RESEARCH PAPER

The pollen-specific R-SNARE/longin PiVAMP726 mediates fusion of endo- and exocytic compartments in pollen tube tip growth

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Abstract

The growing pollen tube apex is dedicated to balancing exo- and endocytic processes to form a rapidly extending tube. As perturbation of either tends to cause a morphological phenotype, this system provides tractable model for studying these processes. Vesicle-associated membrane protein 7s (VAMP7s) are members of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family that mediate cognate membrane fusion but their role in pollen tube growth has not been investigated. This manuscript identifies PiVAMP726 of Petunia inflata as a pollen-specific VAMP7 that localizes to the inverted cone of transport vesicles at the pollen tube tip. The endocytic marker FM4-64 was found to colocalize with yellow fluorescent protein (YFP)-PiVAMP726, which is consistent with PiVAMP726 containing an amino-acid motif implicated in endosomal localization. At high overexpression levels, YFP- PiVAMP726 inhibited growth and caused the formation of novel membrane compartments within the pollen tube tip. Functional dissection of PiVAMP726 implicated the N-terminal longin domain in negative regulation of the SNARE activity, but not localization of PiVAMP726. Expression of the constitutively active C-terminal SNARE domain alone, in pollen tubes, generated similar phenotypes to the full-length protein, but the truncated domain was more potent than the wild-type protein at both inhibiting growth and forming the novel membrane compartments. Both endo- and exocytic markers localized to these compartments in addition to YFP-PiVAMP726, leading to the speculation that PiVAMP726 might be involved in the recycling of endocytic vesicles in tip growth.

Key words: Endocytosis, pollen tube growth, SNARE, VAMP7.

Introduction

In angiosperms, to achieve internal fertilization the sperm cells contained with pollen grains must be transmitted a considerable distance from the receptive stigma surface to the ovules. This task is facilitated through the growth of pollen tubes that grow rapidly from the pollen grains and extend through the pistil, directed by cues from various female tissues (Cheung et al., 1995; Johnson and Preuss, 2002; Palanivelu et al., 2003; Okuda et al., 2009). These tubes extend by a process known as tip growth – a highly polarized process in which vesicles containing cell-wall materials are delivered to the tube apex establishing a restricted growth zone (Derksen et al., 1995; Moscatelli and Idilli, 2009). Regulation of this growth is complex and a large number of factors, including Rop/Rac GTPase (Li et al., 1999; Fu et al., 2001; Cheung and Wu, 2008), Ca²⁺ (Miller et al., 1992; Pierson et al., 1994) calcium-dependent protein kinase (Yoon et al., 2006; Myers et al., 2009), F-actin (Steer and Steer, 1989; Derksen et al., 1995; Gu et al., 2005), and phospholipase C (Dowd et al., 2006; Helling et al., 2006), have been implicated in the process. A characteristic V-shaped cone of transport vesicles, which points back towards the pollen grain, is found at the...
tip of growing pollen tubes (Lancellle and Hepler, 1992; Derksen et al., 1995). Despite being occupied by a high density of transport vesicles, this region is devoid of large organelles and has been termed the ‘clear zone’ (Derksen et al., 1995). This cone is a result of reverse-fountain cytoplasmic streaming, where the vesicles are transported to the apex along the tube flanks, accumulate in an annulus-shaped region adjacent to the extreme apex, and then turn back and flow rearward through the centre (Bove et al., 2008). Fusion of vesicles with the plasma membrane appears to be restricted to this annulus-shaped region, although more than one passage through the apex may be needed to achieve fusion (Bove et al., 2008).

Interestingly, the rate of secretion delivers several-fold more membrane to the growing apex than is required for tube extension, suggesting that retrieval and recycling regulates the membrane economy of growing pollen tubes (Steer and Steer, 1989; Derksen et al., 1995). The use of styryl FM dyes, which are taken up by endocytosis and progressively label internal membrane compartments, have made it possible to dissect endo- and exocytic pathways in vivo, revealing that both secretory and endocytic vesicles contribute to the V-shaped apical formation and suggesting that most of the internalized membrane is redirected to the secretory pathway (Parton et al., 2001; Camacho and Mahiö, 2003; Bolte et al., 2004; Moscatelli et al., 2007; Bove et al. 2008; Zonia and Munnik, 2008). Further, fluorescent recovery after photobleaching analyses with FM dyes have revealed that exocytosis does not involve the entire apical domain, being absent within the annular region of membrane fusion, which is consistent with endocytosis occurring at the extreme apex (Bove et al., 2008; Zonia and Munnik, 2008; Moscatelli and Idilli, 2009).

