blue MRF' (Stratagene, La Jolla, CA) and plated on LB agar plates with 100µg mL^{-1} ampicillin. Plasmids extracted from XL-blue MRF' were amplified with pGAD vector primers (GAD-F, 5'-TACCACTACAATGGATGATC-3', GAD-R, 5'-GCACAGTTGAAGTGAACTTG-3') to verify insert size and, amplified fragments were digested with 4-base cutting restriction enzyme, *Taq* I, to group positive clones in classes based on resulting digestion patterns. Positive clones were back-transformed into the AH109 yeast strain with the bait construct to confirm the interaction.

Sequence analysis

Nucleic acid and protein databases were searched with obtained sequences using BLASTP and BLASTN algorithms. All publicly available sequences were obtain from

GenBank through NCBI (http://www.ncbi.nlm.nih.gov/), phylogenetic analysis were carried out using DAMBE and Clustclaw software, the resulting phylogenetic tree and sequence alignment were manually fine-tuned and verified (Xia and Xie, 2001). A Prosite scan was performed through ExPASy PROSITE (http://us.expasy.org/prosite/) and PlantsP Feature Scan (http://plantsp.genomics.purdue.edu/plantsp/html/feature_scan.html). Potential CDPK phosphorylation sites were inferred as previously described (Harper and Harmon, 2005).

RT-PCR analysis

Total RNA was extracted from 100 μg of *P. inflata* tissues using Concert reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The final RNA pellets were resuspended in 50 µl of diethyl pyrocarbonate-treated water, and the RNA concentration was determined spectrophotometrically. The integrity of the RNA samples was verified by electrophoresis on 1.2% denaturing gels. Reverse transcription, 3' RACE amplification and purification of PCR products were performed as described in McCubbin et al., 2004 with PiVAMP721 gene-specific primers, F: 5'-ATGGGGCAACAAACGTTGA-3' and R: 5'-GGACCGAAGTTAACAAAA-3'. Briefly, the RNA was converted to cDNA using Superscript II (Invitrogen). Two microliters of each RT reaction was used as template in a 50 µl PCR reaction with gene-specific primers. PCR were then performed with the resulting cDNA products following 30 cycles of 95^{o}C for 30s, 60^{o}C for 30s and 72^{o}C for 2min. Each reaction was analyzed by Tris-acetate-EDTA-agarose gel electrophoresis and stained with ethidium bromide before visualization under UV illumination.

Constructs of plasmids for pollen expression

All constructs were generated by PCR, cloned into pGEM-T Easy vector (Promega, Madison, WI) and sequenced, prior to cloning into vectors for expression in pollen. An *EcoR* I site within the coding region of PiVAMP721 were removed by using primers, ERI remF: 5'-AGTATACCGAGTTCTCTGGT-3' and ERI remR: 5'-ACCAGAGAACTCGGTATACT-3' to synonymously change the relevant codon. The coding region of PiVAMP721 was converted to encode *Pvu* II at the 3' end and *Sac* I at the 5' end using primers LO/PVUII: 5'-CAGCTGATGGGGCAACAAACGTTG-3'; LOCTER: 5'-TTAAAAACAATTGAAGCCAGG-3'. An N terminal longin domain alone fragment was generated by PCR using the primers, LO/PVUII: 5'-CAGCTGATGGGGCAACAAACGTTG-3' and LO/NT-R: 5'-TTATTCTTCAGGATGTTCAACAC-3'. The C terminal domain alone construct was generated using by PCR using primers LODNT: 5'-CAGCTGAACAAGGAATTTGGG-3'; and LOCTER: 5'-TTAAAAACAATTGAAGCCAGG-3'.

Pollen expression constructs were generated in pBluescript (KS) (Life Technologies) using the pollen specific promoter *Lat52* (Twell et al., 1989). pBS*Lat52*-YFP was generated by cloning the coding region of eYFP (Clonetech, Palo Alto, CA) behind the *Lat52* promoter in pBluescript (KS) as previously described (Yoon et al., 2006). All YFP fusion constructs were subsequently generated by cloning appropriate fragments into the *Sac* I and *Pvu* II site to generate in frame fusions. All yeast 2 hybrid constructs were generated by cloning appropriate fragments into the *Sac* I and *Sma* I site

of pGADC1 (activation domain) and pGBDC3 (bait). Standard recombinant DNA methodology was used in all cloning steps.

Transient expression in pollen

Transient expression of GFP fusion constructs in pollen were performed as previously described (Yoon et al., 2006). Briefly, *Petunia inflata* pollen was collected from freshly dehisced anthers (10 flowers/bombardment), and suspended by gentle vortexing in 200 μl of pollen germination medium (PGM) (0.01% H_3BO_3, 0.02% $MgSO_4$, 0.07% $CaCl_2$, 15% PEG-4000, 2% sucrose). The pollen was spotted onto a 2.5 cm sq. piece of positively charge nylon membrane in a 9 cm Petri dish.

Microprojectile bombardment was performed using a PDS-1000/He biolistic system (Bio-Rad, Hercules, CA). Gold particles (1.1 μm) were prepared according to the manufacturer's protocol using 2 μg of plasmid DNA/0.5 mg of particles. Co-bombardment was achieved by coating particle with 2 μg of each plasmid construct. Bombardment were performed at 28-inches Hg chamber vacuum, using a 1100 psi rupture disk, 0.25 inch gap distance and 1 inch particle travel distance. After bombardment, the pollen was washed from the nylon membrane into a Petri-dish with 4 ml PGM and cultured on an orbital shaker at 100 rpm for 4 hrs at 30^{o}C. FM 4-64 staining was performed according to Parton et al. (2001). FM 4-64 (Molecular Probes, Eugene, OR) was added into PGM to a final concentration of 10 μM and visualized after 15 min of gentle shaking.

Analysis of transformed pollen tubes

Epifluorescence microscopy for YFP fusion constructs observation was performed using an Orthomat epifluorescence microscopy (Leitz) with a 40X, dry, 0.7 numerical Fluor objective. GFP fluorescence was visualized using a Fluor objective, 480 nm excitation, 500 nm dichroic mirror and >530 nm emission. Images were captured using a Sensys cooled CCD camera (Photometrics, Tucson, AZ). Confocal images were obtained using either Bio-Rad MRC 600 or Zeiss 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) 488 nm excitation and 515-565 nm emission filter was used for GFP signal, 543 nm excitation and 560 nm long-pass emission filter for FM 4-64, 514 nm excitation and 530-560 nm band-pass emission filter for YFP and 458 nm excitation and 450-490 nm band-pass emission filter for CFP. All confocal images were analyzed using Metamorph v4.5 (Molecular Devices Corp., Downlington, PA) and processed using Adobe Photoshop v5.5 (Adobe Systems Inc., San Jose, CA).

RESULTS

Identification of PiVAMP721 and sequence analysis

PiVAMP721 was initially identified during a yeast 2 hybrid library screen using the pollen specific CDPK, PiCDPK2 (Yoon et al., 2006), as bait. Though it was subsequently determined that this clone constituted a false positive in terms of interaction with PiCDPK2, sequence analysis of the cDNA encoded in its prey vector aroused further interest in studying this gene. BLAST search of the cDNA sequence showed that it encodes a full-length 220 amino acid VAMP7 which we subsequently named *P. inflata* (Pi) VAMP721. The sequence alignment shows its gene product contains all the characteristics of VAMP7, namely, an N terminal longin domain; a coiled coil SNARE

domain with arginine at “0” layer; a C terminal transmembrane domain separated from the SNARE domain by a cluster of basic amino acids (Fig. 2 and Fig. 3).

VAMP7 is a family of R or t-SNAREs that functions in membrane fusion. R-SNARE or VAMP can be broadly divided into brevins and longins (Filippini et al., 2001). Although brevins have been extensively characterized in synaptic systems, the longins which can be further divided into VAMP7-like, Sec22-like and Ykt6-like longins have only been recognized rather recently (Rossi et al., 2004a). Intriguingly, plants do not have brevins but possess an expanded family of longins, at least in the case of *Arabidopsis* which has 11 VAMP7-like longins. Mammals, on the other hand, have a family of brevins but rather small number of longins. Humans, for example, has only one of each VAMP7, Sec22, Ykt6 longin. A brief phylogenetic analysis of VAMP7s shown that PiVAMP721 is most closely related to rice VAMP7 (Fig. 4). The closest *Arabidopsis* homologues are AtVAMP725 and AtVAMP726. The AtVAMP721-727, rice VAMP7, pear VAMP7s and PiVAMP721 form a clade, while AtVAMP711-714, human, mouse, rat and fruit fly VAMP7s form another distinct clade (Fig. 4). From the pattern of VAMP7 phylogeny, it is conceivable that plants have a subfamily of VAMP7s that is closely related to animals, but also have another family of distantly related VAMP7s. PiVAMP721 belongs to the latter group. It is interesting to note that AtVAMP711-713 have been shown to be localized to the vacuole, while AtVAMP721-727 are mostly localized on the plasma membrane with AtVAMP723 to the ER and AtVAMP727 to endosome (Uemura et al., 2004). The closest *Arabidopsis* homologues of PiVAMP721, AtVAMP725 and AtVAMP726, both localize to the plasma membrane (Uemura et al., 2004). These AtVAMP7 localizations (PM, endosome, vacuole) are largely consistent

with a potential function in endocytic trafficking as inferred by bioinformatic studies (Kloepper et al., 2007). While the localization of *Arabidopsis* VAMP7s has been briefly studied in leaf protoplasts, their individual functions in their specific native cell types remains speculative.

PiVAMP721 is a pollen specific V-SNARE.

Since we identified PiVAMP721 in a pollen cDNA library, we investigated the expression pattern of PiVAMP721 to determine whether it is pollen-specific. To achieve this, semi-quantative RT-PCR was used. From the results shown in figure 5, expression of PiVAMP721 was found to start in the anthers of 10 mm buds, persist through the latter stages of pollen development and present in mature and in vitro germinated pollen. Furthermore, its expression was not detected in other parts of the flower, or the vegetative tissues analyzed. This expression pattern suggests that it is expressed specifically in pollen and pollen tubes. Notably the closest Arabidopsis homolog AtVAMP725 (At2g32670) has been classified as a pollen specific or pollen enriched gene by genome analyses (Honys and Twell, 2003; Pina et al., 2005).

Localization of YFP-PiVAMP721 and colocalization with endocytic maker FM4-64

Next we addressed the question of PiVAMP721 localization by transient expression. To this end, a YFP-PiVAMP721 fusion construct was made (Fig. 3). R-SNAREs are generally membrane anchored cytosolic proteins. PiVAMP721 contains a putative transmembrane domain. As to the cellular localization of PiVAMP721, our transient expression showed its signal localized mainly to the tip; with a pattern

reminiscent of the previous described V-shape inverted cone of vesicles (Fig. 6A). This is consistent with that predicted inferred by sequence homology and the localization of human VAMP7 and AtVAMP7s.

We used the membrane-impermeable styryl dye, FM 4-64, to investigate whether the tip focused localization of PiVAMP721 was indicative of colocalization with endocytic vesicles. FM dyes are a series of cationic styryl dyes which typically have a lipophilic tail at one end and a highly hydrophilic charged head group at the other. These molecules fluoresce only when associated with the membrane and are widely used as endocytic tracers (Vida and Emr, 1995; Emans et al., 2002). Externally applied FM dyes gradually start to fluoresce on binding to the plasma membrane and are internalized via rapidly moving endosomes, eventually reaching the vacuole. It has been shown that this internalization is dependent on the vesicle transport machinery (Bolte et al., 2004). FM dyes have been successfully used in a number of pollen-related endo/exocytosis studies including petunia pollen (Parton et al., 2001; Camacho and Malhó, 2003; de Graaf et al., 2005; Dowd et al., 2006). FM 4-64 is associated with plasma membrane and internalized through an energy dependent process presumed to be endocytosis (Parton et al., 2001; Wang et al., 2005).

When images of FM 4-64 and YFP labeling were merged, we found that the two signals do colocalize extensively, especially in the V-shaped cone of vesicles at the tube apex (Fig. 6B). This is in contrast to Lat52-YFP controls in which the red FM4-64 signal tends to wrap around the green YFP signal because Lat52-YFP do not show a tip-focusing localization. Uemura et al. has identified a potential localization indicating region between +4 and +6 layers of the SNARE domain of AtVAMP7s, according to this

rule, PiVAMP721 has the PM/endosome localization indicator “RSQAQD” consistent with these observations (Uemura et al., 2005).

The other interesting observation was that high levels of over-expression of YFP-PiVAMP721 (as judged by YFP fluorescence) produced slightly bulging tips in some cases. Coincident with the bulging tips, larger patches of fluorescent signal were observed (Fig. 6D and E). This observation suggested that a high levels of YFP-PiVAMP721 over-expression was perturbing membrane trafficking. The bulging tip phenotype is subtle compared with that observed for genes involved in the regulation of polarity such as that previously described for PiCDPK1 (Yoon et al., 2006). Most of the tubes show slight bulging, very different from the ballooning observed on losing growth polarity. The phenotypic difference suggests the underlying mechanism is likely to be different.

Function of N and C termini of PiVAMP721

The only VAMP7 that has been studied extensively is human Ti-VAMP, which has been shown to be involved in a wide variety of membrane fusion events. Most significantly to this study, human VAMP7 has been shown to be involved in the polar extension of neurites. Over-expression of full-length VAMP7 has no observable effect in neurites, but the over-expression of the N terminal longin domain is strongly inhibitory to growth, while the C terminal SNARE domain promotes neurite outgrowth (Martinez-Arca et al., 2000). From these over-expression experiments and the resolved crystal structure of Ykt6 longin domain, it is generally believed that the longin domain possesses an inhibitory function, which regulates that membrane fusion activity of the C-terminal

domain (Martinez-Arca et al., 2003; Tochio et al., 2001). Hence in the context of mediating membrane fusion, the N terminal longin domain alone behaves in a dominant negative way whereas the C terminal SNARE domain alone is constitutively active. This concept has been supported by both *in vivo* and *in vitro* experimental data (Martinez-Arca et al., 2003; Pryor et al., 2004). The N terminal longin domain has also been suggested to mediate localization in addition to its regulatory function. Domain deletion experiments from both mammals and plants are consistent with a targeting function of longin domain (Martinez-Arca et al., 2003; Uemura et al., 2005). Based on these results we designed an experiment to investigate the functions of the N and C terminal domains of PiVAMP721 in pollen tube growth.

Somewhat surprisingly we found that over-expression of the N terminus led to essentially normal pollen tubes (Fig. 7A and B), both in length and shape. Further, YFP-PiVAMP721-Nter lost the localization exhibited by the full length protein, and instead exhibited a more or less homogeneous distribution across the whole cytosol similar to soluble YFP. In contrast, over-expression of YFP-PiVAMP721-Cter showed a more pronounced phenotype than the full length construct, although the subcellular localization was similar to that exhibited by the full-length fusion (Fig. 7C and D). A tip-focused signal and apical membrane localization can still be seen, however large patches of signal were frequently often observed in the subapical region, and the frequency with which tips bulged was also higher. Co-localization with FM 4-64 signal indicated that FM 4-64 was trapped in these fluorescent patches, suggesting that they were at least partly derived from endocytic vesicles (Fig. 7E and F). Inhibition of pollen tube extension was also seen more frequently with YFP-PiVAMP721-Cter than the full length fusion protein (Fig. 7C

and D, Fig 8A and B), although cytoplasmic streaming was still observed even in severely inhibited pollen tubes. The over-expression of YFP-PiVAMP721-Cter sometimes produced a rather dramatic phenotype, very short tubes with bulging tips where FM 4-64 and YFP-PiVAMP721-Cter colabeled large membrane sacs usually occupying the subapical region.

Although at first glance our results are opposite to those for Ti-VAMP in terms of polar growth, they are consistent with the antagonistic role of N and C terminal of VAMP7 obtained from the human VAMP7 experiments. Taken together, PiVAMP721 and human Ti-VAMP/VAMP7 have a similar localization at the tip of a polar growing cell, the N and C terminal domains from both proteins have opposing effects on polar growth. However, the opposite effects on growth phenotype caused by expressing the truncated N and C termini of these two proteins indicated that PiVAMP721 plays a very different role in pollen growth to that of Ti-VAMP/VAMP7 in axon and dendrite growth.

Co-expression of N and C termini of PiVAMP721

The N terminus of another longin, Ykt6, has been shown to interact with its C terminus and this interaction inhibits the function of the C terminus (Tochio et al., 2001). Although the VAMP7 N terminus was also shown to have inhibitory function, a yeast two hybrid assay of human VAMP7 failed to detect this kind of direct interaction (Martinez-Arca et al., 2003). We also performed yeast two hybrid screen with full-length VAMP7 (minus the transmembrane domain), the N terminus, the C terminus minus transmembrane domain as baits, no direct interaction between N and C termini was detectable (data not shown, see Figure 3 for plasmid constructs). However, the N terminal

longin domain of VAMP7 must communicate with the C terminal SNARE domain somehow to exert its inhibitory function. In support of this notion, Ti-VAMP interacts with an adaptor protein AP3 (Martinez-Arca et al., 2003). We hypothesized that in petunia pollen N and C termini may interact indirectly probably though an adaptor protein. This could account for the fact that no interaction were observed in a yeast two hybrid assay, as yeast may not have these necessary adaptor proteins which are present in petunia pollen.

To test this hypothesis, we co-transformed petunia pollen grain with PiVAMP721 N and C terminal constructs. Taken as a whole, the pollen tubes co-expressing the two separate halves of PiVAMP721 behaved more like transformants expressing full length YFP-PiVAMP721, a V-shaped tip-focusing signal was observed, and large membrane aggregates and bulging tube tips were rarely seen (Fig. 9A). These results indicated that if a proper cellular context is provided, the untethered N and C termini of PiVAMP721 can still function together. Phenotypic variation was observed with some transformants more similar to YFP-Nter transformants and some more to YFP-Cter transformants (Fig. 9B and C). We reasoned that this may be a consequence of variation in the ratio of YFP-Nter and YFP-Cter expressed in particular transformants and sought to investigate this possibility.

Coexpression of N and C termini of PiVAMP721 in different ratios

To confirm that the variation observed in YFP-Nter and YFP-Cter cotransformant was caused by different ratios of these two constructs, we mixed YFP-Nter and YFP-Cter plasmids in different ratios prior to precipitating the plasmids onto gold particles for

bombardment. Subtle but reproducible phenotypic differences were observed between 1:1, 1:3 and 3:1 ratios of YFP-Nter and YFP-Cter as show by figure 8A and B. These results showed that as the ratio of YFP-Nter:YFP-Cter was skewed toward increasing YFP-Cter, pollen tube inhibition and the incidence of tip bulging increased. These results suggest that the N terminus of PiVAMP721 can titrate away the effects of the C terminus *in vivo*. This data is consistent with the idea that the C terminus is dedicated to the promote membrane fusion and is constitutively active, while the N terminus plays an inhibitory role in regulating the C-terminus, such that the whole protein performs in a balanced, presumably regulated way.

PiVAMP721 C terminus induced membrane fusions can trap secretory proteins

Lastly we investigated the content of C terminus over-expression induced membrane compartments. We hypothesized that these membrane compartments might contain materials from both endocytic and secretory pathways. We investigated whether a secreted protein marker is trapped inside these membrane compartments. To achieve this, we co-expressed PiVAMP721 C terminus with CFP tagged invertase. Invertase is a family of secreted cell well enzymes, GFP tagged invertase has been successfully used previously and our CFP version of invertase a showed similar localization consistent with published results (de Graaf et al., 2005; Fig 10A). When co-expressed with PiVAMP721 C terminus, some cell wall localization of invertase can still be seen, the majority of the CFP signal however, was observed to be inside the induced membrane fusions (Fig. 10B). Intriguingly, this indicated that over-expression of PiVAMP721 disrupted both endocytosis and secretory pathway.

DISCUSSION

Pollen tubes are particularly useful in studying endocytosis and signaling. Tube growth is dependent upon active endo/exocytosis, membrane and cell-wall building materials necessary for tube extension are deposited and simultaneously membrane components are recycled. Previous studies as well as studies from other polar growth models, such as axons, root hairs, phage pseudopods and yeast budding, indicate that extensive endo/exocytosis and membrane fusion processes are involved (Camacho and Malhó, 2003; Preuss et al., 2004; Samaj et al., 2004). Signal transduction is vital to this polar growth process as every step of membrane budding and fusion is tightly regulated as exemplified by the classical calcium controlled synaptic vesicle release. Ca^{2+} signaling, Rop GTPase, actin dynamics have been shown to involve in pollen tube growth (Taylor and Hepler, 2000). Plant endocytosis is believed to follow the plasma membrane, endosome to vacuole pathway, TGN (*trans*-Golgi network) and PVC (prevacuolar compartment)/MVB (multivesicular body) have been proposed to correspond to early and late endosome (Lam et al., 2007; Müller et al., 2007). A number of molecular markers for these compartments such as GTPase and SNAREs have also been identified (Ueda et al., 2003; Uemura et al., 2004). Despite recent progress in our understanding of plant endocytosis, the main endocytic R-SNARE proteins, VAMP7s, have not previously been characterized at molecular level. It should be also noted that this endocytosis model has largely been developed based on results from generalized cells such as leaf protoplasts and BY-2 cells, to what extent it can be applied to specialized cells remains to be tested.

For example, the exact nature and morphology of early and late endosomes in pollen tubes remain elusive (Samaj et al., 2006).

The R-SNARE PiVAMP721 identified in this study has only one homolog in yeast and mammals but many homologs in *Arabidopsis*. There are 14 R-SNAREs (11 VAMP7s, 2 Ykt6s and 1 Sec22) among the 54 SNARE genes identified in the *Arabidopsis* genome (Sanderfoot et al., 2000; Uemura et al., 2005). Of particular interest is that *Arabidopsis* does not possess any brevin type R-SNAREs but a rather larger number of longins, whereas in mammals there are a number of brevins (6) but only three longins (1 VAMP7, 1 Ykt6 and 1 Sec22). Longins differ from brevins in that they possess an additional N terminal longin domain. Localization and regulatory functions have been proposed for the longin domain (Rossi et al. 2004a; Uemura et al. 2005). One attractive hypothesis proposed is that longins are the prototype R-SNAREs and animal brevins, by the loss of the inhibitory longin domain, achieve faster membrane fusion as required by neurological transmission events (Rossi et al., 2004b; Proux-Gillardeaux et al., 2005). Systematic sequence analysis suggests that R SNAREs can be divided into R.I, Sec22 group for ER trafficking, R.II Ykt6 group for ER-Golgi and intra-Golgi trafficking, R.III VAMP7 group for endosomal trafficking, and R.IV brevin for secretion (Kloepper et al., 2007). Although VAMP7s are assigned to endocytic trafficking from this classification, VAMP7 can mediate secretion from lysosomes (Luzio et al., 2007). Presumably plants also have to use VAMP7 for secretion, as they do not even possess a R.IV type brevin, although this remains to be confirmed.