In recent years, it has become clear that it is more appropriate to regard endo- and exocytosis as cyclic processes where vesicles are continuously formed, released, transported, docked, fused, and recycled, rather than independent processes (Battey et al., 1999; Bonifacino and Glick, 2004; Samaj et al., 2005; Viotti et al., 2010). Endocytic pathways intersect the secretory and biosynthetic processes during recycling to the PM and protein transport to the PM (Sutter et al., 2006a; Viotti et al., 2010). Based on colocalization of the trans-Golgi network (TGN) and endocytosed PM markers, there is now compelling evidence that the TGN or a subdomain of it acts as an early endosome in plant cells (Dettmer et al., 2006; Lam et al., 2007; Chow et al., 2008; Viotti et al., 2010). In pollen tubes, the exact nature and morphology of early and late endosomes remains elusive (Samaj et al., 2006), but the existence of a ‘rapid recycling’ mechanism, such that endocytic vesicles are directly re-employed in secretion, has been hypothesized to operate at the tube tip (Monteiro et al., 2005; Samaj et al., 2006).

There is surprisingly little information available concerning the molecules that mediate membrane fusion events in tip-growing plant cells, in particular SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors). SNAREs are usually small proteins (150–300 amino acids), possessing a membrane-anchored C-terminus and a cytosolic SNARE motif that functions in membrane fusion. The proposal that specific classes of SNAREs are sufficient to mediate and guide the specificity of membrane fusion events (Söllner et al., 1993) has gained substantial empirical support (see Södhof and Rothman, 2009 for review). Although it is reasonable to assume that defects in SNARE function would affect tip growth, the only SNAREs reported to exhibit defective pollen function are members of the syntaxin family. Syp2 and Syp4 SNARE subfamilies of syntaxin-like proteins are thought to participate in vesicle trafficking between the vacuole and the TGN (Sanderfoot et al., 2001) and recently it was reported that Arabidopsis SYP124, which is pollen specific, mediates exocytic membrane fusion at the pollen tube apex (Silva et al., 2010).

Traditionally SNAREs have been classified as v- or t-SNAREs, depending on their localization to either transport vesicles (v) or target (t) membranes (Fasshauer et al., 1998). More recently SNAREs have been subdivided into Q- and R-SNAREs based on the presence of a conserved glutamine or arginine polar residue, respectively, at the position of the so-called ‘zero’ ionic layer of the four-helix bundle. Although generally Q-SNAREs are t-SNAREs and R-SNAREs are v-SNAREs, this classification is misleading as these proteins can also mediate homotypic membrane fusion (Bock et al., 2001). 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, regulatory ‘longin domain’ of 120–140 amino acids at their N-terminus (Filippini et al., 2001). Longins are essentially brevins with an extended N-terminal domain implicated in the regulation of membrane fusion events (Martinez-Arca et al., 2003). Brevins have been extensively characterized in synaptic systems, but longins, which are subdivided into VAMP7-like, Sec22-like, and Ykt6-like, have been recognized relatively recently (Rossi et al., 2004a). Intriguingly, plants do not possess brevins but possess an expanded family of longins. For example, Arabidopsis has 11 VAMP7-like longins, whereas the human genome has only one of each class.

This manuscript reports the identification and characterization of a VAMP7-longin class SNARE gene expressed specifically in Petunia inflata pollen (PiVAMP726). The PiVAMP726 protein was shown to localize to transport vesicles in the apical dome of pollen tubes. Dissection of the functional domains of PiVAMP726 provided evidence suggesting that its SNARE domain-containing C-terminus mediates fusion between transport vesicles at the pollen tube tip, while the N-terminus longin domain negatively regulates this function. Interestingly, endo- and exocytic markers are colocalized to novel membrane compartments induced by PiVAMP726 overexpression, suggesting that, at least when overexpressed, this protein mediates fusion between endocytic and secretory vesicles. This data is consistent with a recently proposed model of rapid vesicle recycling in pollen tubes (Samaj et al., 2006) leading to the hypothesis that PiVAMP726 might play a role in mediating vesicle fusion and recycling at the pollen tube tip.
Materials and methods
Sequence analysis
Nucleic acid and protein databases searches were performed using BLASTP and BLASTN algorithms. Sequences were obtained from GenBank through NCBI (http://www.ncbi.nlm.nih.gov). A Prosite motif scan was performed through ExPasy PROSITE (http://us.expasy.org/prosite). The GenBank accession number for PiVAMP726 is HM535664.