Two close homologs of PiVAMP721, AtVAMP725 (At2g32670) and AtVAMP726 (At1g04760) have been localized to the plasma membrane and the

endosome in a recent study (Uemura et al., 2004). Humans have a single VAMP7 which is involved in a wide arrange of membrane fusion events, besides mediating neurite outgrowth, it is also involved in degranulation in mast cells (Hibi et al., 2000), transport from late endosomes to lysosomes in fibroblasts (Advani 1999; Ward et al., 2000), raft-mediated transport to the apical membrane in polarized epithelial cells (Lafont et al., 1999), phagesome sealing and pseudopod extension in macrophages (Braun et al., 2004). It is not particularly surprising for Ti-VAMP to be involved in so many diverse processes as humans have only one VAMP7. These processes include both endocytic and exocytic events, but it is worth noting some of the confirmed exocytic events are lysosome to plasma membrane trafficking (Luzio et al., 2007). Plants, on the other hand, have a family of VAMP7s with different expression patterns and localizations (Uemura et al., 2004). It is conceivable and perhaps likely that each particular plant VAMP7 performs a narrow range of functions performed by human VAMP7 in a tissue-specific manner, a situation analogous to that already demonstrated for *Arabidopsis* syntaxins (Sanderfoot et al., 2001). In the case of PiVAMP721, we first sought to determine its subcellular localization and then whether it is involved in endocytosis.

Our observations support the hypothesis that PiVAMP721 localizes to transport vesicles. In the YFP fusion expression images, we consistently observed strong florescence in the clear zone of pollen tube tip. This observation is consistent with the localization of human VAMP7 which was reported to locate at the leading edge of growing axons (Martinez-Arca et al., 2000). The fluorescence of the fusion protein colocalized with FM 4-64 in the apical V-shaped cone indicating that YFP-PiVAMP721-labeled structures are transport vesicles, which are most likely endocytic in nature (de

Graaf et al., 2005). The only way for FM4-64 to get inside of cell is through endocytosis, but in cells engaged in vigorous secretion such as pollen tubes and root hairs, FM 4-64 quickly stains the clear zone (Parton et al, 2001, Voigt et al., 2005). We consistently observed that the PiVAMP721 signal as a fluorescent haze beyond the resolution of light microscopy in the apical region while membrane structures large enough to be discerned were visible in the subapical region. This localization is in consistent with the proposed endocytosis pathway. In root hairs, endocytic vesicles in apical region are below light microscope resolution while the early endosome at the apical and subapical region is around 500 nm, the late endosome is about 2 µm and located in the shank (Ovecka et al., 2005). High levels of PiVAMP721 over-expression leads to substantial enlargement of the labeled membrane compartments suggesting that high levels of this protein may have increased membrane fusion between the labeled structures. The N terminal longin domain, has been proposed to be important in encoding for the localization of VAMP7s. In this study, however, its' removal had no discernable effect on localization, as YFP-PiVAMP721-Cter exhibited a localization indistinguishable from that of the full-length fusion protein, although we cannot rule out subtle differences such as they localized to different set of vesicles. In contrast the YFP-PiVAMP721-Nter fusion protein was visible through the cytoplasm, again suggesting that the N-terminal domain of PiVAMP721 does not play a role in localization. The seemingly correct localization of YFP-PiVAMP721-Cter, which is essentially a brevin molecule, may simply indicate that endocytic vesicles are the default localization of these proteins (Brandizzi et al., 2002; Kloepper et al., 2007).

Several lines of evidences lead support the hypothesis that the vesicles labeled by PiVAMP721 are endocytic. As mentioned above, VAMP7s have been proposed to

mediate endocytic traffic and PiVAMP721 colocalized with the endocytic marker FM4-64. In addition, over-expression of PiVAMP721 has the exact opposite effect on polar growth compared with over-expressing human VAMP71 in neurites. Human VAMP7 is involved in exocytic events, increasing the rate of exocytic fusion led to an increase in growth rate. In contrast, over-expression of PiVAMP721 reduced growth rate consistent with perturbation of an alternative process. The N-terminal domain of longins are generally believed to possess a negative regulatory function, as well as determining localization. Though the N-terminal domain of PiVAMP721 does not appear to play any role in localization, our results support that this domain plays a negative regulatory role. The N terminal longin domain of human VAMP7 has dominant negative effects (inhibition of neurite outgrowth, inhibition of endosome fusion with lysosomes) and its C terminal SNARE domain has constitutive active effects (Martinez-Arca et al. 2000). The results form our experiments suggest similar activities for the domains of PiVAMP721. Over-expression of the PiVAMP721 SNARE motif-containing C terminus produced a more severe phenotype (increased growth inhibition, increased frequency of aberrant membrane sacs within the tip). The over-expression of the N terminus, hypothesized to have an inhibitory effect on SNARE complex formation, did not however, result in a significant phenotype. Nonetheless, when co-expressed, the N terminus can apparently titrate the effects of over-expression of the C terminus. These results are consistent with the C terminus being constitutively active, promoting membrane fusion (as visualized by an increased in the frequency and size of membrane sacs at the tip) in a manner that is inhibitory to pollen tube extension. Co-expressing the N-terminal domain of PiVAMP721 was observed to decrease both the growth inhibition and size/frequency of aberrant

membrane compartments in a manner dependent on the ratio of the N- and C-termini, consistent with the N-terminus negatively regulating the constitutively active C-terminus. The disparity between the results that the N-terminus alone did not significantly affect pollen tube growth in vitro, in contrast to the result for human VAMP7 seems likely to be related to localization. The longin domain of human VAMP7 but PiVAMP721 is targeted to the "correct" localization hence the VAMP7 longin domain is concentrated to the physiologically relevant membrane. In contrast the longin domain of PiVAMP721 is diffused throughout the entire cytosol and hence its' concentration at the site of physiological relevance is relatively, but dramatically, reduced. Our results do suggest that the free longin domain of PiVAMP721 can negatively regulate its' free C-terminal SNARE domain, we speculate that as the truncated SNARE domain does not have a longin domain attached (unlike the endogenous wild type protein) in this case the free longin domain does not have to compete with the fused N-terminal domain, hence a lower concentration of longin domain is likely to be required to generate a visible phenotypic effect.

The bulging pollen tubes tips observed in the over-expression experiments are not uncommon phenotype, loss of polarity mutants frequently have such phenotypes but these tend to be considerably more severe (e.g. Yoon et al., 2006). We do not think that loss of polarity is a likely cause in this case. A more likely alternative explanation is that interfering with membrane trafficking, through the promotion of vesicle fusion within the tube tips, leads to these vesicles and their cargo being trapped in the tip rather than recycled. The membrane compartments visible at the tip may cause tip to bulge either as a results of physical force or by perturbing osmotic balance.

In view of our results, we propose that PiVAMP721 mediates membrane fusion events between endocytic vesicles, endocytic vesicles and early endosomes, and/or endocytic vesicles and exocytic vesicles. The first two types of vesicle fusion are of regular endocytic in nature while the endocytic and exocytic vesicle fusion is the so called rapid recycling. Both types of vesicle traffic have been observed in pollen tubes and root hairs using endocytic markers (Parton et al., 2001; Ovecka et al., 2005; Wang et al., 2006; Samaj et al., 2006). The clear zone was thought to be filled predominantly with secretory and endocytic vesicles, but recent evidence presents a more complex picture. For example, in root hairs, endocytic vesicles may stay within the clear zone for up to 20 min and contact the plasma membrane several times during this time. The putative early endosome may also stay inside the clear zone. Endocytic vesicles may fuse to early endosomes and presumably also bud off from endosomes (Ovecka et al., 2005; Voigt et al., 2005). Besides the recycling “secretory” function, the inherent secretory nature of the early endosome or TGN should also be noted. This suggests a close relationship between secretory and endocytic pathways, the "rapid recycling pathway" of endocytosis is often evoked to explain this phenomenon, though it may also be accounted for by extensive material exchange between secretory and endocytic vesicles (Monteiro et al., 2005). In a recent report, endosidin1, an endocytosis inhibitor, was found to disrupt endocytosis in a selective way. For example, PIN2, AUX1 and BR1 were found to localize to endosidin1-induced membrane compartments while PIN1 and PIN7 were not affected (Robert et al., 2008). This indicated that there are at least two possible ways to recycle plasma membrane proteins and endosidin1 blocks a pathway that is an earlier, presumably faster, recycling pathway but it did not affect the later pathway.

Of particular relevance to the results we obtained in this study is a recent model proposed by Samaj et al (2006). This model hypothesizes that the TGN represents a tip-localized vesicular compartment that integrates secretion and endocytosis at the growing tip of tip growing cells. Significantly this compartment was proposed to have dual exo- and endocytic functions. Our results provide for the first time, empirical molecular evidence for this model. Based of this hypothesis, the PiVAMP721 induced membrane compartments we observed represent modified TGN, the size of its' compartments having been enlarged as a result of excess vesicle fusion. In particular, the colocalization of a secretory enzyme, invertase, along with FM4-64 to the PiVAMP721-Cter induced membrane compartment supports the hypothesis that PiVAMP721 may mediate vesicle fusion between endocytic and secretory vesicles in this early rapid recycling pathway.

Although data present in this paper are obtained from transient over-expression experiments, we think it is physiologically relevant for the following reasons. Transient expression has been used successfully in studying the localization and function of a number of proteins, in fact, our method and protein constructs are similar with those of Martinez-Arca et al. study in which the function of human Ti-VAMP was first characterized (Martinez-Arca et al. 2000). The various constructs employed produced very different phenotypes, providing further evidence that the phenotype we observed were not simply due to over-expression.

If PiVAMP721 is indeed functions in endo- exocytic vesicle fusion within the TGN as we hypothesize, it not only represents the first R-SNARE/longin functionally characterized in pollen, but is also the first to which this function has been ascribed. It will be of great interest to further find out the interacting SNARE partners of

PiVAMP721 and show their interaction *in vitro* as has been successfully demonstrated in mammalian systems (McNew *et al.* 2000). An additional question worthy of further study is the *in vivo* function of PiVAMP721, the stylar content presented in the PiVAMP721-specific compartments might give us valuable information about pollen-pistil interactions, with respect to pollen tube nutrition and also guidance cues provided by the style to direct growth.

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Figure legends

Figure 1. Classification of SNARE proteins with emphasis on R-SNAREs.

Base on the presence of conserved arginine or glutamine at the "0" layer of ionic interaction, SNAREs are classified into R and Q-SNAREs (Fasshauer *et al.*, 1998). Previously, they were classified as v- and t-SNAREs base on their presence at the vesicle and target membrane. R-SNAREs or VAMPs can be divided into short "brevins" and longer "longins" dependent upon the presence or not of a N terminal longin domain (Filippini et al., 2001). SNAREs can also be divided into RG or RD-SNAREs based upon the presence of conserved arginine glycine pair or arginine aspatate pair at the "0" layer (Rossi, Picco, Vacca *et al.*, 2004b). Longins are all RG-SNAREs and they can be further classified into Sec22-like (R.I), Ykt6-like (R.II), and VAMP7-like (R.III) longins base upon the homology to Sec22, Ykt6 and VAMP7 of their longin domain (Rossi et al., 2004a, Kloepper et al., 2007). PiVAMP721 discussed in this paper belongs to VAMP7-like longin. Brevins can be classified into RG-brevins such as VAMP1, 2 and RD-brevins such as VAMP4. Functional classification of VAMPs is according to Kloepper et al., 2007.

Figure 2. Sequence alignment of PiVAMP721 and other VAMP7s.

Sequence alignment of PiVAMP721 with selected VAMP7s, human synaptobrevin 1, a well-studied RD-brevin is also shown for comparison. Conserved amino acids are highlighted in grey. Features such as longin domain, coiled-coil SNARE domain, "0" layer arginine with heptad register and transmembrane domain are marked with symbols shown in the legend. Potential phosphorylation site, N-myristoylation site

and special amino acids used in protein construct of PiVAMP721 are also shown. Abbreviations: At, *Arabidopsis thaliana*; Dm, *Drosophila melanogastor*; Hs, *homo sapiens*; Mm, Os, *Oryza sativa*; Pp, *Pyrus pyrifolia*; Rn, *Rattus novernicus*; Sc, *Saccharomyces cerevisiae*; Tm, *Triticum monococcum*; Xl, *Xenopus leavis*. The NCBI accession numbers of the proteins are: AtVAMP711, O49377; AtVAMP712, Q9SIQ9; AtVAMP713, NP_196676; AtVAMP714, Q9FMR5; AtVAMP721, Q9ZTW3; AtVAMP722, P47192; AtVAMP723, Q8VY69; AtVAMP724, O23429; AtVAMP725, O48850; AtVAMP726, Q9MAS5; AtVAMP727, Q9M376; AtYkt61, Q9ZRD6; AtYkt62, Q9LVM9; DmVAMP7, NP_610524; HsYkt6, NP006546; HsVAMP7, NP_005629; MmVAMP7, NP_035645; Os VAMP7-like-1, CAD70274; PpVAMP7, AAQ15287; RnVAMP7, NP_445983; TmVAMP7, AAS88558; ScYkt6, NP012725; XlVAMP7, AAH77586.

Figure 3. Domain features of PiVAMP721 and protein constructs used in this study

PiVAMP721 consists of a longin domain, a coiled-coil SNARE domain and a transmembrane domain. For YFP fusion proteins, YFP was N terminal fused to the full-length, N terminus up to E127, C terminus from L106. For yeast 2 hybrid analysis, full-length is refer to M1 to K195, N terminus up to E 127, C terminus from L106 to K195. For *E. coli* expression, His6 tags were fused to same constructs used in yeast 2 hybrid.

Figure 4. Phylogenetic tree of VAMP7s

Ykt6 family longins are used as an outgroup. For phylogenies of all SNAREs and Arabidopsis SNAREs, see Sanderfoot et al., 2000, Uemura et al., 2004, Hook and Means, 2001.

Figure 5. Expression pattern of PiVAMP721.

Expression patterns of PiVAMP721. Total RNA was extracted from different stages of anther, mature pollen, germinating pollen tubes, leaves and roots. First strand cDNA was generated by reverse transcription from each total RNA. PCR amplification was performed by specific primers for each transcript.

Figure 6. Localization of PiVAMP721 and phenotypes of PiVAMP721 transformants.

A. Transient expression of YFP-PiVAMP721 in petunia pollen tube. Yellow channel represent the YFP signal, light image of pollen tube was also shown for comparison. Note the strong V-shaped cone signal at the tip (arrow head). B. colocalization of FM4-64 and YFP-PiVAMP721, green channel represent the YFP signal, red channel represent the FM signal, yellow channel represent the merging of the above two channels. Note the green and red signal are largely colocalized especially at the V-shaped apical region. C and D. representative confocal images of YFP-PiVAMP721 pollen tubes over-expressing YFP-PiVAMP721. Yellow channel represent the YFP signal, note the appearance of larger signal patches and the plasma membrane localization of PiVAMP721 (arrow head). Images were generated using confocal microscope. Scale bar = 5 μm.

Figure 7. Localization and phenotypes of N terminal longin domain and C terminal SNARE domain.

A and B. petunia pollen tube transformed with YFP-PiVAMP721-N terminus. Note no apical inverted cone signal. C and D. Severe phenotype of YFP-PiVAMP721-C terminus transformants. E and F. colocalization of FM4-64 and YFP-PiVAMP721-C terminus. green channel represent the YFP signal, red channel represent the FM signal, yellow channel represent the merging of the above two channels. Note the green and red signal are largely colocalized especially at the V-shaped apical region. Strong YFP signal patch caused by C terminus over-expression can be seen and FM4-64 and YFP-PiVAMP721Cter colabeled these membrane aggregation. Note the tube shape change and plasma membrane localization (arrow heads). Images were generated using confocal microscope. Scale bar = 5 µm.

Figure 8. Effects of different ratios of PiVAMP721 N and C termini co-expression on petunia pollen tube growth

Statistics of pollen tube length (A) and bulging tips (B) relative to wild type tubes are shown. C, YFP-PiVAMP721-Cter; F, YFP-PiVAMP721; N, YFP-PiVAMP721-Nter; 1N:3C, YFP-PiVAMP721-Cter and YFP-PiVAMP721-Nter in a 1:3 ratio; 1N:1C YFP-PiVAMP721-Cter and YFP-PiVAMP721-Nter in a 1:1 ratio; 3N:1C: YFP-PiVAMP721-Cter and YFP-PiVAMP721-Nter in a 3:1 ratio.

Figure 9. Phenotypes of pollen tubes co-expressing of PiVAMP721 N and C termini

FM 4-64 staining of petunia pollen tube co-transformed with YFP-PiVAMP721 N and C termini. These double transformants can have an YFP-PiVAMP721-like phenotype (A) or like N (B) and C (C) terminus. Images were generated using confocal microscope. Scale bar = 5 μm.

Figure 10. Co-expression of Lat52-CFP-Invertase with PiVAMP721 C terminus in pollen tube

A. Co-expression of Lat52-CFP-Invertase with YFP vector in pollen tube, blue channel represent CFP signal and yellow channel represent YFP signal. Most invertase signal is seen at the surface of pollen tube presumably at cell wall. B. Co-expression of Lat52-CFP-Invertase with PiVAMP721 C terminus in pollen tube. Blue channel represent CFP signal and yellow channel represent YFP signal. Most invertase signal can be seen inside the membrane fusions induced by PiVAMP721 C terminus over-expression. Images were generated using confocal microscope. Scale bar = 5 μm.

Figure 1. Classification of SNARE proteins with emphasis on R-SNAREs.

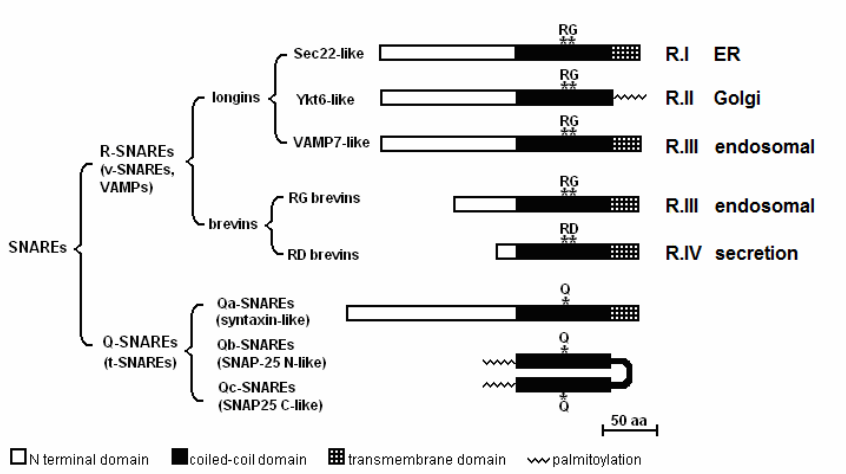


Figure 2. Sequence alignment of PiVAMP721 and other VAMP7s.

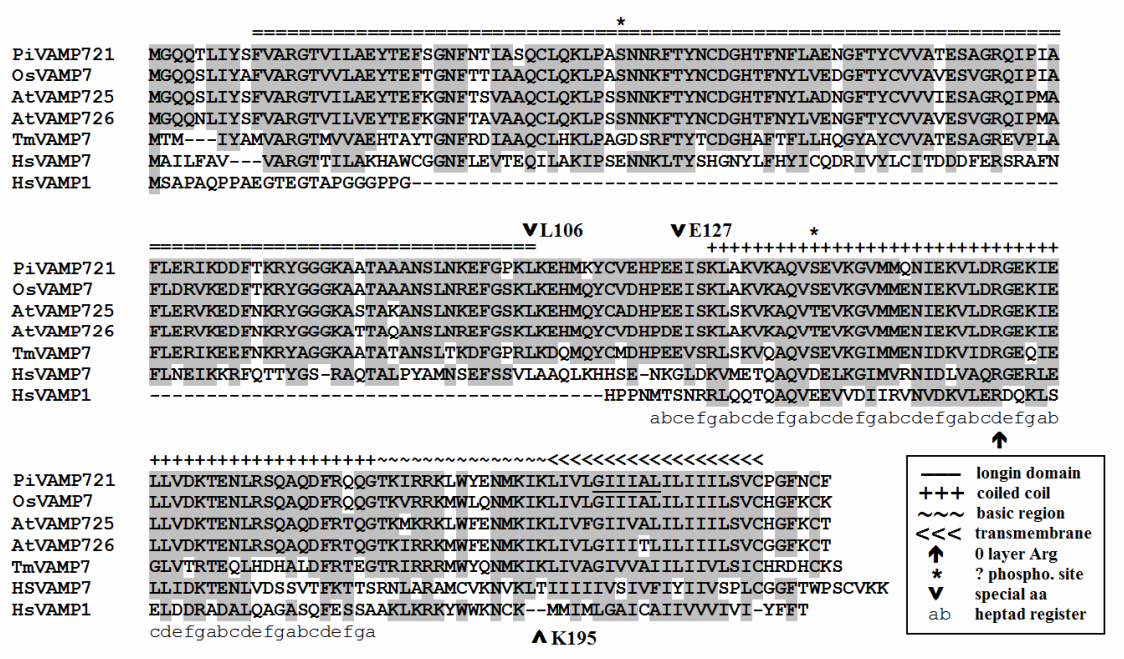


Figure 3. Phylogenetic tree of VAMP7s.

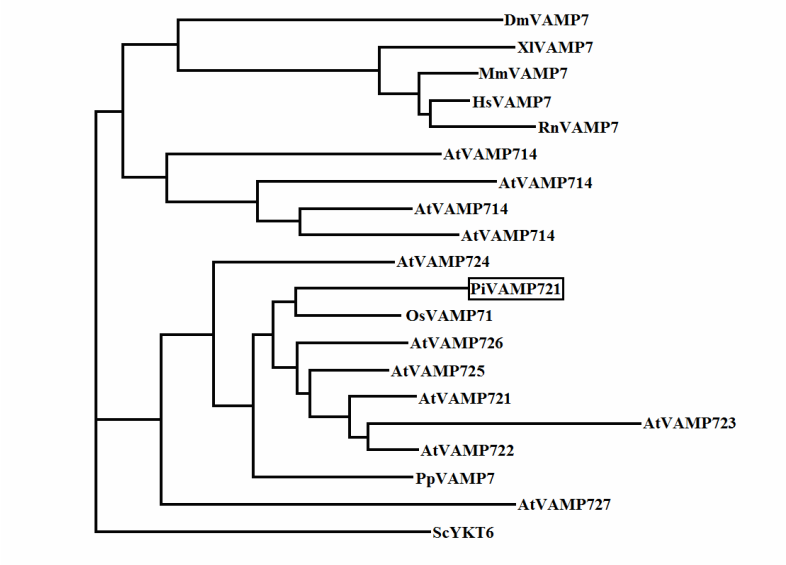


Figure 4. Domain features of PiVAMP721 and protein constructs used in this study

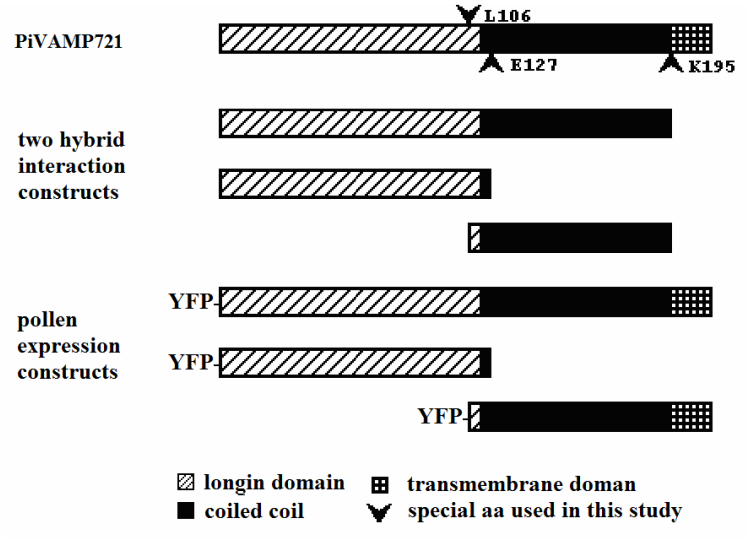


Figure 5. Expression pattern of PiVAMP721.

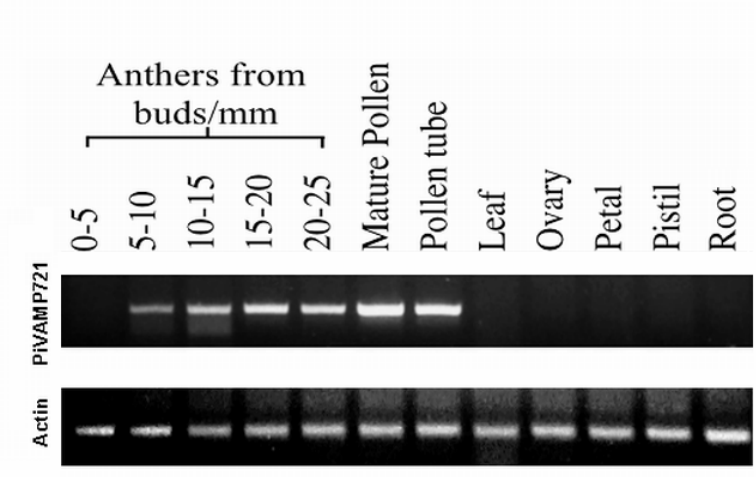


Figure 6. Localization of PiVAMP721 and phenotypes of PiVAMP721 transformants.

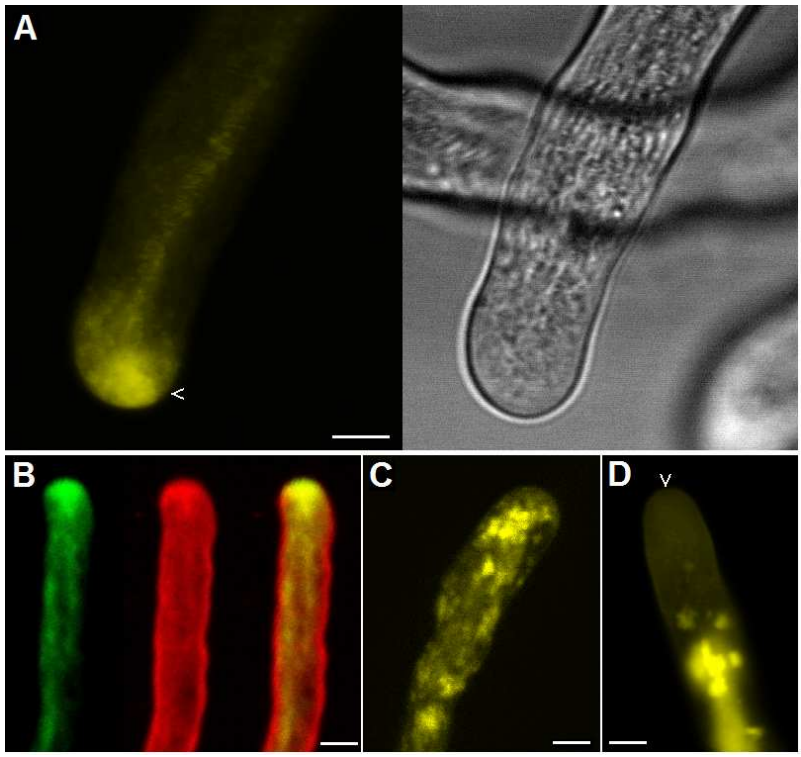


Figure 7. Localization and phenotypes of N terminal longin domain and C terminal SNARE domain.

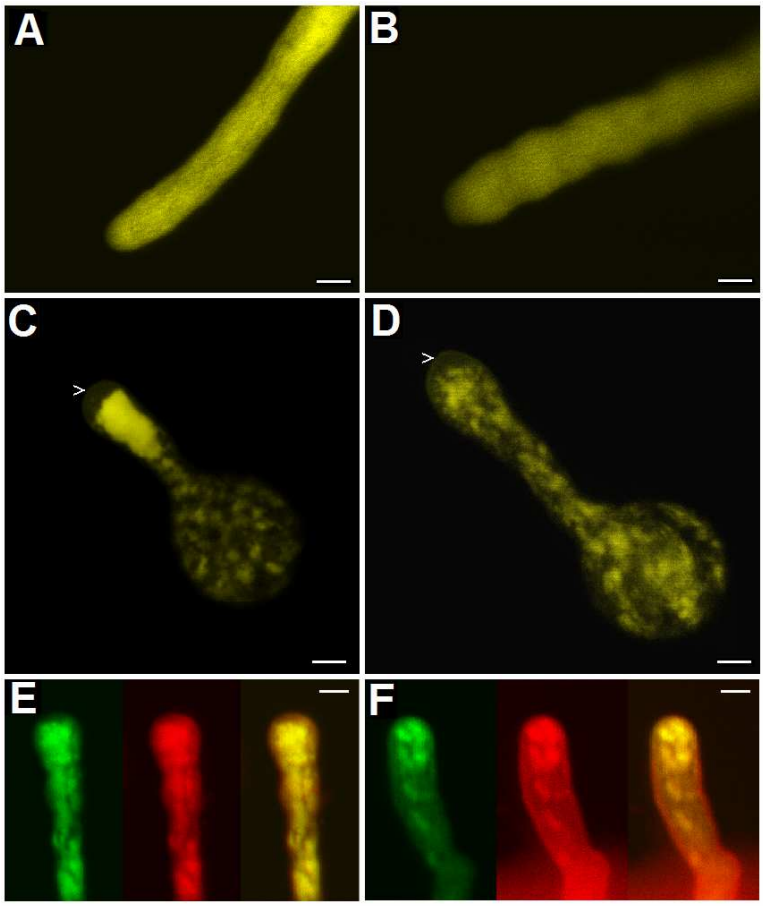
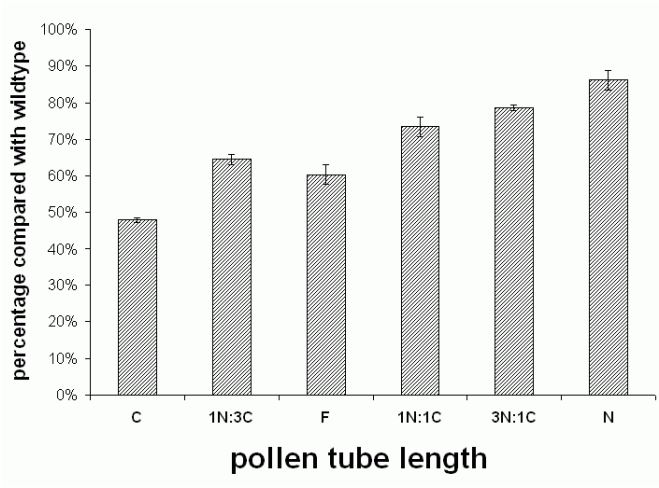


Figure 8. Effects of PiVAMP721 on petunia pollen tube growth.

A.



B.

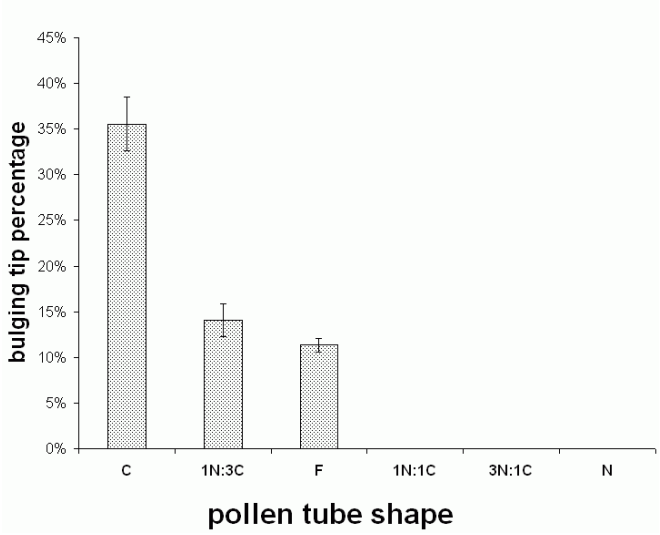


Figure 9. Phenotypes of pollen tubes co-expressing of PiVAMP721 N and C termini

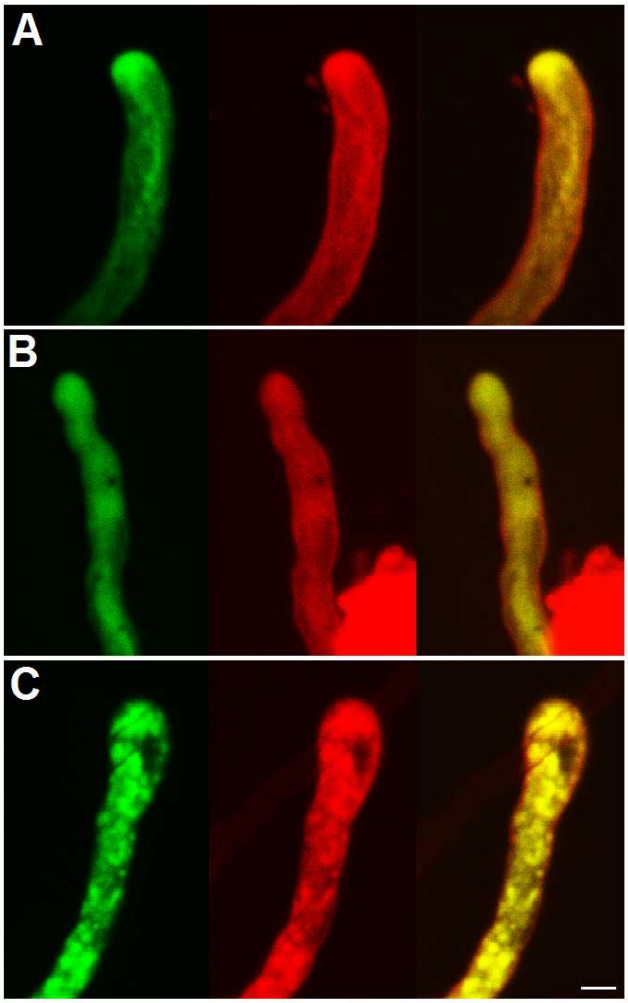
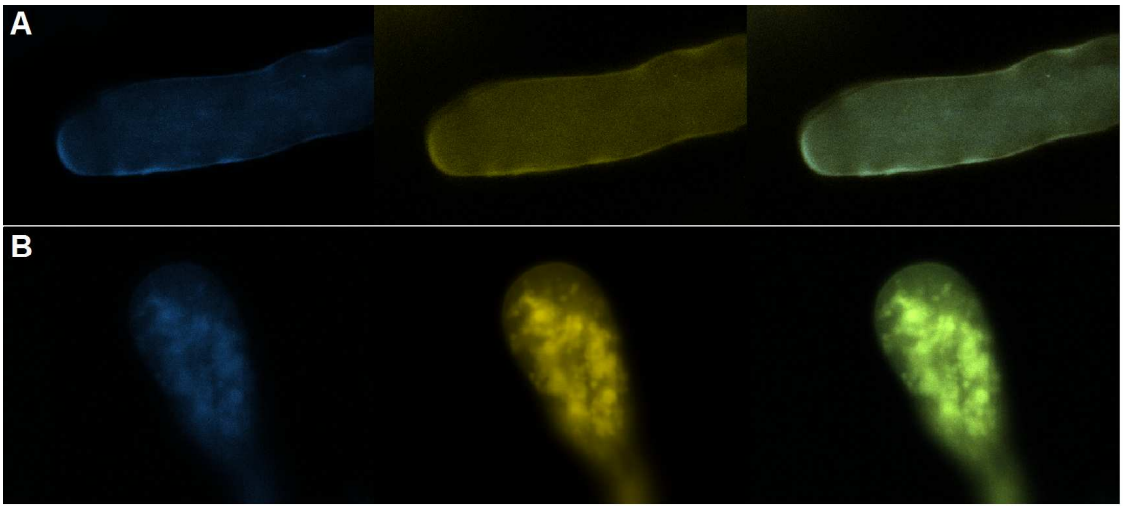


Figure 10. Co-expression of Lat52-CFP-Invertase with PiVAMP721 C terminus in pollen tubes



CHAPTER 2B

Investigation into the potential role of phosphorylation in the regulation of the function of PiVAMP721 in pollen tube growth

Abstract

SNARE proteins mediate membrane fusion events in endocytosis and endocytosis. This function is regulated and facilitated by a number of regulatory factors. The activity of both and SNAREs and their regulators can also be regulated by phosphorylation. Here we provide evidence that Ser-42, part of a putative phosphorylation site in the PiVAMP721 N-terminal login domain, may possess an important regulatory function. Expression of a non-phosphorylatable mutant (S42A) in pollen tubes led to a phenotype consistent with up-regulation of the activity of the full length protein whereas expression of the phospho-mimetic mutation (S42E) had a significantly reduced phenotype. These results suggested that the function of PiVAMP721 may be regulated by phosphorylation.

Introduction

Biological membrane bound organelles are characteristic of eukaryotes. Through compartmentalization by membrane, enzymes, ions, and other materials can be concentrated and confined in a close environment. However, an inherent problem accompanying this compartmentalization is a need to exchange material between different compartments. One way to solve this problem is through vesicle trafficking and membrane fusion. Recent evidence suggests that membrane fusion events may more common than previously thought. Multiple intermediate stages may exist and usually there is more than one pathway to reach a particular destination (Gundelfinger et al., 2003;

Titorenko and Mullen, 2006). Besides the classical endomembrane system, exocytosis and endocytosis, other organelles such as mitochondria and peroxisomes are also engaged in vesicle trafficking between themselves and to endomembrane system (Schrader and Yoon 2007; Titorenko and Mullen, 2006). A common theme in these myriad membrane fusion events is that they are mediated by a family of closely related SNARE proteins. SNARE proteins are believed to have evolved with the emergence of the endomembrane system in eukaryotes and undergone multiple rounds of genomic wide duplications (Kloepper et al., 2008). The mechanism for SNARE-mediated membrane fusion is the formation of stable four-helix bundle by four SNARE domains contributed by three or four SNARE proteins (Weber et al., 1998). This SNARE complex is transformed from *cis* (some SNARE at vesicle and some at target membrane) at the beginning stage of membrane fusion to *trans* (all SNARE on the same membrane) at the end (Fiebig et al., 1999).

SNAREs can be classified into Q and R SNAREs based on the conserved amino acid residue at the SNARE domain. Q and R SNAREs can be further divided structurally and functionally (Fasshauer et al., 1998). For example, R SNARE can be divided into at least 4 subgroups based on their function and whether they have an N terminal login domain. R.I is Sec22 like logins and involved in ER trafficking, R. II is Ykt6-like longins and involved in ER to Golgi and intra-Golgi traffic, R. III is VAMP7-like longins involved in endosome traffic and R. IV is synaptobrevin like brevins involved in secretion (Kloepper et al., 2007).

SNAREs are abundant in plant and *Arabidopsis* has 54, including 14 R-SNAREs (Uemura et al., 2004). One particular feature of *Arabidopsis* SNAREs is that there has

been expansion of the R.III VAMP7 SNARE family compared with animals, but R.IV synaptobrevin type SNAREs are absent (Sanderfoot et al., 2000; Rossi et al., 2004). This somewhat surprising information suggests that plants have some unique features in their membrane trafficking. Although general expression pattern and localization of *Arabidopsis* SNAREs have been conducted, individual functional characterization of SNARE proteins in their proper physiological context is lacking (Sanderfoot et al., 2000; Uemura et al., 2004).

We described an R.III SNARE PiVAMP721 in chapter 2A and present results that suggest that it is involved in endo- exocytosis in pollen. We further showed its membrane fusion function is mediated through its C terminal SNARE domain and this function is negatively regulated by its N terminal longin domain. One of major research interest in our lab is in protein kinase signaling in pollen tube growth, particularly with regard to calcium signaling through calcium dependent protein kinases. We initially identified PiVAMP721 as a false positive in a GAL4 based 2 hybrid screen using a pollen specific CDPK as bait. Such screens are not, however always reliable for detecting interactions between cytoplasmic proteins and there are numerous reports of the activities of SNARE proteins being demonstrated regulated by phosphorylation (for review see Gerst, 2003), by a variety of different protein kinases including the calcium regulated protein kinase C and calmodulin activated kinases I and II in animal cells. As a result we decided to investigate whether potential sites of phosphorylation by CDPKs that were identified in PiVAMP721 were significant to the function of this protein. The experimental approach we employed was to mutate putative serine phosphorylation sites and assess the effect of these changes on PiVAMP721 function by expressing them in pollen tubes. Here we

provide evidence that PiVAMP721 function may be regulated by the phosphorylation status of S-42. Serine 42 lies in the N-terminal longin domain of PiVAMP721, which we previously showed possesses a negative regulatory function (chapter 2). A non-phosphorylatable mutation of this amino acid produces a phenotype similar to expressing the constitutively active C-terminal SNARE domain, whereas expression of a phospho-mimetic mutant led to a phenotype which was less severe than that seen for the wild type full length PiVAMP721.

Material and Methods

Plant material

Petunia inflata and *Petunia hybrida* were grown under green house conditions with a supplemental light regime of 16 hr light and 8 hr dark.

Constructs of plasmids for pollen expression

All constructs were generated by PCR, cloned into pGEM-T Easy vector (Promega) and sequenced, prior to cloning into vectors for expression in pollen or yeast two hybrid analysis. The *EcoR* I site within the coding region of PiVAMP721 were synonymously changed to code the same amino acids using primes, ERI remF: 5'-AGTATACCGAGTTCTCTGGT-3' and ERI remR: 5'-ACCAGAGAACTCGGTATACT-3'. The coding region of PiVAMP721 was converted to encode *Pvu* II at the 3' end and *Sac* I at the 5' end using primers LO/PVUII: 5'-CAGCTGATGGGGCAACAAACGTTG-3'; LOCTER: 5'-TTAAAAACAATTGAAGCCAGG-3'. Serine 42 mutation was generated using primers

S/A, S42AF: 5'-TTGCCTGCAGCAAATAATAG-3' and S42AR: 5'-CTATTATTTGCTGCAGGCAA-3'; S42E mutation was generated using primers S42EF: 5'-TTGCCTGCAGAAAATAATAG-3' and S42ER: 5'-CTATTATTTTCTGCAGGCAA-5'.

Pollen expression constructs were generated in pBluescript (KS) (life Technologies) using the pollen specific promoter *Lat52* (Twell et al., 1989). pBS*Lat52*-YFP was generated by cloning the coding region of YFP (Clonetech, Palo Alto, CA) behind the *Lat52* promoter in pBluescript (KS) as previously described (Yoon et al., 2006). All YFP fusion constructs were subsequently generated by cloning appropriate fragments into the *Sac* I and *Pvu* II site to generate in frame fusions. Standard recombinant DNA methodology was used in all cloning steps.

Transient expression in pollen

Transient expression of fluorescent protein fusion constructs in pollen was performed as previously described (Yoon et al., 2006). Briefly, *Petunia inflata* pollen was collected from freshly dehisced anthers (10 flowers/bombardment), and suspended by gentle vortexing in 200 μl of pollen germination medium (PGM) (0.01% H_3BO_3, 0.02% $MgSO_4$, 0.07% $CaCl_2$, 15% PEG-4000, 2% sucrose). The pollen was spotted onto a 2.5 cm sq. piece of positively charge nylon membrane in a 9 cm Petri dish.

Microprojectile bombardment was performed using a PDS-1000/He biolistic system (Bio-Rad, Hercules, CA). Gold particles (1.1 μm) were prepared according to the manufacturer's protocol using 2 μg of plasmid DNA/0.5 mg of particles. Bombardment were performed at 28-inches Hg chamber vacuum, 1100 psi rupture disk, 0.25 inch gap

distance and 1 inch particle travel distance. After bombardment, the pollen was washed from the nylon membrane into a Petri-dish with 4 ml PGM and cultured on an orbital shaker at 100 rpm for 4 hrs at 30^{o}C.

Analysis of transformed pollen tubes

Epifluorescence microscopy for YFP fusion constructs observation was performed using an Orthomat epifluorescence microscopy (Leitz) with a 40X, dry, 0.7 numerical Fluor objective. All images were analyzed using the Metamorph v4.5 (Molecular Devices Corp., Downlington, PA) and processed using Adobe Photoshop v5.5 (Adobe Systems Inc., San Jose, CA).