Reverse-transcription PCR analysis
Total RNA was extracted from 100 μg P. inflata tissues using Concert reagent (Invitrogen) according to the manufacturer’s instructions. RNA pellets were dissolved in 50 μl RNase-free water, and quantified spectrophotometrically. RNA was converted to cDNA using an oligo-dT primer and Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Aliquots (2 μl) of each reverse-transcription reaction was used as template in a 50 μl PCR reaction volume with gene-specific primers (F, 5′-ATGGGGAACAAACGTTGA-3′; R, 5′-GGACCGAAGTTGAAACAC-3′). Amplifications were performed with the cycling program of 25 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 2 min. A P. inflata actin gene (GenBank accession number JQ012917) was used as a control for loading, using primers Actin-F (5′-ATGGCTGAAGTTGAG-3′) and Actin-R (5′-TGGAGGATATCCACGTTC-3′) and the same cycling parameters. Each reaction was analysed by agarose gel electrophoresis and stained with ethidium bromide before visualization under UV illumination.

Construction of plasmids for pollen expression
All constructs were generated by PCR, cloned into pGEM-T Easy vector (Promega, Madison WI, USA), and verified by DNA sequencing, prior to cloning into vectors for expression in pollen. NeoI and EcoRI sites within the coding region of PiVAMP726 were removed by PCR to synonymously change amino acid codons. The resulting coding region of PiVAMP726 was then converted to encode the restriction sites for PvuII at the 3′ end and SacI at the 5′ end using primers LO/PVUII (5′-CAGCTGATGGGCAACAAACGTTGA-3′) and LO/NT-R (5′-TTTTTCTCGAGATGTCACAC-3′). The N-terminal longin domain (PiVAMP726/LD) fragment was generated by LO/PVUII (5′-CAGCTGATGGGCAACAAACGTTG-3′) and LO/NT-R (5′-TTTTTCTCGAGATGTCACAC-3′). The C-terminal domain fragment (PiVAMP726/SD) was generated with LODNT (5′-CAGCTGATGGGCAACAAACGTTG-3′) and LO/NT-R (5′-TTTTTCTCGAGATGTCACAC-3′).

Pollen expression constructs were generated in pRSET-C (Invitrogen) using the pollen-specific promoter Lat52 (Twell et al., 1989). pRSET-C Lat52-yellow fluorescent protein (YFP) was generated by cloning the coding region of YFP (Clontech) behind the Lat52 promoter in pRSET-C Lat52GUSNos. All fluorescent fusion constructs were subsequently generated by cloning appropriate fragments into the Pvu II and Sac I sites to generate in frame fusions. Lat52-invertase-cyan fluorescent protein (CFP) encoding region from Lat52-invertase-GFP (de Graaf et al., 2005) with that of CFP. All constructs were verified by sequencing.

Transient expression in pollen
Transient expression of fluorescent protein fusion constructs in pollen were performed as previously described (Yoon et al., 2006). Briefly, P. inflata pollen was collected from freshly dehisced anthers (10 flowers/bombardment), and suspended by gentle vortexing in 200 μl pollen germination medium (PGM) containing 0.01% (w/v) H3BO3, 0.02% (w/v) MgSO4, 0.07% (w/v) CaCl2, 15% (w/v) polyethylene glycol-4000, 2% (w/v) sucrose, and 20 mM MES (pH 6.0). The pollen-containing PGM were then transferred to a 2.5-cm2 piece of positively charged nylon membrane on top of a pre-wetted Whitman filter in a 9-cm diameter Petri dish.

Microprojectile bombardment was performed using a PDS-1000/He biolistic system (Bio-Rad). Gold particles (1.1 μm) were prepared according to the manufacturer’s protocol using 1 μg plasmid DNA/0.5 mg of particles. Co-bombardment was achieved by coating particle with 1 μg of each plasmid construct (unless otherwise indicated). Bombardments were performed in a 25 inch Hg chamber vacuum, using an 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 3–4 h at room temperature. FM 4-64 staining was performed according to Parton et al. (2001) as adapted for P. inflata by Dowd et al. (2006). 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 visualization was performed using an Orthomart epifluorescence microscope (Leitz) with a ×40, 0.7 numerical aperture, dry 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 a Bio-Rad MRC 600 or a Zeiss 510 laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) with the following parameters: for GFP, 488 nm excitation, 515–565 nm emission filter; for FM 4-64, 543 nm excitation, 560 nm long-pass emission filter; for YFP, 514 nm excitation and 530–560 nm band-pass emission filter; and for CFP, 405 nm excitation and 450–490 nm band-pass emission filter. Colocalization studies were performed using the multumode function of the Zeiss 510 meta confocal microscope and the settings described above. Confocal images were analysed using Metamorph v. 4.5 (Molecular Devices, Downington, PA, USA) and processed using Adobe Photoshop v. 5.5 (Adobe Systems, San Jose, CA, USA). Pollen tube lengths were measured manually using printed photomicrographs. For each experiment, a minimum of 50 independent transformants were assessed to derive consensus phenotypes.

Results
Identification of PiVAMP726
A full-length cDNA clone of PiVAMP726 was initially identified as a false positive in the course of a yeast 2-hybrid pollen cDNA library screen. A BLAST-P search of the translated cDNA sequence revealed that this clone encoded a full-length 220-amino-acid vesicle-associated membrane protein 7 (VAMP7) homologue, here termed P. inflata VAMP726 (PiVAMP726). Sequence analysis showed that the translated gene product contain all the characteristics of a VAMP7 family member: an N-terminal longin domain (PS50859); a coiled-coil v-SNARE domain with arginine at the ‘zero’ layer (PS50892); and a C-terminal transmembrane domain separated from the SNARe domain by a cluster of basic amino acids.

A BLAST-P search against Arabidopsis thaliana identified AtVAMP726 (E value 1e-137) as the most similar of the 11 Arabidopsis VAMP7-like longins to PiVAMP726. The VAMP72 derivatives to which this gene belongs are unique
to plants, as opposed to the VAMP71 class which are also found in animals (Sanderfoot, 2007). AtVAMP711–713 have previously been reported to localize to the vacuolar membrane, whereas AtVAMP726–727 localize largely to the plasma membrane, with the exceptions of AtVAMP723 and AtVAMP727 which localize to the ER and endosome, respectively (Uemura et al., 2004). As these localizations of Arabidopsis VAMP7s were investigated by transient expression of GFP fusions constructs in Arabidopsis leaf protoplasts, their localization and function(s) in specific or more specialized cell types such as pollen tubes is largely speculative.

Expression and localization of PiVAMP726

Using semi-quantitative reverse-transcription PCR, this study investigated the expression profile of PiVAMP726 through pollen development and examined whether it was expressed in other tissues. PiVAMP726 mRNA was first detectable in anthers from 10 mm buds (which contain microspores entering pollen mitosis I), increases to pollen maturity, and remains high after in vitro pollen germination. Expression was not detected in other floral organs or in vegetative tissues assayed (Fig. 1), hence expression of PiVAMP726 is most likely pollen specific. Notably, AtVAMP726 (GenBank accession At1g04760), the Arabidopsis VAMP7 with highest sequence similarity to PiVAMP726, has been classified as pollen specific or pollen enriched in genome wide analyses (Honys and Twell, 2003; Pina et al., 2005), suggesting that AtVAMP726 may be the functional homologue of PiVAMP726 in A. thaliana.

PiVAMP726 localization

N-terminal fluorescent protein fusions of VAMP7-like proteins have previously been shown to fluoresce and retain functionality (Martinez-Alca et al., 2000) and have been used to investigate VAMP7 localization in plants (Uemura et al., 2004, 2005). Hence, to investigate the cellular localization of PiVAMP726, the present study generated an N-terminal YFP-PiVAMP726 fusion construct for expression in pollen tubes. Transient transformation by biolistic bombardment has been previously used to study localization of VAMP7s (Uemura et al., 2004), as well as widely employed to study protein localization (including SNAREs) in pollen tubes (for example, see de Graaf et al., 2005; Dowd et al., 2006; Yoon et al., 2006; Silva et al., 2010). Although this approach does not afford direct assessment of transgene transcript levels, protein expression levels can be crudely assessed by the fluorescence of fusion proteins and manipulated by controlling the quantity of DNA used to coat gold particles (Dowd et al., 2006) as well as manipulating the length of time between bombardment and DNA analysis. A substantial benefit of this approach is that it facilitates the rapid generation of a large number of independent transformants with which to assess phenotypic change.

Analysis of pollen tubes transiently expressing YFP-PiVAMP726 at low levels (as judged by fusion protein fluorescence) revealed that the fluorescence signal was visible primarily as an inverted V-shaped cone at the tube tips (Fig. 2A, B). R-SNAREs are membrane-anchored v-SNAREs and the amino acid sequence of PiVAMP726 encodes a C-terminal transmembrane domain typical of this class, which is consistent with YFP-PiVAMP726 localizing to the membrane of the previously reported inverted cone of transport vesicles cone centred on the growing tip (Parton Fig. 1. Reverse-transcription PCR analysis of PiVAMP726 expression. Developmental expression of PiVAMP7 through pollen development and floral tissues as indicated. Expression of a Petunia inflata actin gene (GenBank accession JQ012917) is shown as a control.