Results

Effects of Ser 42 non-phosphorylatable and phosphorylation mimic mutation

There are two CDPK phosphorylation consensus sites in PiVAMP721, Ser-42 in the longin domain and Ser-139 located at the SNARE domain. We first wanted to assess the effects of Ser 42 phosphorylation to PiVAMP721 function. PiVAMP721/S42A and PiVAMP721/S42E were constructed and used in transient in vivo expression studies. The serine to alanine substitution created a non-phosphorylatable PiVAMP721, while the serine to glutamate substitution created a constitutive phosphorylation mimic. As shown in figure 1, PiVAMP721/S42A transformants consistently exhibited shorter pollen tubes, more membrane fusion and a higher incidence of bulging pollen tube tips. In other words, their phenotype was similar to that observed when the constitutively active truncated, PiVAMP721-C terminus was expressed alone (chapter 2A). In contrast,

PiVAMP721/S42E expressing pollen tubes grew longer, no bulging tips were observed and the frequency with which membrane sacs were visible in the pollen tube tip was reduced. Combined, these results suggest that S42 phosphorylation has a negative effect upon the ability of PiVAMP721 to mediate membrane fusion.

Discussion

SNARE hypothesis as originally proposed suggested that SNARE proteins are necessary and sufficient to mediate specific membrane fusions (Rothman, 1994). This was found to be partly true at least *in vitro* (Weber et al., 1998; Chen et al., 1999). It is estimated that yeast SNAREs can form more than 300 different combinations of SNARE complexes, but only 9 of these complexes is competent in mediating membrane fusion (Malasm et al., 2008). However, it is equally clear from *in vitro* experiments that SNARE proteins interact with each other promiscuously and cannot provide the specificity for membrane fusion solely by themselves (Yang et al., 1999). These experiments also showed although SNARE proteins alone are able to mediate membrane fusion *in vitro*, it happens at a much slower pace than *in vivo*, usually several orders of magnitude slower (Mcnew et al.; 2000; Parlati et al., 2000; Brunger, 2006). Other regulators are believed to work together with SNAREs to guide the specificity and facilitate the membrane fusion process (Hong, 2005; Brunger, 2006; Malasm et al., 2008).

One important way to regulate SNARE function is through phosphorylation and dephosphorylation. SNARE regulators and SNARE themselves are found to be phosphorylated by multiple kinases (Gerst, 2003). The phosphorylation of SNAP-23, SNAP-25, syntaxin and VAMP/synaptobrevin have been reported. (Nielander et al., 1995;

Hirling and Scheller, 1996; Marash and Gerst 2001, 2003). Among these, the phosphorylation of SNAP-23 is best studied and has been shown to be important in promoting SNARE assembly in vivo (Cabaniols et al., 1999).

The Q-SNARE syntaxins have a long N terminal extension like VAMP7 longins, but unlike the profiling-like fold structure of longin domain, syntaxin N termini assume a three helix bundle and interact reversibly with its SNARE domain (Munson et al., 2000; Rizo and Sudhof 2002, Rossi et al., 2004). Syntaxin can thus assume an incompetent "closed state" in which the SNARE domain is blocked and a competent "open" state (Misura et al., 2000). The transition between open and closed states is the major way to regulate syntaxin function as demonstrated in both neurons and yeast (Misura et al., 2000; Fiebig et al., 1999; Gerst, 2003; Margittai et al., 2003). One way to trigger the transition is by N terminal phosphorylation/dephosphorylation. When the N terminus of syntaxin is phosphorylated by kinases such as PKA, it assumes the close state, when dephosphorylated by phosphatase, its N terminal become open state. This kind of state transition triggered by phosphorylation is demonstrated functionally in yeast (Marash and Gerst 2001, 2003; Gerst, 2003). The longin domain of VAMP7 is also believed to negatively regulate its function as is the cases for syntaxins. This regulatory function itself must be switched off to activate the molecule, one method through which this might be achieved is through phosphorylation/dephosphorylation of the longin domain. Our results with serine 42 mutations in PiVAMP721 support this hypothesis as serine to alanine substitution renders the protein in a constitutive active state similar to over-expression of its C terminus alone. This non-phosphorylatable mutation may lock the protein in an open state and cannot transit into a closed state by phosphorylation. In

contrast, the phospho-mimetic serine to glutamate substitution may trap the protein in a closed state in which it cannot be turned on by phosphatase. The phenotypes of the transformants reported here are consistent with this hypothesis.

Although VAMP7 phosphorylation has not been reported, VAMP/synaptobrevin can be phosphorylated (Nielander et al., 1995). VAMP/synaptobrevin can be phosphorylated *in vitro* by multiple kinases including calcium/calmodulin-dependent protein kinase II and casein kinase II. However, phosphorylation status did not affect its efficiency to incorporate into SNARE complexes and the significance of VAMP/synaptobrevin phosphorylation has not been well studied (Nielander et al., 1995; Hirling and Scheller, 1996).

The function of SNARE proteins is regulated and modulated by a number of mechanisms in mammalian cells and yeast, this is likely also to be true in plants. Given the absence of brevins and expansion of the VAMP7/longin family in plants, it is likely that there has been a diversification and increase in the mechanisms needed to regulate longin domains. We explored the potential effects of PiVAMP721 phosphorylation in pollen tube growth and our results indicate PiVAMP721 phosphorylation may affect its overall function and in turn pollen tube growth. Although the phosphorylation of PiVAMP721 needs to be confirmed by other methods and the potential kinases responsible still remain to be identified.

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Figure legends

Figure 1. Reprehensive images of PiVAMP721 S42A and S42E transformed pollen tubes

A. Images of wild type PiVAMP721, a short pollen tube and slightly bulging tip was shown, light image was also shown. B. Transient expression of PiVAMP721 S42A, pollen tubes exhibit a shorter pollen tube and some pollen tube tips were bulging. C. transient expression of PiVAMP721 S42E, pollen tubes are longer and no bulging tips. For statistics of pollen tube length and shape, see figure 5.

Figure 2. Pollen tube length and shape in various PiVAMP721 mutations

Pollen cultured *in vitro* for 4 hrs after biolistic bombardment. Transformants expressing each mutant construct were observed using Orthomat epifluorescence microscope (Leitz) with 40X, dry, 0.7 numerical aperture Fluor objective. At least 50 individual transformants expressing each mutant construct were counted and measured. The bar in A indicates percentage of pollen tube length compared to that of wild type. The bar in B indicates percentage of bulging pollen tube tips.

Figure 1. Reprehensive images of PiVAMP721 S42A and S42E transformed pollen tubes.

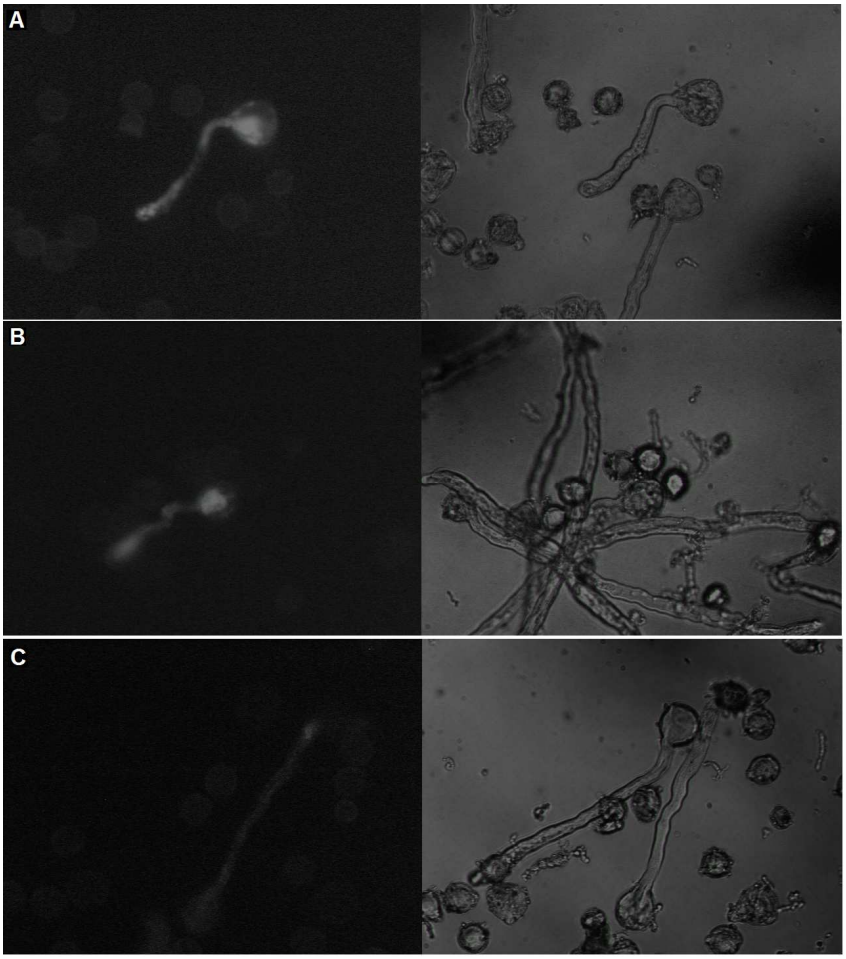
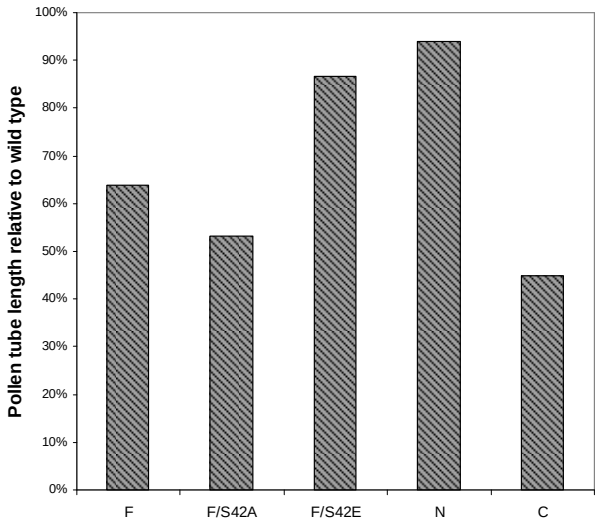
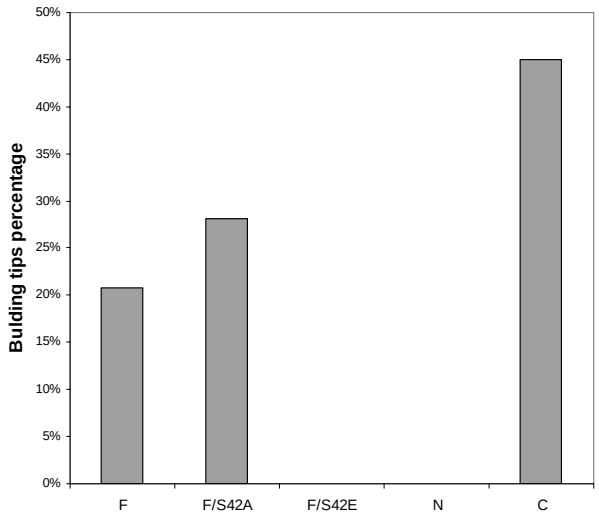


Figure 2. Pollen tube length and shape in various PiVAMP721 mutations

A.



B.



CHAPTER 3

Investigation into the role of peroxisomal proteins, PiSCP1 and PiCDPK2, in pollen tube growth in *Petunia inflata*

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*, these authors contribute equally to this research.

Abstract

The role of peroxisomes in pollen tube growth is poorly understood. Here we report the identification of two peroxisome proteins, PiSCP1and POiCDPK2, which we show to be important in pollen tube growth. PiSCP1 is a small novel protein which was isolated in a yeast 2 hybrid screen to identify potential CDPK substrates. Wild-type PiSCP1 had no obvious effect on pollen tube growth, but non-phosphorylatable and phospho-mimetic mutations of its two potential phosphorylatable serine residues inhibited tube growth. We further divided PiSCP1 into A, B and C domains based on sequence conservation with Arabidopsis homologs and synapsin. A and C portions retain peroxisome localization and were inhibitory to pollen tube growth while B is cytosolic and had no effect on pollen tube growth. PiSCP1 interacts with two previously described pollen specific CDPKs, PiCDPK1 and PiCDPK2 in yeast two hybrid assays, and can also rescue loss of growth polarity induced by over-expressing PiCDPK1. However, PiCDPK1 localizes to the plasma membrane, whereas here we show that PiCDPK2, like PiSCP1 localized to peroxisomes, suggesting that the interaction between these two molecules may be physiologically relevant *in vivo*.

Introduction

Peroxisomes are small organelles virtually ubiquitous to eukaryotes. Bioinformatic studies suggest that peroxisomes contain proteins of both eukaryotic and alaph-proteobacteria origin (Gabaldón et al., 2006). This inconspicuous single-membrane bound organelle is versatile and participates in a variety of metabolic pathways. Although plant peroxisomes have the signature function of β-oxidation and scavenge the resulting hydrogen peroxide, they also have a classical plant-specific function in photorespiration and some unique features. Plant peroxisomes are able to differentiate in specific cell types and each type contains a unique set of enzymes corresponding to the specific requirements of that cell type (Nishimura et al., 1996). Peroxisomes can bud from pre-existing peroxisomes or arise *de novo*, they also have the ability to dramatically to increase their number and size. Peroxins are protein factors responsible for these functions as well as peroxisome protein import (Brown and Baker, 2003; Platta and Erdmann, 2007). All peroxisome proteins have to be imported from elsewhere as peroxisomes have neither a genome nor protein translation machinery. The signaling role of peroxisomes has recently gained considerable attention, besides their obvious metabolic functions, evidence suggests that plant peroxisomes also participate in stress response and plant pathogen defense (Titorenko and Rachubinski, 2004; Baker et al., 2006). Plant peroxisomes are involved in the biosynthesis of hormones and signaling molecules such as indole acetic acid (IAA), jasmonic acid (JA), salicylic acid and NO (Baker et al., 2006; Prado et al., 2004).

The role of peroxisomes in pollen tube growth is poorly understood. It has long been known pollen has oil bodies and peroxisomes, but the relationship between these

organelles is unclear and whether pollen peroxisomes are specialized is still under investigation (Zhang et al., 1994; Piffanelli et al., 1998). Theoretically speaking, the potential roles of peroxisomes in pollen tube growth include, reserve mobilization by β-oxidation for energy, developmental signaling as peroxisomes have been shown to produce signaling molecules such as GABA, JA, IAA and NO (Prado et al., 2004, Baker et al., 2006) and structural functions in membrane production. Though few peroxisomal mutants have been reported to possess an abnormal pollen tube growth phenotype, two interesting mutants have been identified recently. *cts* mutants have reduced fertilization due to impaired pollen tube growth which results in unfertilized ovules and aborted embryos. *In vitro* pollen tube growth experiments have shown that *cts* pollen tubes have comparable growth rate to wild type in normal growth media, but germination and growth were reduced if an exogenous carbon source was absent in growth media. *CTS* was found to encode for an ATP-binding cassette transporter required for substrate import in peroxisome for β-oxidation. CTS was proposed to be necessary in fulfilling the energy requirement during pollen tube growth since pollen represents a symplastically isolated sink (Footitt et al., 2007). In a second peroxisome mutant, *amp2/amc*, reduced male transmission efficiency was reported to be due to pollen tube overgrowth and sperm discharge disruption at the final stage of male-female gametophyte reception (Mano et al., 2006; Boisson-Dernier et al., 2008). Pollen tube reception is impaired only when both parents bore the *amc* mutation. *amc/amp2* encodes for Pex13 which is essential for peroxisomal protein import and *AMC* mutation disrupts PTS1/2-dependent protein import. These reports suggest that functional peroxisomes in either the male or female

gametophyte are essential for proper pollen-tube reception and subsequent sperm discharge.

Here we report the identification of a peroxisomal protein specifically expressed in pollen tubes, a small novel protein, PiSCP1. We further demonstrate that a previously described calcium dependent protein kinase, PiCDPK2 also localizes to peroxisomes. PiSCP1 was first identified as a putative substrate for petunia PiCDPK1 in a yeast 2 hybrid screen. We previously reported that PiCDPK1 and PiCDPK2 as pollen specific CDPKs of *Petunia inflata* (Yoon, 2006; Yoon et al., 2006). PiCDPK1 was shown to be important in the regulation of pollen tube growth polarity and localized to the plasma membrane. PiCDPK2 in contrast was implicated in pollen tube growth, but not polarity and shown to localize to an undefined internal membrane compartment (Yoon et al., 2006) Here we report show that PiSCP1-YFP and PiCDPK2-YFP labeled organelles colocalize with peroxisome markers. Further we investigate the role of PiSCP1 in pollen tube growth through the use of non-phosphorylatable and phospho-mimetic forms of putative phosphorylation sites, as well as the functions of putative functional domains of PiSCP1 ascribed based on its sequence conservation when compared with Arabidopsis homologues and putative homology to the neuronal protein synapsin.

Material and methods

Plant material

Petunia inflata and *Petunia hybrida* were grown under green house conditions with a supplemental light regime of 16 hr light and 8 hr dark.

Yeast two hybrid library screening

We used the modified system for yeast two hybrid library screening (James et al, 1996). The yeast strain AH109 (MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::$GAL1_{UAS}$-GAL_{TATA}-HIS3, MEL1, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ) was used in the screen. AH109 was sequentially transformed first with pGBD/ΔN-PiCDPK1 bait construct, and then with a *Petunia inflata* pollen cDNA library in pGAD424 by the lithium acetate method (Gietz et al., 1995). Transformation was plated onto synthetic drop-out (SD) medium lacking leucine, tryptophan and histidine. After 7 days, cells were replicated onto SD medium lacking leucine, tryptophan, histidine and adenine, and positive transformants were subjected to a colony lift assay as described in yeast protocol handbook (Clontech, Palo Alto. CA). Yeast plasmid DNA was extracted from positive clones and transformed into competent XL1-blue MRF' (Stratagene, La Jolla, CA) and plated on LB agar plate with 100µg mL^{-1} ampicillin. Plasmids extracted from XL-blue MRF' were amplified with pGAD vector primers (GAD-F, 5'-TACCACTACAATGGATGATC-3', GAD-R, 5'-GCACAGTTGAAGTGAACTTG-3') to verify the insert size and amplified fragments were digested with 4-base cutting restriction enzyme, *Taq* I, to group positive clones in classes based on resulting digestion patterns. Positive clones were back-transformed into the AH109 yeast strain with the bait construct to confirm the interaction.

Isolation of full-length PiSCP1 cDNA

A *Petunia inflata* flower cDNA library was constructed in λZapII (Stratagene) as previously described (Skirpan et al., 2001). This library was screened to isolate the full-

length PiSCP1 cDNA. A 500bp partial cDNA of PiSCP1 obtained by yeast two hybrid screening was used to synthesize radio-labeled probes using the RTS RadPrime DNA labeling (Life Technologies, Gaithersburg, ML) and hybridized to filters for 16 hrs in hybridization buffer at 62^{o}C. After hybridization, filters were washed sequentially with 2X SSC buffer, 0.1% (w/v) SDS; 0.5% (w/v) SDS and 0.1% SSC buffer, 0.1% (w/v) SDS for 20 min per wash. Positive plaque were excised and subjected to secondary screening. Hybridization plaques were purified and *in vivo* excision was performed following the manufacture protocols and the full-length PiSCP1 cDNA was sub-cloned in pGAD vector (Clontech).

RNA gel blot analysis

Total RNA was extracted from 100 mg of *P. inflata* pollen. 15µg of total RNA was separated on a formaldehyde gel and stained with ethidium bromide to verify equal loading. The RNA was then transferred to Biodyne B membrane (Life Technologies) for hybridization. DNA probes were prepared using the RTS RadPrime DNA labeling (Life Technologies) and hybridized to filters for 16 hrs in hybridization buffer at 62^{o}C. After hybridization, filters were washed sequentially with 2X SSC buffer, 0.1% (w/v) SDS; 0.5% (w/v) SDS and 0.1% SSC buffer, 0.1% (w/v) SDS for 20 min per wash. Autoradiography was carried out at -70^{o}C with an intensifying screen. Autoradiographs were scanned using a LIDE flat bed scanner (Canon USA Ins., New York, NY).

Plasmid constructs for recombinant proteins

Standard molecular cloning methods were used and all plasmid constructs were confirmed by restriction digestion and DNA sequencing. The vector pREST-B (Invitrogen, Carlsbad, CA) was used to express the full-length PiCDPK1 and PiSCP1 fusion construct having a 6X His tag on its N-terminus. The coding regions of PiCDPK1 and PiSCP1 were amplified by PCR using the following primers:

PiCDPK1-NcoI-5', 5'-CCATGGGGAACTGTTGTTCAAG-3';

PiCDPK1-NcoI-3', 5-CCATGGCAACAAATGACTCCCTCC-3';

PiSCP1- NcoI-5', 5'-CCATGGAGATGAGTTTAAGTTGCTTG-3';

PiSCP1- NcoI-3', 5-CCATGGCTAAGATGAGTTTAGTTGCTTG-3'. Amplified DNA fragments were cloned into pGEM-T Easy vector (Promega, Madison, WI), digested with *Nco* I and subcloned into the *Nco* I site of the pRSET-B vector.

To create plasmid constructs for transient expression, we amplified PiCDPK1 and PiSCP1 coding sequences by PCR using following primers. For the Lat52-PiCDPK1-GFP construct PiCDPK1-NcoI-5' and PiCDPK1-NcoI-3' were used. For the Lat52-PiSCP1-GFP construct, PiSCP1- NcoI-5' and PiSCP1- NcoI-3' were used. Amplified DNA fragments were cloned into pGEM-T Easy vector (Promega), digested with *Nco* I and subcloned into the *Nco* I site of the pBluescript (KS) vector (Stratagene).

The dominate negative construct of PiCDPK1 has been previously described in Yoon et al., 2006. Deletion mutations of PiSCP1 were generated by PCR using following combination of primers. For the PiSCP1-A deletion construct, PiSCP1-Ncol-5' and PiSCP1-NT-NcoI-R (5'-GTCGACCATGGACAGGCTATTCTCA-3'). For the PiSCP1-B domain construct, PiSCP-MF-NcoI (5'-GGATCCATGGGATGAGAATAGCCTGTG-3') and PiSCP-MR-NcoI (5'-GTCGACCATGGGACCGCTGTTATG-3'). For the C

domain construct PiSCP1-NcoI-3' and PiSCP1-CT-NcoI (5'-GGATCCATGGAGTTTCCCAGCCCA-3'). Following PCR, amplified fragments were cloned into the *Nco* I site of the Lat52-GFP expression vector to generate Lat52-PiSCP-A-GFP, Lat52-PiSCP-B-GFP and Lat52-PiSCP-C-GFP.

Non-phosphorylatable mutants of PiSCP1 (S16A and S92A) were generated by overlapping PCR using PiSCP-S16AF (5'-AGGACAGATGCAGATAACGAA-3'), PiSCP-S16AR (5'-TTCGTTATCTGCATCTGTCCT-5'), PiSCP-S92AF (5'-AGGGATTGGGCATTTGAGGAT-3') and PiSCP-S92AR (5'-ATCCTCAAATGCCCAATCCCT-3') to replace serine-16 and serine-92 with the codon for alanine. Phospho-mimetic mutants of PiSCP1 (S16E and S92E) were generated by overlapping PCR using PiSCP-S16EF (5'-AGGACAGATGCAGATAACGAA-3'), PiSCP-S16ER (5'-TTCGTTATCTGCATCTGTCCT-5'), PiSCP-S92EF (5'-AGGGATTGGGAATTTGAGGAT-3') and PiSCP-S92ER (5'-TTCGTTATCTTCATCTGTCCT-5') to replace serine-16 and serine-92 with the codon for aspartate. After mutagenesis full-length PiSCP1 constructs containing the mutation were regenerated using PiSCP1-Ncol-5' and PiSCP1-NcoI-3' to remove the stop codon and introduce an *Nco* I site. The amplified fragments were cloned into the *Nco* I site of the Lat52-GFP expression vector to generate C-terminal GFP fusion constructions, Lat52-PiSCP1/S16A-GFP, Lat52-PiSCP1/S92A-GFP, Lat52-PiSCP1/S16E-GFP and Lat52-PiSCP1/S92E-GFP.

For colocalization experiments, Lat52-CFP version of each constructs was generated by amplifying CFP fragment from eCFP (Clontech) using

CFP-N-NCO 5'-CCATGGTGAGCAAGGGC-3' and

CFP-C-SAC 5'-GAGCTCTTATGTCAGTTGGTCATG-3' and cloned into the Lat52 expression vector to replace the coding region of GFP. The PTS2-CFP fusion protein was generated by amplifying the 5' 174 base pairs of *Arabidopsis ped1* thiolase that encode the N-terminal PTS2 signal sequence using primers Perox-F 5'-ACCATGGAGAAAGCGATCGA-3' and Perox-R 5'-TCCATGGATAGTGGAGTCCTATG-3' and cloned into the *Nco* I site of Lat52-CFP vector (Johnson and Olsen, 2003).

Expression and purification of PiCDPK1 and PiSCP1

E. coli cells (BL21) transformed with either pRSET-PiCDPK1 or pREST-PiSCP1 were grown overnight at 37^{o}C in 2 ml of LB/ampicillin (100 µg /ml), transferred to 250 ml of the same media and cultured until the OD_{600} was 0.7. Expression was induced by the addition of 1 mM Isopropyl-thio-β-D-galactoside for 2 hr at 30^{o}C. Cells were harvested, resuspended in ice-cold lysis buffer (10 mM) and sonicated. The fusion proteins were purified by affinity chromatography with nickel resin (Sigma). After loading the column with the recombinant proteins, it was washed with wash buffer (20 mM imidazole, 0.5 mM NaCl, 50 mM phosphate buffer, pH 6.0, 10% glycerol, 1 mM PMSF) and eluted with elution buffer. Protein concentration was determined using Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). The purity and integrity of the recombinant proteins was assessed by SDS-PAGE.

In vitro binding assay and In vitro phosphorylation assay

The binding assay was performed by mixing 2 µg of 6X His-tag-PiCDPK1 fusion protein attached to phenyl sepharose (Sigma) beads with 2 µg of 6X His-tag-PiSCP1 fusion protein in the presence of 500 µl of binding buffer (20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT and 0.1% Triton X-100) with 1 mM Ca^{2+}. Samples were rotated at 20 rpm for 2 hr at 4^{o}C, pelleted and washed three times with washing butter (20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.3 M NaCl, 1 mM DTT and 0.1% Triton X-100). The proteins were eluted in 1X SDS sample buffer and resolved by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). The pulldown samples of 6X His-tag-PiCDPK1 and 6X His-tag-PiSCP1 fusion proteins were analyzed by Western blotting using monoclonal Anti-polyHISTIDINE Clone HIS-1 antibody (Sigma) and anti-mouse IgG alkaline phosphatase conjugated secondary antibody (Sigma).

0.6 µg of 6X His-tag-PiCDPK1 fusion protein was incubated with 0.5 µg of6X His-tag-PiSCP1 fusion protein in phosphorylation buffer (50 mM HEPES, pH 7.0, 1 mM $MgCl_2$, 1 mM DTT) with either 50 µM Ca^{2+}or 1 mM EGTA. The reaction was initiated by the addition of 10 µCi of [γ-^{32}P] ATP and incubated for 10 min at room temperature. The reaction was terminated by the addition of 5X SDS sample buffer and electrophoresed on a 10% SDS-PAGE. The gel was blotted to PVDF membrane and exposed to X-ray film.

Transient expression in pollen

Transient expression of GFP fusion constructs in pollen were performed as previously described (Yoon et al., 2006). Briefly, *Petunia inflata* pollen was collected

from freshly dehisced anthers (10 flowers/bombardment), and suspended by gentle vortexing in 200 µl of pollen germination medium (PGM) (0.01% H_3BO_3, 0.02% $MgSO_4$, 0.07% $CaCl_2$, 15% PEG-4000, 2% sucrose). The pollen was spotted onto a 2.5 cm sq. piece of positively charge nylon membrane in a 9 cm Petri dish.

Microprojectile bombardment was performed using a PDS-1000/He biolistic system (Bio-Rad). Gold particles (1.1 µm diameter) were prepared according to the manufacturer's protocol using 2 µg of plasmid DNA/0.5 mg of particles. Co-bombardment was achieved by coating particle with 2 µg of each plasmid construct. 0.5 to 1 µg of plasmid DNA were used for colocalization purpose. Bombardment were performed at 28-inches Hg chamber vacuum, 1100 psi rupture disk, 0.25 inch gap distance and 1 inch particle travel distance. After bombardment, the pollen was washed from the nylon membrane into a Petri-dish with 4 ml PGM and cultured on an orbital shaker at 100 rpm for 4 hrs at 30^{o}C.

Pollen tubes were stained with FM 4-64 according to Parton et al. (2001). FM 4-64 (Molecular Probes, Eugene, OR) was added into PGM to a final concentration of 10 µM and visualized after 15 min of gentle shaking.

Analysis of transformed pollen tubes

Epifluorescence microscopy for GFP observation was performed using an Orthomat epifluorescence microscope (Leitz) with a 40 X, dry, 0.7 numerical aperture. GFP fluorescence was visualized using a Fluor objective, 480 nm excitation, 500 nm dichroic mirror and >530 nm emission. Images were captured using a Sensys cooled CCD camera (Photometrics, Tucson, AZ).

Confocal images were obtained using either a Bio-Rad MRC 600 or Zeiss 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) 488 nm excitation and 515-565 nm emission was used for GFP signal, 543 nm excitation and 560 nm long-pass emission for FM 4-64, 514 nm excitation and 530-560 nm band-pass emission filter for YFP and 458 nm excitation and 450-490 nm band-pass emission filter for CFP. All confocal images were analyzed using the Metamorph v4.5 (Molecular Devices Corp., Downlington, PA) and processed using Adobe Photoshop v5.5 (Adobe Systems Inc., San Jose, CA).

Results

Identification of PiSCP1

PiSCP1was identified in a GAL4 yeast two hybrid screen using the kinase domain of PiCDPK1 as bait. The kinase domain of PiCDPK1 with a 6 amino acid N-terminal deletion was used as the bait construct to avoid possible problems resulting from putative myristoylation and palmitoylation sites at the N-terminus. A full screen of approximately 2×10^7 clones was performed using this construct. After several rounds of selection and appropriate controls, 4 classes of interacting clones were identified and sequenced. The single true positive cDNA class was transformed back into yeast, and re-assayed for activation of ß-gal, alone and in combination with the bait construct. These controls confirmed that the interaction was reproducible and not a result of auto-activation or genomic mutation. The clone obtained was a partial cDNA, and was used to screen a pollen cDNA library to identify a full-length cDNA that we named *P. inflata* Small

CDPK interacting Protein 1 (PiSCP1). PiSCP1 encodes a protein of 103 amino acids with a deduced molecular mass of 11.7 KDa.

Analysis Amino acid sequence analysis of PiSCP1

BLAST searches revealed significant homology to 2 *Arabidopsis* expressed genes (Fig. 1A), but no gene of known function. Alignment with the *Arabidopsis* genes revealed these three genes are similar in length and contained conserved regions at the N- and C-termini separated by a highly variable central region. PiSCP1 contains several potential sites of phosphorylation by CDPK, based on the consensus Basic-X-X-S/T (Cheng et al., 2002; Harper and Harmon, 2005), the N- and C- terminal conserved regions each possess one site of potential serine phosphorylation (Harper and Harmon, 2005). Interestingly, both serine sites were conserved in the *Arabidopsis* homologs (Fig. 1A). One of the *Arabidopsis* homologs (At5g46770) is classified as pollen-enriched by microarray analysis (Pina et al. 2005).

As none of PiSCP1 homologues has known function, we surveyed the literature in search of proteins with which PiSCP1 might share functional analogy. We noticed that PiSCP1 shares a low level of sequence identity with neuronal proteins called synapsins (Fig. 1B). Synapsins are predominately expressed in neurons, which like pollen tubes, grow by tip-growth. These proteins were first identified as components of the exocytotic pathway which governs the secretion of neurotransmitters in neurons, but have since been shown to also be involved in the regulation of tip growth in these cells (Kao et al., 2002). Synapsins are regulated by phosphorylation by a number of protein kinases including PKA, CamKII and MAPK, and their association with synaptic vesicles and actin

microfilaments is regulated by phosphorylation (Greengard et al., 1993; Jovanovic et al., 1996). Three vertebrate synapsins (synapsins I, II, and III) have been identified to date and are composed of three distinct domains. The small N-terminal A domain is highly conserved in all synapsins, this is followed by a variable B domain, and a large central C domain that is also highly conserved. A serine residue in the A domain of human synapsins I has been demonstrated to function as a phospho-switch which controls the dynamic association of synapsins with synaptic vesicles (Masahiro et al., 1999). Sequence comparison between the N-termini of PiSCP1 and human synapsin 1 showed that they share 31% amino acid identity through the entire A domain, and serine 16 of PiSCP1 which is part of a consensus CDPK phosphorylation site, aligns with the phospho-switch of human synapsin I (Fig. 1B). This potential phosphorylation site is also conserved in the two Arabidopsis homologues of PiSCP1.

Expression pattern of PiSCP1

The development regulation of PiSCP1 was examined by RNA gel blot analysis using the partial cDNA obtained by yeast two hybrid screening as a probe (Fig. 2). The transcript detected was found to be pollen-specific, expression starting late in pollen development (10 mm buds = pollen mitosis I), peaking in mature pollen and remaining high in pollen tubes. This expression pattern is almost identical to that of PiCDPK1 and PiCDPK2 and is consistent with these genes being involved in a common signaling pathway(s) (Yoon et al., 2006).

PiCDPK1 interact with and phoshporylates PiSCP1 in vitro in a Ca^{2+}-dependent manner

To confirm the interaction between PiCDPK1 and PiSCP1, we performed an *in vitro* binding assay with purified fusion proteins of PiCDPK1 and PiSCP1 (Fig. 3A). Each protein was expressed as a 6X His tagged fusion protein in *E. coli* and purified using Ni-NTA^{+} resin. PiCDPK1 was then further purified by hydrophobic interaction chromatography using phenylsepharose. PiSCP1 was then incubated with phenylsepharose-bound His tagged PiCDPK1 in the presence of 1 mM Ca^{2+}. After washing, the resin-bound fraction was washed, eluted and subjected to Western blot using an anti-His tag monoclonal antibody. As shown in Figure 3A, PiSCP1 was pulled down only in the presence of PiCDPK1, confirming its' physical interaction with PiCDPK1.

We next assay whether the physical interaction between PiCDPK1 and PiSCP1 led to phosphorylation of PiSCP1 *in vitro*. As shown in Figure 3B, full-length PiCDPK1 was found to phosphoylate full-length PiSCP1 in a calcium-dependent manner. In the absence of calcium, no phosphorylation of PiSCP1 was detected whereas strong phosphorylation of PiSCP1 was detected upon calcium activation of PiCDPK1.

PiSCP1 localized to peroxisome

Transient expression of a PiSCP1-GFP fusion construct in growing pollen tubes showed that the fusion protein localized to internal membrane compartments mostly small spherical organelles and some slightly larger organelles (Fig. 4A). These organelles move rapidly with cytoplasmic streaming. Colocalization with FM 4-64 shows these small organelles largely stay out of the apical clear zone and are not stained by FM 4-64 (Fig. 4A). This indicates these organelles are most likely not part of the endocytic pathway.

The localization of PiSCP1 to these punctuate spherical compartments is reminiscent of peroxisome localization in pollen (Dammann et al., 2003). To test whether PiSCP1 does indeed localize to peroxisomes, a colocalization experiment was performed using PiSCP1-YFP and cyan fluorescent protein (CFP)-tagged with the peroxisome PTS2 targeting signal from the *Arabidopsis* thiolase. Thiolase is the classical PTS2 peroxisomal protein that was first identified in yeast (Glover et al., 1994). Co-expression this peroxisome marker with a vector encoding soluble YFP alone confirmed that in our hands the localization of this marker was identical to published peroxisome localization and that we were able to image CFP and YFP fluorescence separately without "bleed-over" (Fig 4B). When we co-expressed PTS2-CFP with PiSCP1-YFP at low levels (as indicated by strength of fluorescence), extensive colocalization was indeed observed (Fig. 4C and D), though there are some larger compartments emitting PiSCP1-YFP signal that did not show PTS2-CFP fluorescence. When PiSCP1 was highly over-expressed, the discrepancy between these signals became stronger and most YFP signal is not colocalize with peroxisome (Fig. 4E and F).

PiCDPK2 localized to peroxisomes and colocalized with PiSCP1

Another CDPK identified in our laboratory, PiCDPK2s has previously been shown to exhibit a similar punctuate localization pattern to PiSCP1 (Yoon et al. 2006). Previously we showed that its N-terminal is essential to its association with membrane. A truncated version of PiCDPK2 in which its N terminal 6 amino acids is deleted largely lost its membrane localization (Yoon et al. 2006). This deletion removed both putative palmitoylation and myristoylation sites and demonstrated that lipid modification is

required for its correction localization. A sequence comparison of the PiCDPK2 N terminus and known CDPKs localized by lipid modification is shown in Fig 5. Since the nature of membrane associated by PiCDPK2 is still unclear, we further performed colocalization experiment between PiCDPK2-YFP and peroxisomal marker. Although PiCDPK2-YFP did colocalize with PTS2-CFP, the shape of the peroxisomes appeared to be changed by its presence (Fig 6A and B). There was also more background signal causing the colocalization to be somewhat less clean than that of PiSCP1. A colocalization assay between PiCDPK2 and PiSCP1 was also performed, and not surprisingly, PiCDPK2-YFP was found to colocalize with PiSCP1-CFP (Fig 6C and D), the peroxisome morphological change caused by PiCDPK2 expression seemed unchanged by the co-expression of PiSCP1. Over-expression of PiCDPK2 caused mild inhibition of pollen tube growth, the co-expression of PiCDPK2 and PiSCP1 did not cause obvious phenotypic change (Yoon et al. 2006).

PiSCP1 interacts with PiCDPK2

Although PiSCP1 was originally identified in a yeast 2 hybrid screen with PiCDPK1 kinase domain as the bait, this does not preclude interaction between PiSCP1 with PiCDPK2, indeed as substrate specificity of CDPKs are probably more influenced by localization rather than amino acid sequence (Yoon, 2006) the co-localization of PiSCP1 with PiCDPK2 is arguably more significant than its in vitro interaction with PiCDPK1. Unfortunately we have not been successful in our attempt to express active PiCDPK2 in *E. coli*, hence to investigate the putative interaction between PiSCP1 and PiCDPK2, we decided to employ the yeast two hybrid system. We found that PiSCP1 did

indeed interact with the N-terminal variable/kinase domain of PiCDPK2 (Fig. 7). This indicated that PiSCP1 interacts with the N-terminal variable/kinase domains of both pollen expressed CDPK isoforms despite the fact that these two regions combined are only 21% identical at the amino acid level (Yoon, 2006). That two CDPK isoforms interact with the same substrate in yeast two hybrid system is not an unusual observation, in fact, PiRhoGDI1, another protein identified in out laboratory is behaved in the same fashion (Yoon, 2006). As PiSCP1 and PiCDPK2 both localized to peroxisomes most likely this is the interaction of physiological relevance.

Effects of co-expression of PiSCP1 and PiCDPK1 or PICDPK1-DN

Pollen tubes over-expressing PiSCP1 did not generate a discernable phenotype (Fig. 8A). However, in co-bombardments over-expression of PiSCP1 rescued the PiCDPK1 loss of polarity phenotype (in 82% of co-transformants) (Fig.8 B), but not the PiCDPK1-DN (Fig. 8C) phenotype. Phenotypic rescue was confirmed to result from co-expression by using a GFP fusion of each gene with the wildtype version of the other (data not shown).

Effect of over-expressing deletion constructs of PiSCP1 in pollen tube

As PiSCP1 and the two Arabidopsis homologues shared two highly conserved regions split by a central region of low amino acid sequence conservation, we next chose to investigate whether the regions of conservation were functionally significant to PiSCP1. We designed an in vivo assay to assess the function of each segment of PiSCP1

individually. Three deletion mutant constructs were generated, A (amino acids 1-30), B (amino acids 29-67) and C (amino acids 65-103), and generated Lat52 promoter driven, C-terminal GFP-fusion constructs. This allowed us to express each domain in pollen tubes and assess their effect(s) upon pollen tube growth.

In contrast to full-length wild type PiSCP1 which over-expression does not cause discernable changes, some of these truncated constructs had dramatic effects on pollen tube growth. PiSCP1/A-GFP severely inhibited pollen tube growth and localized to fast moving structures in a similar manner to the full-length protein (Fig. 9A). PiSCP1/C-GFP exhibited a similar localization to P1SCP1/A-GFP and inhibited pollen tube growth but less than PiSCP1/A-GFP (Fig. 9C). Over-expression PiSCP1/B-GFP did not substantially effect pollen tube growth, further this portion of PiSCP1 was not associated with any membrane compartment but rather exhibited cytosolic localization (Fig. 9B). To confirm the peroxisomal localization of A and C portions of PiSCP1, YFP versions fusion constructs were generated for all three segments and co-expressed with Lat52-PTS2-CFP. Although the result did confirm the peroxisomal localization of A and C, the above mentioned “background” problem seemed to be more severe than for wild type PiSCP1 particularly for the A domain construct, suggesting either that the A portion alone was not as effective as the full-length protein for targeting to peroxisomes, or possibly that the peroxisomal integrity was compromised in some way (Fig. 9A and C).

Co-expression of PiSCP1/A-GFP and PiSCP1/C-GFP constructs with PiCDPK1 both rescued the PiCDPK1 phenotype to some extent (Fig. 10A and C). Co-expression of the PiSCP1/A-GFP with PiCDPK1 severely inhibited pollen tube growth, but the tubes grew in a largely polar manner and did not balloon as is observed in tubes over-

expression PiCDPK1 alone (Fig. 10A, Yoon et al., 2006). Co-expression of PiSCP1/C-GFP with PiCDPK1 gave a phenotype where pollen tubes apparently grew fairly normally for a short distance, then polarity was lost and the tips ballooned (Fig. 10C). In contrast, The PiSCP1/B-GFP did not have any effect on the PiCDPK1 over-expression phenotype and the phenotype generated by co-expression PiSCP1/B-GFP with PiCDPK1 was indistinguishable to that of PiCDPK1, i.e. there was no rescue (Fig. 10B).

Effects of over-expressing non-phosphorylatable (S16A and S92A) and phospho-mimetic (S16E and S92E) mutants of PiSCP1 in pollen tubes

To investigate whether PiSCP1 might be regulated by phosphorylation by a CDPK in vivo, we scanned the protein for consensus phosphorylation motifs. Putative CDPK phosphorylation sites were identified in the conserved regions A and C of PiSCP1 (one in each). To assess whether the serine residues involved (Ser-16 and Ser-92) might be phospho switches, full length mutant PiSCP1 constructs were made in which S16 and S92 was converted to the non-phosphorylatable amino acid alanine (PiSCP1/ SI6A and S92A) and phospho-mimetic amino acid glutamate (PiSCP1/S16E and S92E). Double mutations, PiSCP1/S16A92A and PiSCP1/S16E92E, were also generated. These constructs were introduced into pollen by biolistic bombardment to assess the effects of their expression *in vivo*.

All mutants generated significant pollen tube growth inhibition, and on average grew to only ~50-65% of the length of wild type tubes. All however, retained the punctuate localization pattern observed for the wild type protein (Fig. 11 and Fig. 12). To confirm these mutations did not alter localization, colocalization experiments were

conducted with PTS2-CFP. The results showed all phospho-mimetic and non-phosphorylatable mutant of PiSCP1 retained peroxisomal localization (Fig. 11). This result suggests that the phosphorylation status of PiSCP1 did not alter its sub-cellular localization. However, rendering the protein non-phosphorylatable or mimicking constitutive phosphorylation at either or both ser-16 and ser-92 severely perturbed pollen tube growth, consistent with reversible phosphorylation of these residues playing a critical role in PiSCP1 function in relation to pollen tube growth.

PiSCP1 may dimerize in vivo

We also used PiSCP1 as the bait construct in a yeast two hybrid screen. One positive clone obtained turned out to be PiSCP1 itself. This interaction was found to be real and reproducible. Furthermore, when the various phosphorylation site mutant forms of PiSCP1 were tested, activation of reporter gene was obtained in all cases (Fig. 13). This result suggested that PiSCP1 may function as a dimer *in vivo* and its phosphorylation status did not affect dimer formation.