Fig. 2. Localization of YFP-PiVAMP726 in pollen tubes. (A,B) Fluorescence micrograph (A) of a transformed pollen tube expressing low levels of YFP-PiVAMP726 (1 μg plasmid DNA/transformation) and the light image (B): the fluorescent signal labels the inverted V-shaped cones of vesicles at the pollen tube tip. (C–E) Fluorescence images of pollen tubes expressing higher levels of YFP-PiVAMP726, within which larger membrane compartments are visible largely in the pollen tube tips (3 μg plasmid DNA/transformation). Bars = 5 μm.
et al., 2001; Camacho and Mahló, 2003; Dowd et al., 2006). Although at low expression levels YFP-PiVAMP726 localized only to structures below the limit of resolution of confocal microscopy, at higher expression levels (as indicated by fusion protein fluorescence) YFP-PiVAMP726 was associated with larger compartments, primarily at the tube apices (and sometimes extending down the tube shank) (Fig. 2C–E). The presence of these larger compartments was associated with inhibition of growth (mean ± SD tube length 60 ± 4.7% relative to the wild type) and at low frequency of bulging of the pollen tube tips (11 ± 1.3% of transformants), bulging being defined as the tube diameter in the tip region being >120% of that in the tube shank). Although growth in pollen tubes exhibiting this phenotype was inhibited, they maintained viability as active cytoplasmic streaming was observed even in severely inhibited tubes. Given the established role of SNARE proteins in membrane fusion, a likely explanation of this phenotype is that high-level overexpression of PiVAMP726 promoted membrane fusion beyond endogenous rates, leading to the formation of larger membrane compartments observed. The tip bulging phenotype observed was subtle relative to previously reported phenotypes associated with loss of polarity (e.g. Li et al., 1999; Dowd et al., 2006; Yoon et al., 2006) and is more reminiscent of that associated with perturbing vesicle trafficking by overexpressing Rab11b GTPase (de Graaf et al., 2005; Zhang and McCormick, 2010). As a result, it is speculated that the disturbance in morphology is related more to effects on the rate of extension or possibly osmoregulation than the regulation of polarity per se.

A potential localization-indicating region has been identified between the +4 and +6 layers of the SNARE domain of Arabidopsis VAMP7s (Uemura et al., 2004). This region of PiVAMP726 bears the motif RSQAQD, suggested to be indicative of plasma-membrane/endosome localization (Uemura et al., 2004). To empirically assess whether the vesicles to which YFP-PiVAMP726 were endocytic in nature, the marker FM 4-64 was used. 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 membranes and are widely used as endocytic tracers (Vida and Emr, 1995; Emans et al., 2002). Externally applied FM dyes gradually start to fluoresce when they bind to the plasma membrane and are internalized via energy-dependent endocytosis, eventually reaching the vacuole (Parton et al., 2001). In pollen tubes, FM 4-64 initially labels the plasma membrane and the inverted cone of transport vesicles centred on the growing tip (Parton et al., 2001, Dowd et al., 2006; Zonia and Munnik, 2008). These vesicles are believed to represent an endocytic pool associated with polar extension of the growing tip (Parton et al., 2001; de Graaf et al., 2005; Monteiro et al., 2005; Dowd et al., 2006; Moscatelli and Idili, 2009).

The images obtained using FM 4-64 were consistent with those previously published for P. inflata pollen tubes (Dowd et al., 2006), fluorescence from this dye being observed at the plasma membrane and the inverted apical cone of transport vesicles. Merging the fluorescent signals of FM 4-64 and YFP-PiVAMP726 demonstrated that they overlap extensively in the apical cone (Fig. 3A), but in contrast to