Discussion

Pollen tube growth has been a focus of intensive research for some time, but the importance of peroxisomes in polar growth has only been noticed recently. In fact, pollen peroxisomes have been virtually ignored beside mutants identified for other reasons. However, as shown by *amc* and *cts* mutants, peroxisome function is essential to pollen tube growth (Footitt et al., 2007; Boisson-Dernier et al., 2008). Based on our current knowledge, peroxisomes are likely to be involved at least in the following aspects of

pollen tube growth, energy source from fatty acid oxidation, male-female gametophyte interaction and NO signaling (Prado et al., 2004; Footitt et al., 2007; Boisson-Dernier et al., 2008). The two peroxisomal proteins presented in this research could provide us some insights into the roles played by peroxisomes in pollen tube growth.

Peroxisomal targeting of PiSCP1

Peroxisomes do not contain a genome, all peroxisome proteins are encoded in the nucleus, translated on ribosomes and must be transported into peroxisome. Pex proteins form the peroxisome protein import complex for the importation of matrix proteins and the insertion of peroxisome membrane proteins (Brown and Baker, 2003; Platta and Erdmann, 2007). There are at least four known targeting signal for proteins to be sorted to peroxisomes. The peroxisomal targeting signal type 1 (PTS1) which is an extreme C-terminal tripeptide and PTS2 which is an N terminal nona-peptide are used for matrix protein import. mPTS1 which is a positive amino acid cluster adjacent to membrane spanning domain and mPTS2 which is mPTS1 plus an ER signal peptide are used for peroxisome membrane protein targeting (Reumann, 2004; Mullen and Trelease, 2006; Platta and Erdmann, 2007). The identification of mPTS2 indicates there is a close relationship between peroxisome and the ER (Titorenko and Rachubinski, 2001). It is worth noting there are some well known peroxisome proteins such as catalase, *Arabidopsis* sarcosine oxidase and interestingly some Pex proteins themselves, which do not have any of these typical targeting signals (Reumann et al., 2007).

To fully understand the function of peroxisomes, it is essential to know which proteins are targeted to them. Bioinformatic analysis is one of the most promising large-

scale approaches to characterize the plant peroxisome proteome. In a recent genomic analysis, about 550 PTS1/2-containing sequences were retrieved from various higher plant species. Among these, 280 are from *Arabidopsis*, only about 50 are known peroxisome matrix proteins (Fukao et al., 2002; Kamada et al., 2003). Proteomics is another approach to identify potential peroxisome proteins, an additional 27 novel peroxisome proteins have been identified in a recent study. Among them, 14 lack predicted PTS1/2 sequences, demonstrating there are still unknown targeting sequence(s) or unidentified targeting pathways to peroxisomes (Reumann et al., 2007).

There were no typical peroxisome membrane and matrix targeting signals identified in PiSCP1, the observation that the N and C terminus YFP fusions still target to peroxisomes suggest that peroxisome targeting information may be stored into more than one location in this protein, this is not unprecedented as multiple distinct targeting sequence have previously been reported for peroxisome membrane proteins (Jones et al., 2001; Murphy et al., 2003). It should also be noted that an internal PTS1-like tripeptide PRL is located at the -20 of carboxyl terminal of PiSCP1, interestingly this internal PTS1-like motif is conserved in the 2 *Arabidopsis* homologs (Fig. 1A). Pumpkin catalase was shown to localize to peroxisomes via its internal PTS1-like QKL sequence at -10 (Kamigaki et al., 2003; Oshima et al., 2008). Alternatively, PiSCP1 may interact tightly with a protein partner and be targeted to peroxisome indirectly. This so called “piggy-back” mechanism was first described to explain the apparent import of oligomeric proteins. In 1994, Glover et al. showed that a truncated version of yeast peroxisomal thiolase imported into peroxisome correctly despite the lacking of the N-terminal PTS2, however, this mutant thiolase mislocalized to the cytosol in a yeast strain in which the

native thiolase gene was disrupted (Glover et al., 1994). Similar results were also obtained for plant isocitrate lyases (Lee et al., 1997). In the case of the HIV viral protein Nef, its peroxisomal localization was found to be dependent on the expression of full-length human thioesterase II (hTE). If the extreme C-terminal PTS1, SKL, in the hTE is deleted, both proteins mislocalized to plasma membrane (Cohen et al., 2000). The piggy-back mechanism has frequently been invoked to explain peroxisome localized proteins that lack an obvious targeting signal (Saleem et al., 2006). The functional characterization of PiSCP1 performed in this research indicated it interacts with CDPKs provides one potential "piggy-back" partner. The piggy-back mechanism can also provide an alternative explanation for the peroxisomal localization of various forms of PiSCP1 deletions and mutants. Even if some of these deletions and mutations lost the peroxisome targeting ability, they can still localize to peroxisomes as long as they retain the ability to interact with endogenous PiSCP1.

Peroxisomal targeting of PiCDPK2

PiCDPK2 does not possess any known PTS or mPTS targeting signal either. Since we had previously shown that myristoylation at the PiCDPK2 amino terminus is involved in its subcellular localization, it is reasonable to conclude that lipid modification is necessary for this localization. Lipid modifications such as myristoylation and palmitoylation are very common among CDPKs and a number of CDPKs have been shown to associate with membrane by means of myristoylation and palmitoylation (Ellary-Ivey et al., 2001; Martin and Busconi, 2000; Rutschmann et al., 2002; Lu and Hrabak, 2002; Chehab et al., 2004). It is estimated that the localization of 27 *Arabidopsis*

CDPKs among 37 total is mediated by N-terminal myristoylation and palmitoylation (Hrabak et al., 2003). One *Arabidopsis* CDPK, CPK1 is localized to peroxisomes in this fashion (Dammann et al. 2003). Kinases localized to peroxisomes are not uncommon, beside CPK1, another *Arabidopsis* kinase GPK1 is also localized to peroxisomes, and during a recent *Arabidopsis* peroxisome gene expression profiling, an additional 4 protein kinases were found to localize to peroxisomes (Fukao et al., 2002, 2003). 20 kinases or putative kinases have been predicated to localize to peroxisomes according to the Araperox database (Reumann et al. 2004).

N-terminal myristoylation is important in mediating membrane association, but recent evidence suggests that palmitoylation plays another arguably even more important role. It has been estimated that palmitoylation on its own provides a membrane association that is about 10 times stronger than that provided by a single N-myristoylation. S-palmitoylation of N-myristoylated proteins effectively permanently anchors the protein to the membrane whereas N-myristoylation alone does not promote lengthy membrane association (Hemsley and Grierson, 2008). This has been demonstrated in the small GTPase, ARA6, where N-myristoylation is necessary for membrane association, but palmitoylation is essential for its eventual correct endosomal localization (Ueda et al., 2001). However, it is equally clear from the same experiment that lipid modification alone is not sufficient for correct localization, albeit necessary. The N-terminal myristoylation and palmitoylation of residues cannot direct fusion protein to a correct location, which suggests they are not sorting signals per se. This is also obvious from our N-terminal amino acid comparison of CDPKs, they both contain potential sites of N-terminal myristoylation and palmitoylation but were targeted to very

different locations, the plasma membrane and peroxisomes. In other words, the critical sorting information must be store elsewhere, the N-terminal myristoylation and palmitoylation merely provides a mechanism for achieving tight membrane association. The actual sorting signals of CDPKs remain to be further investigated.

Signal "overflow" problem

The seeming signal "overflow" problems we observed with peroxisomal targeting protein is not unusual, in fact, this problem is mentioned in the seminal Gould et al. (1989) report in which the extreme carboxyl tripeptide PTS1 signal was first described. In our hands, the general rule seems to be the less DNA used and the less protein get expressed and the cleaner the peroxisome signal, this is in agreement with the observations of others (Gould et al., 1989). When using biolistic bombardment, one can control the amount of DNA which is precipitated onto gold particles, but as the efficiency of transformation varies between individual cells, there is always a relatively broad spectrum of expression levels among transformants. In these circumstances discussing the relative efficiency of peroxisome targeting of between construct would be unjustified, nonetheless we are able to conclude with confidence that PiSCP1 and PiCDPK2 are targeted to peroxisomes.

There are several not necessarily mutually exclusive explanations that may be account for the observation of signal "overflow". First, peroxisome targeting is mediated by protein factors, eg. Pex proteins, and they can be saturated. That more than one import pathway exists can explain why one signal is clear while another signal overflows. If this is the case, this could suggest that PiSCP1 and PiCDPK2 import into peroxisomes in a pathway that is independent from that PTS2 import pathway. Second, these overflow

phenomena might suggest an existing traffic pathway, for example, vesicles trafficking from ER or intermediate structures which are thought to be exist between ER and peroxisome (Titorenko and Mullen, 2006; Mullen and Trelease, 2006). We frequently observed some larger signal patches intermediate between classical sphere and rod-like peroxisome signal, this was observed in PiSCP1-YFP as well as PTS2-CFP signal. We reasoned that if a given protein is over-expressed, the vesicular structures involved in its transportation may accumulate enough YFP/CFP signal to be visible, whereas these structures do not normally accumulate sufficient fluorescence to be detectable. Finally, the overflow phenomena in some proteins might reflect genuine dual localization. Several *bona fide* peroxisome proteins exhibit dual localization, for example, human Pex 14 protein functions as a docking site for peroxisomal protein import but is also targeted to the nucleus where it functions as a transcriptional corepressor, *Arabidopsis* Pex16 protein has been found to be coexist at steady state in the ER and peroxisomes (Gavva et al., 2002; Karnik and trelease, 2005). Some images of PiCDPK2 were reminiscent those of Pex16 localization in the literature (Karnik and trelease, 2005). CDPKs localizing to more than one cellular compartment have also been reported. McCPK1 from ice plant was shown to change its subcellular localization from plasma membrane to the nucleus, endoplasmic reticulum and actin filament in response to reductions in humidity (Chehab et al., 2004). It is possible that PiCDPK2 may have more than one localization or change its localization under certain conditions. It would be interesting to explore this possibility by co-expressing an ER marker with PiCDPK2.

Functional significance of PiSCP1 and PiCDPK2 in pollen tube growth

Functional characterization data presented in this research suggested that PiSCP1, a CDPK-interacting peroxisomal protein is an important component of a calcium regulated signaling pathway(s) in pollen tube growth. Analysis of three distinct regions of PiSCP1 based on homology to two *Arabidopsis* homologs, tentatively suggest that the three regions identified have distinct functions that contribute to the overall function of PiSCP1 in pollen tube growth. We also showed that point mutations of critical serine residues in two potential CDPK phosphorylation sites severely affected PiSCP1 function. Combined with the result PiSCP1 interacts with both PiCDPK1 and PiCDPK2 in yeast two hybrid assays and that PiSCP1 can rescue the over-expression phenotype caused by PiCDPK1, provides evidence that PiSCP1 is regulated by CDPK-mediated phosphorylation *in vivo*.

The truncated N-terminal PiSCP1/A and C-terminal PiSCP1/C regions were found to retain the ability to localize to peroxisomes. But in contrast to the full-length protein, PiSCP1/A severely inhibited pollen tube growth, as did PiSCP1/C to a lesser degree, suggesting that these protein fragments compete with the endogenous protein to disrupt process(es) that is/are critical to pollen tube extension. The finding that PiSCP1 can dimerize provides one possible route through which these truncated proteins might inhibit growth. That these regions are functionally significant is consistent with the observation that they are highly conserved between PiSCP1 and the Arabidopsis homologues. In contrast to the other two regions, the central PiSCP1/B region did not localize to any membrane compartment, rather it assumed a cytosolic localization and further did not adversely affect pollen tube growth. Interestingly co-expressing the A or C regions of PiSCP1 partially rescued the loss of polarity phenotype caused by over-

expressing PiCDPK1, but lead to somewhat different pollen tube phenotypes. The A-region rescued polarity, but tube extension was severely inhibited. The C-domain apparently rescued polarity and allowed normal tube growth for a short period, but eventually the pollen tubes stopped growing and the tips ballooned. Coexpressing the B region had no effect on loss of polarity caused by over expressing PiCDPK1. The ability to rescue the PiCDPK1 phenotype is most likely caused by the possession of one or more CDPK phosphorylation sites in a region – resulting in a titration of excess PiCDPK1 activity away from endogenous substrate(s). Most importantly, these results are consistent with the presence of phosphorylation sites in both the A and C regions of PiSCP1, but not the B region.

Synapsins possess a conserved serine residue in the A-domain at the N-terminus and it has recently been shown that phosphorylation of this serine residue in human synapsin I is critical to neurite outgrowth. Expression of a non-phosphorylatable mutant form of synapsin was found to inhibit tip growth in neurites whereas expression of a phospho-mimetic form of synapsin increased neurite extension (Kao et al., 2002). The phosphorylation at this site has been demonstrated to control the dynamic association of synapsins with synaptic vesicles, synapsin phosphorylation triggering release of synaptic vesicles from the actin cytoskeleton and freeing them to fuse with the plasma membrane. As PiSCP1 possessed a potential CDPK phosphorylation site at the N-terminus that aligned with this phospho-switch of synapsin I, this prompted us to speculate that phosphorylation of this residue may be important in the regulation of PiSCP1 and investigate possible functional analogies between synapsin and PiSCP1. The results from PiSCP1/A and C suggested both regions are important and there is another conserved

serine in the PiSCP1/C which is also part of a CDPK consensus site. We generated non-phosphorylatable and phospho-mimetic mutants to investigate whether either or both of these two serine residues have might be important to the function of PiSCP1 in pollen tube growth. Pollen tubes transiently expressing these non-phosphorylatable and phospho-mimetic mutants show varying degrees of growth inhibition in contrast to the absence of phenotype seen when over-expressing the wild type protein. All mutants retained peroxisomal localization. This result suggests that both serine residues are important to the function of PiSCP1. Pollen tube growth is a delicate system involving the interplay of many signaling pathways, but in a polar growing cell, the range of phenotypes possible is rather more limited and perturbation of signaling pathways generally leads to either growth inhibition or loss of polarity. The similar phenotypes generated by non-phosphorylatable and phosphorylation-mimetic mutants, is not particularly surprising, and does not necessarily indicate a general loss of function of PiSCP1. In fact, these results are consistent with PiSCP1 and PiCDPK2 are potential interacting partners as various mutant forms of PiCDPK2 also generated similar phenotype (Yoon et al., 2006). These phenotypes do suggest that the serine residues under investigation are important to PiSCP1 function, but are not particularly useful in attempting to hypothesize what that function might be.

Organelle movement in plant cells was thought to be somewhat passive as they are moving along with cytoplasmic streaming. Although cytoplasmic streaming has been associated with actin, the physiological significance of this seemingly random movement remains to be determined. There are an unusually large number of myosin genes present in *Arabidopsis* genome. Recently, a number of myosins have been associated with

organelle movement and characterized individually (Hashimoto et al., 2005, 2008, Avisar et al., 2008, Peremyslov et al., 2008). Results from these experiments demonstrated that though organelles seem to all move with cytoplasmic streaming, in fact their movement and indeed the mechanism by which they move is distinct from both cytoplasmic streaming and each other, disruption of individual myosins has different effects on the movement of different organelles. For example, myosins XI-2 and XI-K are implicated in peroxisome and mitochondria trafficking and disruption of them effects root hair growth (Peremyslov et al., 2008). It should be mentioned that the movement of plant and yeast peroxisome dependent upon actin filament which is in contrast to microtubules-dependent peroxisome movement in animal (Jedd and Chuan 2002; Mano et al., 2002; Mathur et al., 2002). Taken together, this suggests the movement of each class of organelle may be regulated individually and the specific movement of a particular kind of organelle may be physiologically important to this organelle. For peroxisomes, their movement may be involved in NO signaling, as NO is only diffusible in a very short distance from peroxisome (Prado et al., 2004). Thus, in the case of NO signaling, the location of peroxisome is very important. Data presented in this research suggest that PiSCP1 may function in regulating pollen tube peroxisome movement in a similar fashion as synapsin, i.e. by reversible association with actin filament. This function of PiSCP1, in turn, is fine tuned by calcium dependent protein kinase, probably PiCDPK2. Thus connected peroxisome function to the calcium regulated signaling network in pollen tube growth. Phosphorylation by PiCDPK1 is also another viable scenario, as a single substrate being phosphorylated by multiple kinases is far from unusual. In fact, synapsin I is phosphorylated by multiple kinases. This model is consistent with our preliminary data

and is a useful working hypothesis although considerable additional studies are still required.

Recent progress in peroxisome research suggests that the organelles play important roles in signaling and decision making in development. In plant, peroxisomes have established roles in signaling transduction with a number of myosins, dynamin-related proteins, Arf and Rab GTPases and kinases all found to be localized to them (Mano et al., 2004; Lay et al., 2006; Peremyslov et al., 2008; Hashimoto et al., 2008). In fact, 14% of peroxisome proteins are predicated to be involved in signaling transduction according to the Araperox database (Reumann et al., 2004). With a refined characterization of Pex proteins and the newly revised ER-peroxisome maturation and replication model, the peroxisome research field is promising exciting discoveries in the near future (Mullen and Trelease, 2006, van der Zand et al., 2006; Tabak et al., 2008). By the identification of two peroxisomal proteins, PiSCP1 and PiCDPK2, and with their functions connected to calcium signaling, we have uncovered a novel link between peroxisomes and the signaling networks in pollen tube growth. Currently the precise function(s) of these proteins and in pollen tube growth is unclear, as are the pathways in which they participate. A further topic worthy of additional study is nature of the targeting signal of PiSCP1 and PiCDPK1.

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Figure legends

Figure 1. Alignment of PiSCP1 and Arabidopsis homologues and human synapsin1

A. Comparison of amino acid sequences between PiSCP1 and its *Arabidopsis* homologues (At5g46770 and At2g35215). Predicted amino acid sequences were aligned using ClustalW software. Consensus amino acids among all sequences are indicated by bold letters. The conserved serine residue in A-domain is underlined. B. Comparison of amino acid sequence between PiSCP1 and human synapsin 1. The predicted amino acid sequences were aligned using ClustalW software. Consensus amino acids among all sequences are indicated by bold letter. The conserved serine residue in all synapsins is underlined.

Figure 2. Expression pattern of PiSCP1

Expression pattern of PiSCP1 mRNA. 15 µg of total RNA was electrophoresed in a 1% formaldehyde/agarose gel and transferred onto a nylon membrane. The membrane was hybridized with 500bp partial PiSCP1cDNA, washed and subsequently exposed to X-ray film.

Figure 3. PiCDPK1 interacts with and phosphorylates PiSCP1

A. In vitro binding assay with PiCDPK1. 6X His-tag-PiSCP1 fusion protein was incubated with phenyl-sepharose bound 6X His-tag-PiCDPK1 protein in the presence of 50 µM Ca^{2+}. After washing, the His tag resin bound fraction were separated by SDS-PAGE and subjected to Western blotting using anti-His antibody to detect His tag fusion

proteins. B. In vitro phosphorylation assay of PiSCP1 by PiCDPK1 in the presence of 50 µM Ca^{2+} or 1 mM EGTA.

Figure 4. PiSCP1 localizes to peroxisome

A. Images showing pollen tubes expressing full-length PiSCP1-GFP. Pollen cultured for 4 hrs after biolistic bombardment and stained with FM 4-64 for 15 min. Green channel represents PiSCP1-GFP signal and red channel represents FM4-64 signal, yellow channel represents the merging of green and red channels. B. Localization of PTS2-CFP. Blue channel represents PTS2-CFP signal and yellow channel represents Lat51-YFP vector, green channel represents the merging of blue and yellow channels. C and D. Colocalization of PiSCP1-YFP with PTS2-CFP. Yellow channel represents PiSCP1-YFP signal and blue channel represents PTS2-CFP signal, overlay represents the merging of yellow and blue channels. Note although blue and yellow signal do colocalize for the most part, some non-colocalization can also been seen (lines). E and F. when over-expressed, PiSCP1-YFP largely not colocalize with peroxisome signal. Images were generated using a confocal microscope. Scale bar = 5 µm.

Figure 5. Alignment of PiCDPK2 N terminal amino acids sequence with other CDPKs

Comparison of PiCDPK2 N terminal amino acid sequence with other CDPKs which lipid modifications which are confirmed to be important in their localization, including CpCPK1 (Ellard-ivey et al., 1999), AtCPK1 and 2 (Dammann et al., 2003), McCPK1 (Chehab et al., 2004), LeCPK1 (Rutschmann et al., 2002) and PiCDPK1 (Yoon et al.,

2006). The potential myristoylation (G) and palmitoylation (C) sites are indicated by underline and bold letters. The reported localization of these CDPKs was also listed.

Figure 6. Representative pollen tubes expressing PiCDPK2 and co-expressing PiSCP1 with PiCDPK2

A and B. Images showing pollen tubes expressing full-length proteins fused to fluorescent proteins. Yellow channel represents PiCDPK2-YFP signal and blue channel represents PTS2-CFP signal, overlay represents the merging of yellow and blue channels. C and D. Colocalization of PiSCP1-CFP with PiCDPK2-YFP. Yellow channel represents PiCDPK2-YFP signal and blue channel represents PiSCP1-CFP signal, overlay represents the merging of yellow and blue channels. Note the peroxisome signal did not look as clear as co-expression with PiSCP1 or vector alone. Images were generated using confocal microscope. Scale bar = 5 µm.

Figure 7. PiSCP1 interacts with PiCDPK2 *in vivo*

Yeast transformed with the bait and prey constructs was grown on selection media, either SD/-T-L or SD/-T-L-H. A. yeast containing BD-PiCDPK2 and AD-PiSCP1 grow on both media. B and C. Neither BD-PiCDPK2 nor AD-PiSCP1 alone was able to auto-active Histidine reporter gene.

Figure 8. Representative pollen tubes over-expressing PiSCP1 and co-expressing PiSCP1 with PiCDPK1 or DN-PiCDPK1

A. Images showing pollen tubes over-expressing full-length PiSCP1, exhibiting normal pollen tube morphology. B. Images showing pollen tube co-expressing PiSCP1 with PiCDPK1, where PiSCP1 rescued the PiCDPK1phenotype. C. Images showing pollen tube co-expressing PiSCP1 with DN-PiCDPK1 did not rescue the phenotype of DN-PiCDPK1, leading to pollen with “sock-like” tubes.

Figure 9. Representative pollen tubes expressing YFP tagged PiSCP1 deletion mutants with peroxisome targeted PTS2-CFP

A. Colocalization of PiSCP1/A-YFP with PTS2-CFP. Pollen cultured for 4 hrs after biolistic bombardment. Yellow channel represents PiSCP1/A-YFP signal and blue channel represent PTS2-CFP signal, overlay represents the merging of yellow and blue channels. B. Colocalization of PiSCP1/B-YFP with PTS2-CFP. Yellow channel represents PiSCP1/B-YFP signal and blue channel in E represents PTS2-CFP signal, overlay represents the merging of yellow and blue channels. C. Colocalization of PiSCP1/C-YFP with PTS2-CFP. Yellow channel represents PiSCP1/C-YFP signal and blue channel represent PTS2-CFP signal, overlay represents the merging of yellow and blue channels. Images were generated using a confocal microscope. Scale bar = 5 µm.