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**Fig. 3.** Colocalization of FM-4-64 with YFP fluorescence in transformed pollen tubes expressing YFP-tagged PiVAMP726 constructs. (A) Colocalization of FM4-64 and YFP-PiVAMP726: YFP and FM4-64 signals are largely colocalized, especially in the V-shaped apical region. (B) Colocalization of FM4-64 and YFP-PiVAMP726/SD: again YFP and FM4-64 signals largely colocalize, and both FM4-64 and YFP-PiVAMP726/SD labelled the membrane aggregations caused by YFP-PiVAMP726/SD expression. (C) FM 4-64 staining of pollen tube co-transformed with both PiVAMP726/LD and YFP-PiVAMP726/SD. This double transformant has a YFP-PiVAMP726-like phenotype (A), i.e. the YFP-PiVAMP726/LD protein appears to rescue the effects of YFP-PiVAMP726/SD expression. Green, YFP signal; red, FM-64 signal; yellow, overlapping YFP and FM-64 signals. All treatments employed 1 µg plasmid DNA/transformation. Images were generated by confocal microscopy. Bar = 5 µm.
FM-4-64, YFP-PiVAMP726 did not appreciably label the plasma membrane. FM 4-64 also labelled the larger compartments observed in transformants expressing higher levels of YFP-PiVAMP726 (data not shown) and did so only in pollen tubes expressing high levels, confirming colocalization and suggesting that that these compartments were formed de novo as a result of overexpression, rather than signifying altered localization of PiVAMP726.

The presence of the endosome-targeting motif RSQAQD combined with colocalization of PiVAMP726 with an endocytic marker suggests that this protein is involved in events commonly referred to as endocytosis. However, given the cyclic nature of vesicle trafficking as well as the fact that exocytic vesicles are also present in this zone, it was not possible to discount the involvement of PiVAMP726 in secretion.

Functional dissection of PiVAMP726

Although the role of VAMP7s has not previously been investigated in plant cells exhibiting tip growth, human VAMP7/Ti-VAMP has received considerable attention in regard to tip growth in neurons. Overexpression of full-length VAMP7 does not cause a discernable phenotype; however, when its functional domains are expressed separately, the N-terminal longin domain (LD) exhibits an inhibitory/regulatory function in neurite extension by tip growth, whereas the C-terminal, constitutively active SNARE domain (SD) promotes outgrowth (Martinez-Arca et al., 2000, 2003). LDs have also been proposed to play a role in targeting in both mammals and plants (Martinez-Arca et al., 2003; Uemura et al., 2005). Using this information, the present study designed experiments to assess the functions of PiVAMP726 longin and SNARE domains in pollen tubes.

Pollen expression constructs were generated, encoding amino acids 1–127 (YFP-PiVAMP726/LD) and 106–220 (YFP-PiVAMP726/SD) of PiVAMP726, each fused N-terminally to YFP (Fig. 4). Each construct was transiently expressed separately in pollen tubes. The YFP-fused longin domain of PiVAMP726 (YFP-PiVAMP726/LD) did not discernibly affect pollen tube morphology (Fig. 5A–C), but inhibited extension slightly (tube length 86±4.8% relative to the wild type). Fluorescence from the YFP-PiVAMP726/LD fusion protein was visible throughout the cytosol in a similar manner to soluble YFP controls and did not exhibit discernable localization.

In contrast to the longin domain, expression of the YFP-fused SNARE domain of PiVAMP726 (YFP-PiVAMP726/SD) led to a pronounced phenotype (Fig. 5D–F). Pollen tube extension was severely inhibited (tube length 48±1.2% of the wild type). Infrequently, apparently at very low expression levels, a tip-focused internal membrane localization was still observed, but 91% of transformants exhibited large YFP-labelled compartments within the pollen tube tip, often to the extent that the apical region was filled by a single large membrane sac. Bulging pollen tube tips were also observed in 36±5.1% of transformed tubes. FM 4-64 was determined to colocalize with YFP-PiVAMP726/SD to these large subapical membrane sacs (Fig. 3B), suggesting that they are derived at least in part from endocytic vesicles. Although the internal membrane system appeared to be greatly disturbed in tubes expressing YFP-PiVAMP726/SD, and growth was inhibited, these tubes remained viable as active cytoplasmic streaming was still observed even in severely inhibited pollen tubes. These results are consistent with the C-terminal SNARE domain being constitutively active and promoting vesicle fusion as predicted, but did not provide strong evidence for a negative regulatory function for the longin domain, because PiVAMP726/LD only a slightly inhibited tube growth.

An additional experiment was designed to further assess whether the PiVAMP726 longin domain played a negative regulatory role in membrane fusion. In this experiment, YFP-PiVAMP726/LD was co-expressed with YFP-PiVAMP726/SD to determine whether the longin domain could rescue the inhibitory effect of the constitutively active SNARE domain. When the two domains were co-expressed, the majority of transformed tubes exhibited a phenotype similar to full-length YFP-PiVAMP726 transformants, i.e. a tip-focused inverted cone of fluorescence along with some small membrane sacs. Large subapical membrane sacs were rare, the bulging tips typical of YFP-PiVAMP726/SD transformants were not observed (Fig. 3C), and mean tube length was 73±4.6% of wild-type tubes. Phenotypic variation was observed however; some tubes appeared more like YFP-PiVAMP726/LD transformants and some more like YFP-PiVAMP726/SD transformants. This study reasoned that this was likely caused by variations in the ratio of YFP-PiVAMP726/LD:YFP-PiVAMP726/SD expressed in particular transformants. To investigate this possibility, the experiment was repeated, co-transforming the plasmids encoding the two domains in different ratios. As can be seen in Fig. 6, both the growth inhibition and tip