Figure 10. Representative pollen tubes expressing PiSCP1 deletion mutants co-expressed with PiCDPK1

A. Representative image showing that PiSCP1/A-GFP partially rescues the phenotype of PiCDPK1. The transformed pollen tube growth is still severely inhibited but the tubes did not balloon. B. PiSCP1/B-GFP did not rescue the PiCDPK1 phenotype.

Pollen tubes expressing both constructs showed an indistinguishable phenotype from the over-expression of PiCDPK1 alone. C. PiSCP1/C-GFP rescues the phenotype of PiCDPK1. Pollen tubes expressing both constructs grew fairly normally for a short time and then lost polarity and the tips ballooned. All images were generated using epifluorescence microscope.

Figure 11. Representative pollen tubes expressing YFP tagged PiSCP1 non-phosphorylatable and phospho-mimetic mutants

Images showing pollen tubes expressing full-length PiSCP1-YFP mutation constructs. Pollen cultured for 4 hrs after biolistic bombardment and stained with FM 4-64 for 15 min. Yellow channel represents PiSCP1-YFP signal and blue channel represents peroxisome signal, overlay represents the merging of blue and yellow channels. A. PiSCP1/S16A-YFP. B. PiSCP1/S16E-YFP. C. PiSCP1/S92A-YFP. D. PiSCP1/S92E-YFP. Images were generated using a confocal microscope. Scale bar = 5 µm.

Figure 12. Effects of pollen tubes expressing PiSCP1 non-phosphorylatable and phospho-mimetic mutants

Pollen cultured *in vitro* for 4 hrs after biolistic bombardment. Transformants expressing each mutant construct were observed using Orthomat epifluorescence microscope (Leitz) with 40X, dry, 0.7 numerical aperture Fluor objective. At least 50 individual transformants expressing each mutant construct were counted and measured. The bar indicates percentage of pollen tube length compared to that of untransformed wild type pollen tube.

Figure 13. PiSCP1 interacts with wild type PiSCP1 and non-phosphorylatable and phospho-mimetic mutants of PiSCP1 in yeast two hybrid assay

Yeast transformed with the bait and prey constructs was grown on selection media, either SD/-T-L or SD/-T-L-H. A-E. yeast containing BD-PiCDPK2 and AD-PiSCP1 (A) or AD-PiSCP1 (B), AD-PiSCP1 (C), AD-PiSCP1 (D), AD-PiSCP1 (E) grow on both media. F and G. Neither BD-PiCDPK2 nor AD-PiSCP1 alone was able to auto-active Histidine reporter gene.

Figure 1. Alignment of PiSCP1 and its Arabidopsis homologs

A.

```
PiSCP1         MSLSCLTCNVLRRTDSDNEQRGRFNNDENSLCLGRVDRSWSGNLAAPRPSS----------
AT5G46770      MSLNCLSCQALPRTDSNKDVDLSGPGPPRVEINNVLGKTCCVNPIGGRNWRSGNLSPRIYE
AT2G35215      MSLNCLACHILQRTDSDRDMGSRKDSSFKENFATSAFEKMVRNRSSLPVVR----------
                                ^

PiSCP1         ------------QKMTTLTSSDDSNHRHHNSGPMEFPSTPRLLRSPGVRRDWSFEDICRQM
AT5G46770      KIGRPGSSLAHKMKKVKKIHHVRLSGPVGSSPSNVPTRQPKLVRSTGVRRNWSFENLRIEE
AT2G35215      ------------------VNKGHRRLYSADIMVYGELDEPKLVRSSGIRRDWSFEDLKKHK
                                                        ^

PiSCP1         VED
AT5G46770      KRKINIDQGL
AT2G35215      DQLRIEETIKE
```

B.

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Region 1: Synapsin entire A-domain (31.0% identity)
Synapsin 1 (1-28)                  MNYLRRRLSDSNFMANLPNGYMTD-LQRPQ
PiSCP1     (1-37)           MSLSCLTCNVLRRTDSDNEQRGRFNNDENSLCLGRVD

Region 2: Part of Synapsin B-domain (38.5% identity)
Synapsin 1 (56-84)                   VAPAASPAAPSPGSSGGGG-FFSSL-SN
PiSCP1     (38-65)                   RSWSGNLAAPRP-SSQKMTTLTSSDDSN