![Fig. 4. Domain structure of PiVAMP726 and truncated constructs used for pollen transformation: (A) YFP-full-length PiVAMP726; (B) YFP-PiVAMP726/LD (longin domain); (C) YFP- PiVAMP726/SD (SNARE domain).](https://academic.oup.com/jxb/article-abstract/63/8/3083/729771)
bulging phenotypes associated with YFP-PiVAMP726/SD expression were progressively reduced as the ratio was skewed in favour of YFP-PiVAMP726/LD. This phenotypic rescue was not caused by a reduction in YFP-PiVAMP726/SD expression as a consequence of expressing both constructs on the same promoter, as co-expression with other Lat52 promoter driven constructs (such as invertase-CFP; Fig. 7) did cause this affect. Overall, these results suggest that the LD of PiVAMP726 negatively regulated the membrane-fusing capability of the SD, as it alleviated growth inhibition, prevented formation of the large membrane compartments, and reduced the incidence of bulging tips associated with overexpression of the C-terminal SNARE domain alone. Hence, even when expressed separately, a functional regulatory interaction occurs between the longin and SNARE domains. Significantly, the ability of the LD to titrate the membrane fusion phenotype of the SD also suggests that the phenotypes observed are related to the biochemical activity of the proteins rather than being an artefact caused by overexpression.

PiVAMP726-mediated membrane compartments trap secretory proteins

The data presented suggest that PiVAMP726 is likely to be involved in regulating fusion of transport vesicles at the pollen tube tip. As PiVAMP726 colocalized with FM 4-64 and possesses a RSQAQD motif, the vesicles to which they localize are likely to be endocytic in origin; however, whether PiVAMP726 mediates homotypic fusion, fusion of endocytic

Fig. 5. Phenotypes of pollen tubes expressing YFP-PiVAMP726/LD or YFP-PiVAMP726/SD constructs. (A–C) Pollen tubes expressing YFP-PiVAMP726/LD: YFP fluorescence was not localized and was visible throughout the pollen tube cytoplasm. (D–F) YFP-PiVAMP726/SD expressing pollen tubes: YFP fluorescence was localized to internal membrane compartments; at high expression levels (as judged by fluorescence intensity) the YFP-labelled compartment dramatically increased in size, at times occluding the pollen tube tip and was associated with inhibition of tube extension and swelling of tube tips. All images were generated by confocal microscopy. All treatments employed 1 μg plasmid DNA transformation. Bars = 5 μm.

Fig. 6. Effects of different ratios of PiVAMP726/LD and PiVAMP726/SD co-expression on pollen tube growth. (A) Proportion of pollen tube length relative to the wild type. (B) Proportion of bulging pollen tube tips relative to the wild type. Pollen was cultured in vitro for 4 h after bombardment and at least 50 transformants expressing each construct were assessed. X axes indicate DNA ratios used to coat gold particles for bombardment: C, 3 μg YFP-PiVAMP726/SD; 1N3C, 1 μg PiVAMP726/LD and 3 μg YFP-PiVAMP726/SD; F, 3 μg YFP-PiVAMP726; 1N1C, 1 μg PiVAMP726/LD and 1 μg YFP-PiVAMP726/SD; 3N1C, 1 μg PiVAMP726/LD and 3 μg YFP-PiVAMP726/SD; N, 3 μg YFP-PiVAMP726/LD. Error bars represent ±1 standard deviation.
and endocytic and secretory pathways should contain constituents of both endocytic and secretory pathways. Having demonstrated that FM 4-64 localized to these membrane sacs, this study sought to determine whether a secreted protein marker also localized to these compartments. For this purpose, fluorescent protein-tagged invertase was employed. Invertase is a family of secreted cell-wall enzymes and GFP-tagged invertase has previously been successfully used as a secreted protein marker in pollen tubes (de Graaf et al., 2005). To facilitate independent imaging of invertase and YFP-tagged protein fusions within transformants, the GFP fused to invertase in the expression construct (de Graaf et al., 2005) was changed to CFP. Invertase-CFP, when co-expressed in pollen tubes with soluble YFP (as a control), localized mainly to the cell periphery, which was consistent with the previously established cell-wall localization after secretion (Fig. 7A; de Graaf et al., 2005), as expected fluorescence derived from soluble YFP was seen dispersed throughout the cytoplasm and did not appreciably colocalize with invertase-CFP. In contrast, when invertase-CFP was co-expressed with YFP-PiVAMP726/SD, although a detectable but much-reduced invertase-CFP fluorescence was visible in the cell wall, a substantial portion of the fluorescence from this fusion protein now colocalized with the fluorescence of YFP-PiVAMP726/SD to the membrane compartments induced by expression of the PiVAMP726 SNARE domain (Fig. 7B). This colocalization was observed not only in transformed tubes with extreme phenotypes (i.e. where a single large membrane compartment occluded the entire tip region) but also in transformants within which numerous small fusion compartments had been induced and was also observed in pollen tubes co-expressing invertase-CFP and YFP-PiVAMP726 (data not shown). This finding that both secretory and endocytic markers localized to the novel membrane fusion compartments suggests that, at least in tubes overexpressing this protein, PiVAMP726 mediates fusion events between endo- and exocytic compartments within the pollen tube tip, and hence might play a role in endocytic recycling.