Region 2: Part of Synapsin B-domain (38.5% identity)
Synapsin 1 (488-510)    GQQHLSGLGPPAGSPLPQRLPSP
PiSCP1     (66-103)     HRHHNS--GPMEFPSTPRLLRSPGVRRDWSFEDICRQMVED
```

Figure 2. Expression pattern of PiSCP1

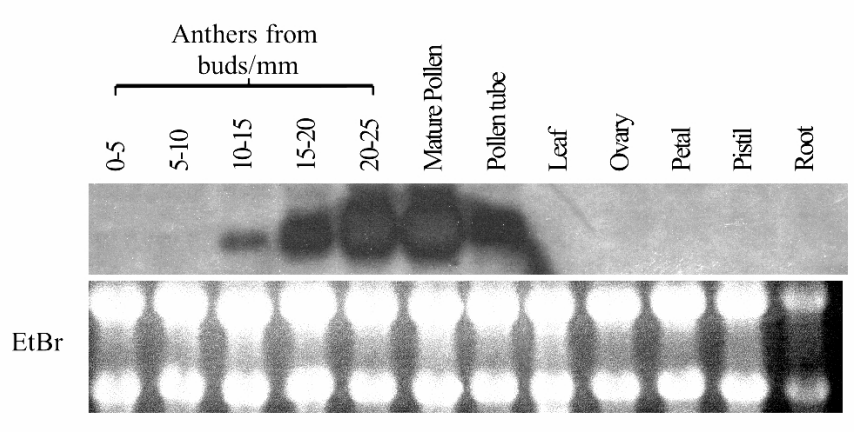


Figure 3. PiCDPK1 interacts with and phoshporylates PiSCP1

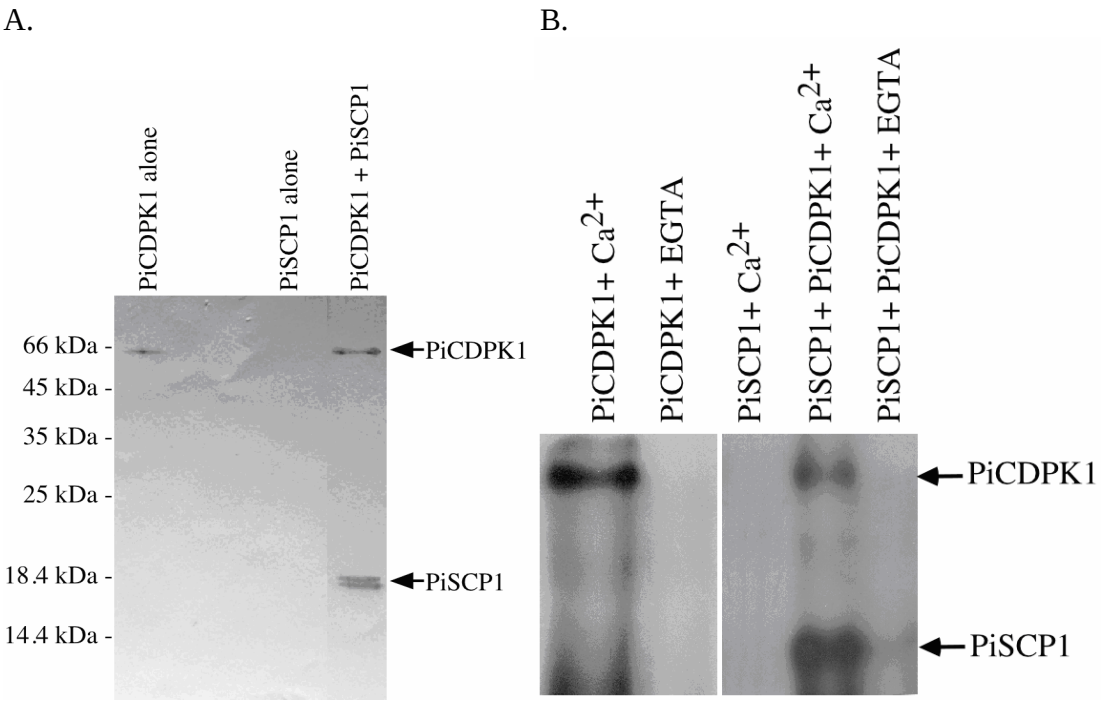


Figure 4. PiSCP1 localizes to peroxisomes

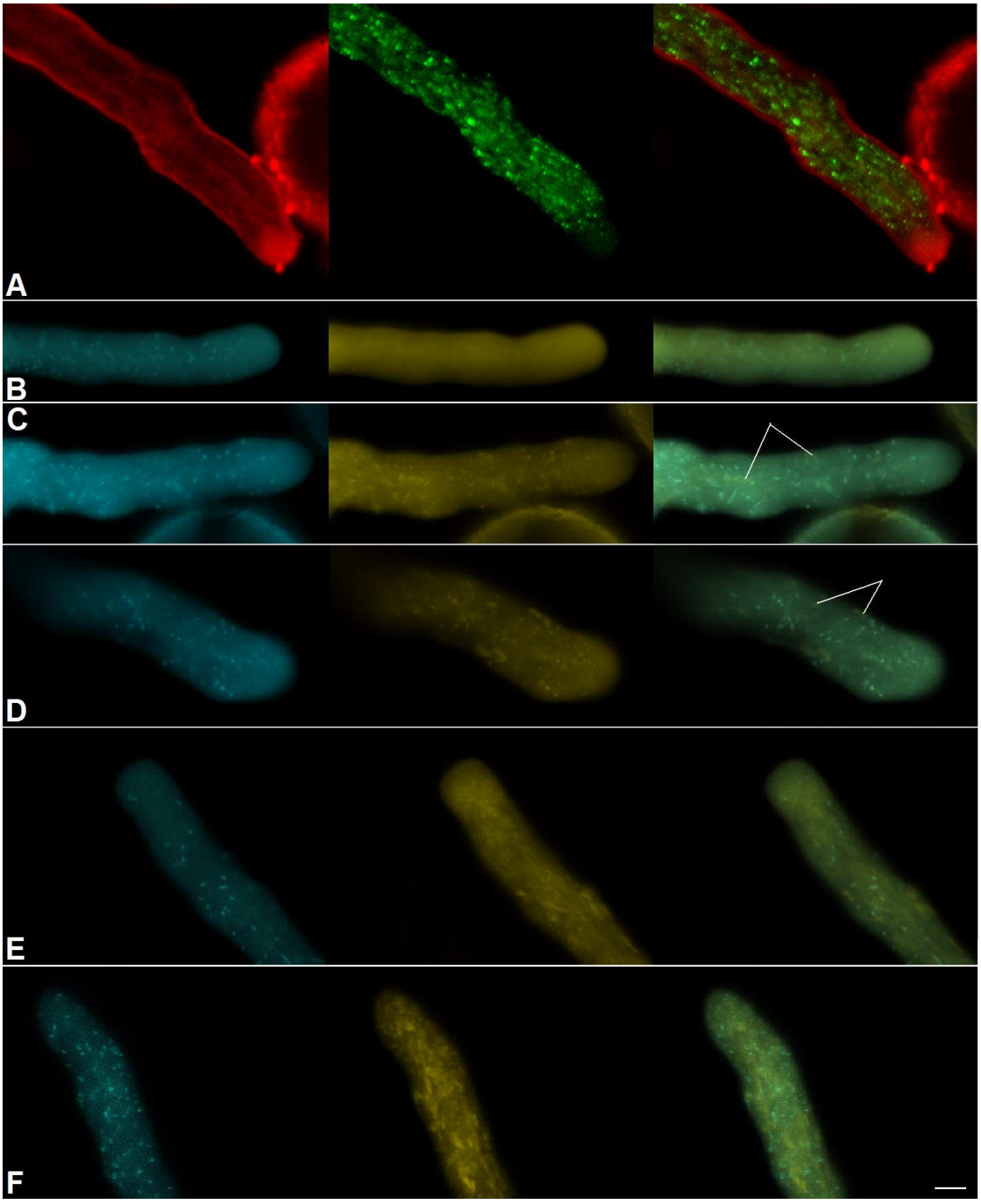


Figure 5. Alignment of PiCDPK2 N terminal amino acid sequences with CDPK of known localization

CpCPK1	MGNT**C**VGPSI	PLASMA MEMBRANE
AtCPK1	MGNT**C**VGPSR	PEROXISOME
AtCPK2	MGNA**C**VGPNI	ER
McCPK1	MGI**C**ASKNKA	PLASMA MEMBRANE
LeCPK1	MGG**C**FSKKYT	PLASMA MEMBRANE
PiCDPK1	MGN**C**CSRGQP	PLASMA MEMBRANE
PiCDPK2	MGT**C**MSVQNA	PEROXISOME

Figure 6. Representative pollen tubes expressing PiCDPK2 and co-expressing PiSCP1 with PiCDPK2

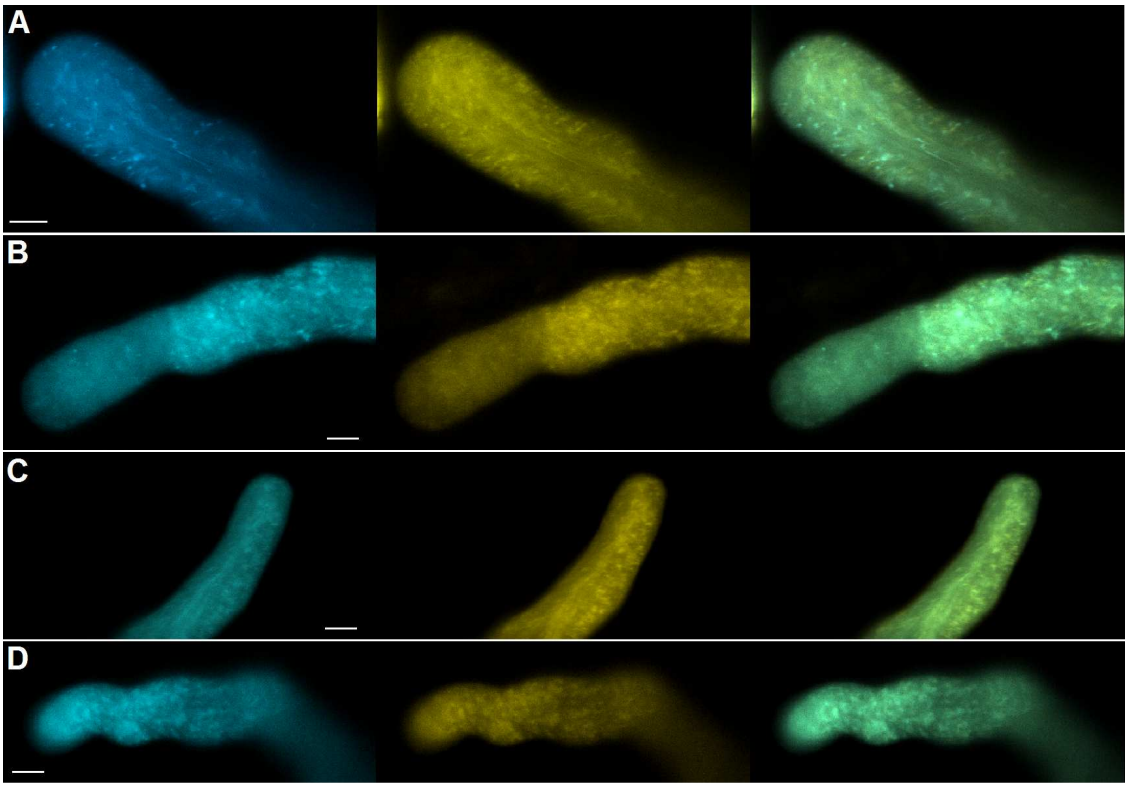


Figure 7. PiSCP1 interacts with PiCDPK2 *in vivo*

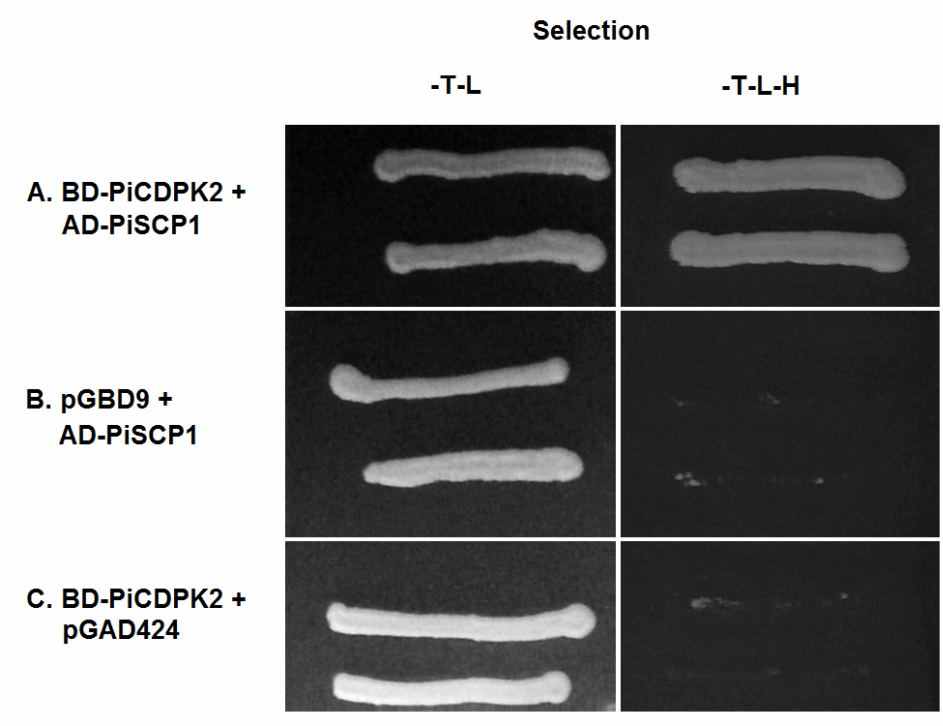


Figure 8. Representative pollen tubes over-expression PiSCP1 and co-expressing PiSCP1 with PiCDPK1 or DN-PiCDPK1

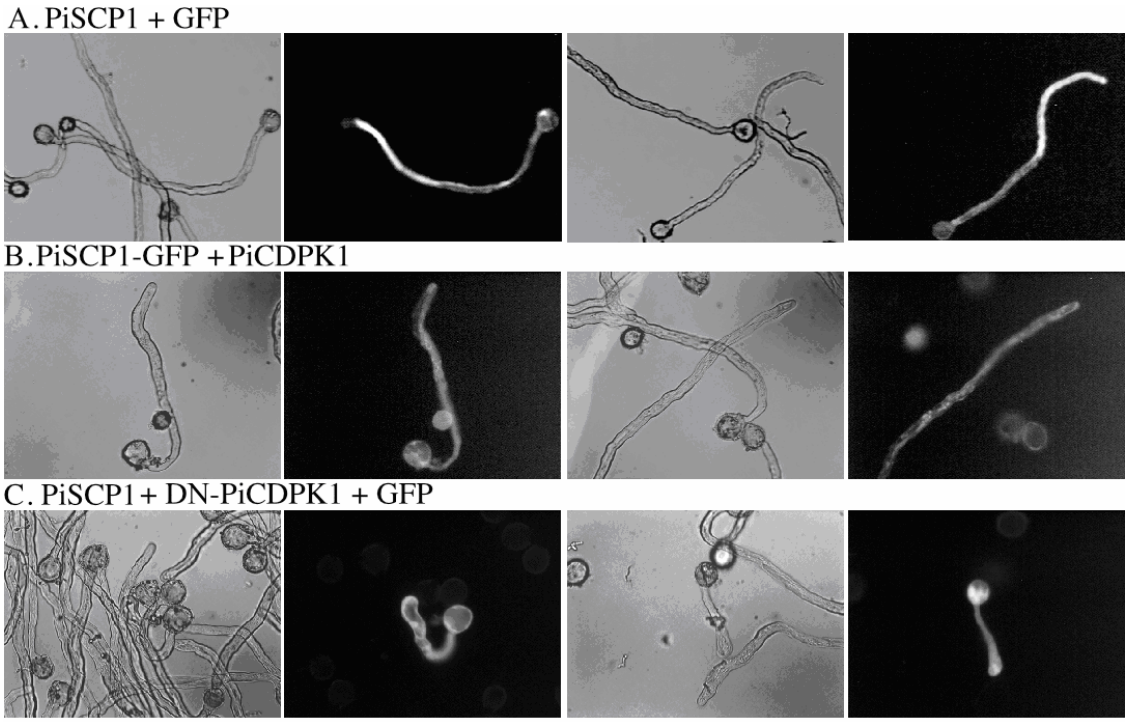


Figure 9. Representative pollen tubes expressing YFP tagged PiSCP1 deletion mutants with peroxisome targeted PTS2-CFP

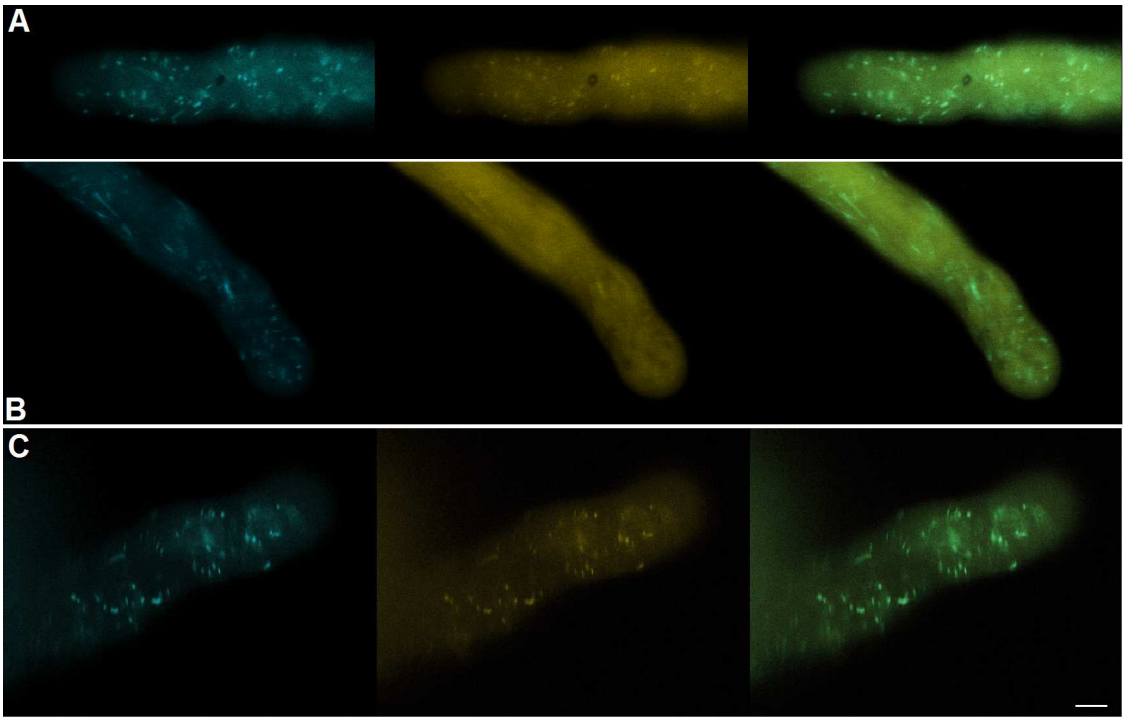


Figure 10. Representative pollen tubes co-expressing PiSCP1 deletion mutants with PiCDPK1

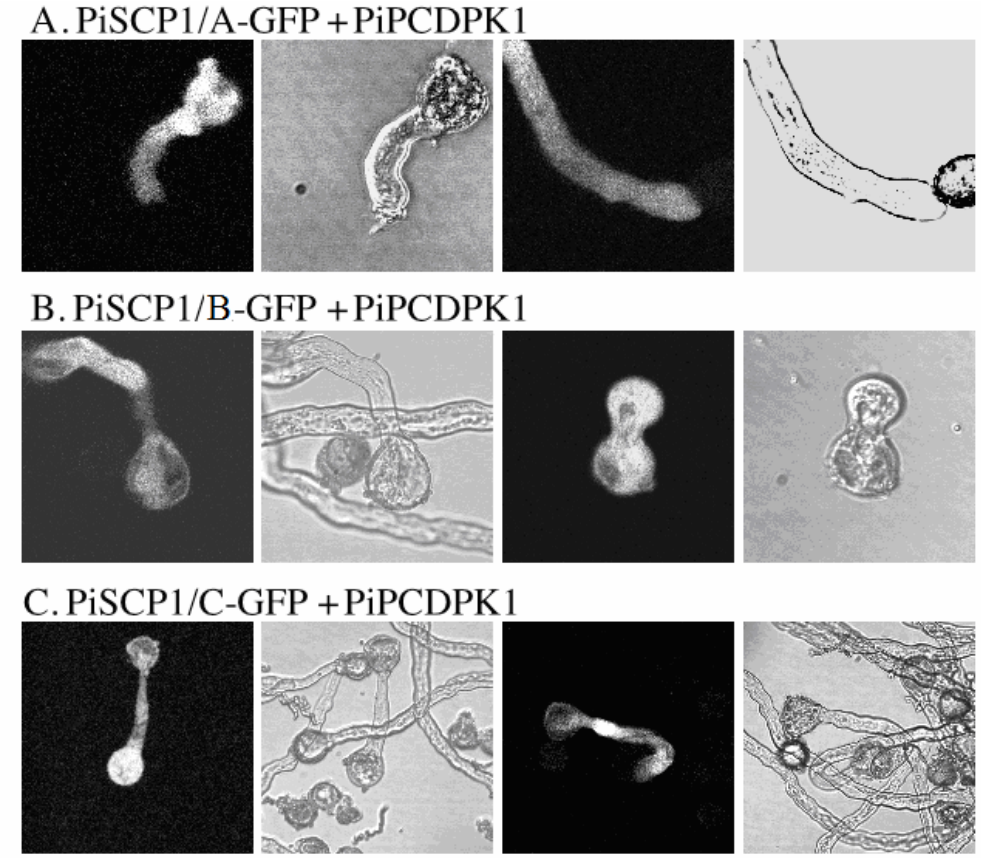


Figure 11. Representative pollen tubes expressing YFP tagged PiSCP1 non-phosphorylatable or phospho-mimetic mutants

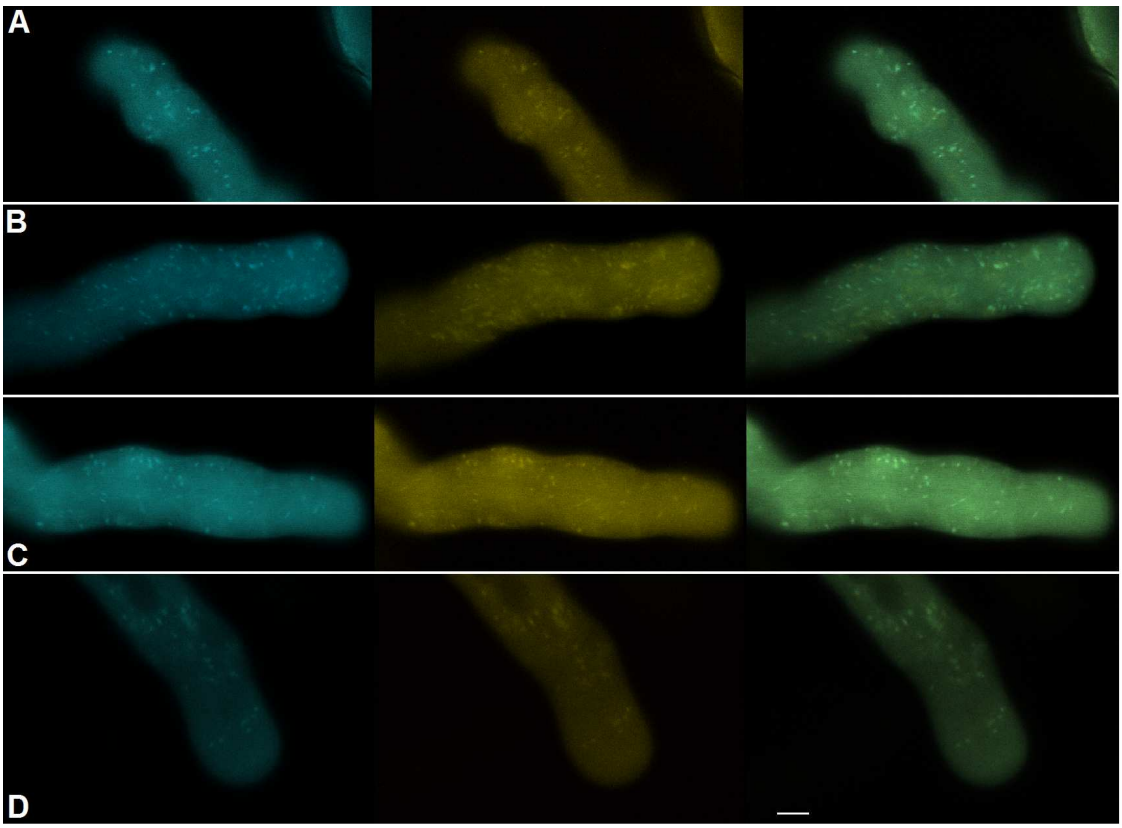


Figure 12. Effects of pollen tubes expressing PiSCP1 non-phosphorylatable and phospho-mimetic mutants

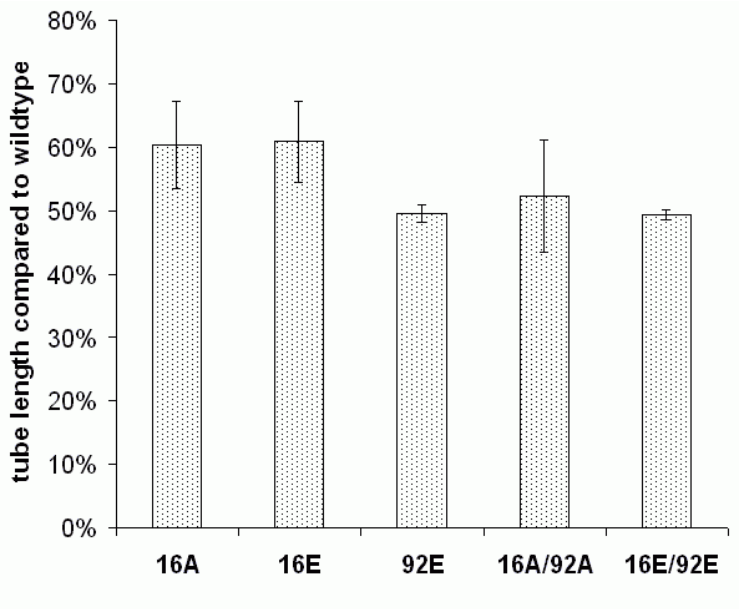
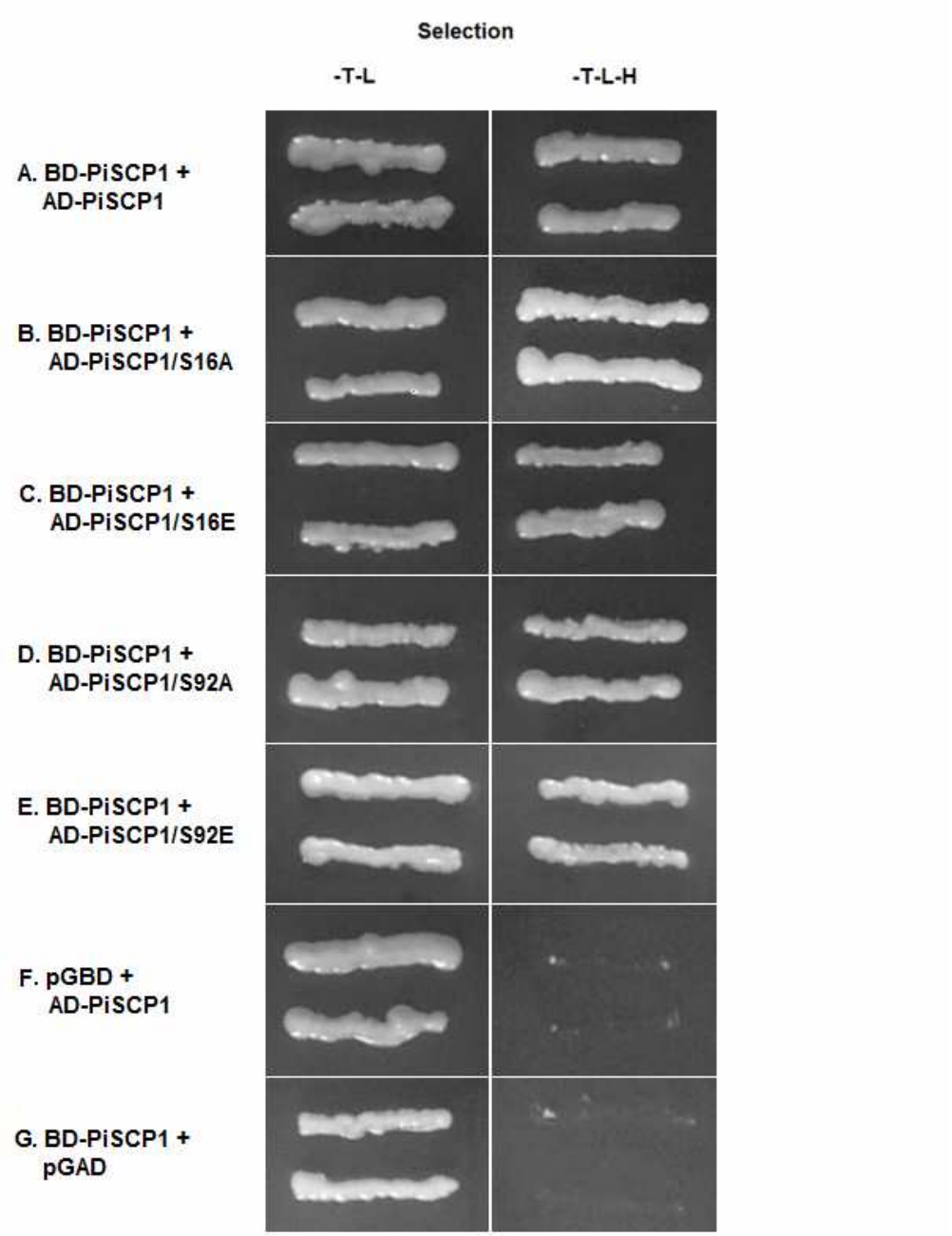


Figure 13. PiSCP1 interacts with wild type PiSCP1 and non-phosphorylatable and phospho-mimetic mutants of PiSCP1 in yeast two hybrid assay



CHAPTER 4

Conclusions and future directions

Conclusions

Endocytosis is essential for the survival of plants, and is especially important to the functions of certain specialized cell types such as guard cells, root hairs and pollen tubes. In pollen tubes, endocytosis is responsible for balancing membrane expansion, deactivating signaling molecules and recycling material back to plasma membrane (Samaj et al., 2006). With more vital dyes and molecular markers are available, endocytosis research is bound to provide us valuable insights for pollen tube growth. Peroxisome research has also recently generated some exciting discoveries. Peroxisomes may give us valuable insights concerning female-male gametophyte interactions. As *amc* and *cts* peroxisome mutants have shown, the presence of some critical factors on either the male or female side is sufficient for successful pollen tube growth and fertilization, the capacity of peroxisomes to generate diffusible NO signal make this a particularly interesting area of study (Footitt et al., 2007; Boisson-Dernier et al., 2008). The identification of myosin isoforms that have peroxisomes as their cargo is another interesting observation and disruption of these myosins leads to a reduction of velocity of peroxisome movement and growth inhibition in root hairs (Hashimoto et al., 2005, 2008, Avisar et al., 2008, Peremyslov et al., 2008).

Bioinformatics has also generated a number of interesting insights for us in endocytosis and peroxisome fields. For example, three categories of R-SNARE proteins, Sec22s are assigned to ER trafficking, Ykt6s to ER-Golgi and VAMP7s to the endosomal trafficking using this approach. (Kloepper et al., 2007). Peroxisomes are found to contain

both proteins of eukaryotic and alaph-proteobacteria origins. Striking sequence and functional similarity between the proteins of eukaryotic origin and ER ERAD system proteins have further been identified (Gabaldón et al., 2006). This implies that peroxisomes have closer relationships to ER and mitochondria than simple functional cooperation, and a number of additional observations are in agreement with this notion (Schrader and Yoon, 2007; Neuspiel et al., 2008; Tabak et al., 2008). However, in plants our understanding of endocytosis and peroxisomes at molecular level is rather scant. The research presented in this dissertation provides some molecular details of protein factors involved in endocytosis and peroxisome and their role in pollen tube growth.

PiVAMP721 belongs to the longin family of SNARE proteins which was only recognized recently (Filippini et al., 2001). The two most well-studied longins are human VAMP7 and yeast Ykt6 (Martinez-Arca et al., 2000; Fukasawa et al., 2004). Based on these studies, we divided PiVAMP721 into PiVAMP721/N which is the longin domain and PiVAMP721/C which is the SNARE and membrane domain. Over-expression results suggested that the full-length protein is slightly inhibitory to pollen tube growth, PiVAMP721/C is highly inhibitory apparently as a result of excessive membrane fusion within the tube tip, while PiVAMP721/N has no discernable effect. We further showed that the N domain is capable of rescuing inhibition caused by expressing PiVAMP721/C. This suggests that the full-length protein behaves in a balanced, regulated fashion with PiVAMP721/N negatively regulating the PiVAMP721/C function, and the PiVAMP721/C regions functions in a constitutively active way. We also showed that the endocytic marker FM4-64 colocalizes with PiVAMP721. This is consistent with the notion that PiVAMP721 functions in an endocytic pathway. Perhaps most significantly a

fluorescent protein marker targeted to exocytic vesicles was also found to colocalize with both FM 4-64 and PiVAMP721 to membrane sacs in the pollen tube tips which were caused by PiVAMP721 over-expression. These sacs most likely represent expansion of membrane enclosed compartments of the TGN, providing that there is a close relationship between endocytosis and exocytosis as has been proposed elsewhere (de Graaf et al., 2005; Ovecka et al., 2005; Samaj et al., 2006). We hypothesized that PiVAMP721 is localized to endocytic vesicles at the initial steps of endocytosis that is from plasma membrane to early endosome. Some of these vesicles are rapidly recycled and merge with the secretory pathway and some vesicles would eventually fuse into early endosomes or TGN. This endocytic function is important as the volume of membrane secreted is larger than that required for tube extension. Consistent with previous reports, the SNARE domain of PiVAMP721 is involved in mediating membrane fusion while its longin domain negatively regulates this function. Once the longin domain is deleted, the constitutively active protein caused large membrane bound structures to be formed in pollen tube tips, presumably as a result of unregulated, excessive vesicle fusion. These structures not only contain endocytic material but may also material from secretory pathway. Hence our findings so far are consistent with the hypothesis that PiVAMP721 is a key component of the machinery that regulates cognate membrane fusion between endocytic compartments and also endo- and exocytic vesicles and our results provide support for a rapid recycling mechanism for endocytic vesicles operating at the tube tip.

PiSCP1 is a novel protein and its homologs in *Arabidopsis* have no known function, although some analogy to human synapsin has been found. We showed it is a peroxisome protein by colocalization with a peroxisome marker. Over-expression of

PiSCP1 also did not generate a significant phenotype. However, PiSCP1 interacted with CDPKs in yeast two hybrid assays and mutation of either of two serine residues, which are potential targets of CDPK activity, caused this protein to substantially inhibit pollen tube growth. This suggests that phosphorylation of either of these two residues may be important to the function PiSCP1. We further characterized PiSCP1 function by dividing it into three parts based sequence conservation to the *Arabidopsis* homologues and potential homology with functional domains of mammalian synapsin. The N- and C-terminal, PiSCP1 A and C domains, retained the ability the localize to peroxisomes and may still retain the ability to interact with CDPK as indicated by the ability to rescue a loss of polarity phenotype caused by over-expressing PiCDPK1 in vivo. The central B domain alone, in contrast appears not to have a function in localization or interaction with CDPK. Based on these observations, we hypothesized that PiSCP1 is a peroxisome protein that may be phosphorylated by multiple calcium-dependent protein kinases at multiple sites. It then channels this signal input from CDPKs into peroxisome and modulate peroxisome function to proper pollen tube growth. Our findings are consistent with this hypothesis but cannot rule out other alternative explanations.

PiCDPK2 is a calcium dependent protein kinase that was identified previously as a pollen specific CDPK involved in pollen tube extension, but not growth polarity (Yoon et al., 2006). Here, we showed that PiCDPK2 is also a peroxisome protein. PiCDPK2 was shown to colocalize with PiSCP1 and interact with it in a yeast two hybrid assay. The function of PiCDPK2 is not clear from over-expression studies (Yoon et al., 2006). We hypothesize that PiCDPK2 is a predominately peroxisomal protein and probably

phoshporylates certain peroxisome proteins including PiSCP1 to coordinates the function of peroxisomes with changing calcium concentrations.

Future directions

Our results are consistent with the hypothesis PiVAMP721 is involved in membrane fusion events involving early endosomes and also early endosomes and exocytic vesicles. However, our FM 4-64 staining can not provide us with definite evidence for the early endosome localization of PiVAMP721. Two options are available to resolve this issue. First, the pulse-chasing FM 4-64 staining employed successfully by Ovecka et al. could provide a better resolution as one can follow the immigration of dyes from PM, early endosome, late endosome to vacuole in this way with less signal background (Ovecka et al., 2005). This being said, differences between root hairs and pollen tubes may prevent the clear identification of early endosome in pollen tubes, as a similar method used by Parton et al. in pollen tubes did not show this information (Parton et al., 2001). Another option is to colocalize PiVAM721 with early endosome markers, but this approach is hindered by the lack of established early endosome markers in pollen tubes. Although Ara6 and Ara7 are established endosome markers, they are mainly used in protoplasts and root hairs and their localization remains to be demonstrated in pollen tubes (Ueda et al., 2001). Preliminary results from our laboratory fail to show clear endosome localization for these markers (Data not shown).

The potential physiological function of PiVAMP721 is also very interesting as endocytosis in pollen tubes growing *in vivo* is likely to be extremely important in pollen-pistil interactions. Endocytic vesicles are likely to contain material from the pistil for energy as pollen tube growth *in vivo* is much faster than *in vitro* (Taylor and Hepler,

1997). But more importantly, endocytosis provides an opportunity for signal molecules or other guidance cues from pistils to enter pollen tubes and in addition a mechanism by which plasma membrane receptors at the pollen tube tip are regulated. Guidance is of vital important to reproductive success for individual pollen tubes. Thus, the elucidation of the contents of pollen tube endocytic vesicles may provide us with insights to the molecular mechanism(s) of this fundamental aspect of pollen biology. By producing stable transgenic plants with tagged PiVAMP721 it may be possible to purify the vesicles to which it localizes. This approach is certainly worthy to be further pursuit although a potential caveat is transgenic lines may be male sterile. An alternative approach would be to use immunoprecipitation to investigate the contents of these vesicles and the interacting partners of PiVAMP721. Potential interacting factors which mighty be identified through this method include Q-SNAREs like syntaxins, Rab GTPase and tethering proteins.

Although we have shown that both PiCDPK2 and PiSCP1 are peroxisome proteins, the sorting signals for these two proteins remain to be identified. We showed both A and C domains of PiSCP1 are still localized to peroxisome, further deletion studies should provide clear picture of the essential sorting signal within these two domains. For PiCDPK2, we know that N-terminal lipid modification is necessary but not sufficient for its localization, deletion studies with the N-terminus fused with various deletions of PiCDPK2 should provide information as to which part of PiCDPK2 contains the information for peroxisome sorting. Colocalization of PiSCP1 and PiCDPK2 with markers for other compartments such as the ER may provide information to the identity of the “overflow” signal.

Although we have shown that PiCDPK2 colocalized with PiSCP1 and interacted in a yeast two hybrid assay, the functional consequences of this potential interaction are unclear and require further exploration. Measuring peroxisome velocity using confocal microscopy is an option to explore the potential subtle peroxisome movement changes. This method has recently been used successfully by Peremyslov et al. in their demonstration of peroxisome movement changes caused by disruption of myosin isoforms (Peremyslov et al., 2008).

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