Discussion

The capability of plant cells to perform endocytosis has only been widely accepted recently, as the high turgor pressure and presence of a rigid cell wall had been thought to make endocytosis energetically unfavourable (Saxton and Breidenbach, 1998). However, a wealth of empirical data now supports the hypothesis that, despite the energetic challenge, endocytosis is not only operational but critical to numerous processes in plant cells (Sanderfoot et al., 2000; Geldner, 2004). Pollen tubes offer significant potential as a model for studying endocytosis. Extensive membrane fusion processes are involved in polar growth, and tightly regulated signal transduction is vital to these processes (Camacho and Malhó, 2003; Preuss et al., 2004; Samaj et al., 2004). Indeed, the list of pathways critical to tip growth in pollen tubes is extensive and includes Ca²⁺ signalling, GTPases, phospholipases, and actin dynamics (Camacho and Malhó, 2003; Preuss et al., 2004; Samaj et al., 2004; Dowd et al., 2006). However, the membrane-fusion machinery in pollen tubes is not well characterized and its elucidation is an important prerequisite to studying how signalling pathways are integrated to control polar growth. Although the data presented in this paper were
obtained using transient overexpression, it is physiologically relevant for the following reasons. Transient expression has been widely and successfully used for studying the localization and function of a wide variety of proteins in pollen tubes (e.g. de Graaf et al., 2005; Dowd et al., 2006; Yoon et al., 2006), including SNAREs (Silva et al., 2010), as well as for studying VAMPs in other cell types – in fact, the present methods and constructs are similar to those of Martinez-Arca et al. (2000) in which the function of human Ti-VAMP was first characterized. Further, in this study the longin domain of PiVAMP726 was found to negatively regulate the effects of the SNARE domain, suggesting that the observed phenotypes were not a simply result of miss-localization caused by overexpression.

The R-SNARE identified in this study has a single homologue in yeast and mammals but many in Arabidopsis. There are 14 R-SNAREs (11 VAMP7s, 2 Ykt6s and 1 Sec22) among the 58 SNARE genes identified in the Arabidopsis genome (Sanderfoot et al., 2000; Uemura et al., 2005; Sutter et al., 2006b). Of particular interest, Arabidopsis does not possess brevin-type R-SNAREs but has an expanded family longins relative to mammals. An attractive hypothesis poses that longins are the prototype R-SNAREs and animal brevins have lost the inhibitory longin domain as a result of selective pressure to achieve faster membrane fusion required for neurological transmission events (Rossi et al., 2004b). Given that plants have a family of VAMP7s with different expression patterns and localizations (Uemura et al., 2004), it is conceivable that each particular plant VAMP7 performs a narrow range of the functions performed by human VAMP7, in a situation analogous to that demonstrated for Arabidopsis syntaxins (Sanderfoot et al., 2001).

Two close homologues of PiVAMP726, AtVAMP725 (At2g32670) and AtVAMP726 (At1g04760), have been reported to localize to the plasma membrane and endosomes when expressed in leaf protoplasts (Uemura et al., 2004). As PiVAMP726 is apparently pollen specific and the localization of this SNARE class had not previously been studied in tip-growing cells, its subcellular localization in growing pollen tubes was assessed. At low expression levels, YFP-PiVAMP726 was visible as a fluorescent haze localized to an inverted cone at the pollen tube tip. Given the established association of R-SNAREs with vesicle membranes and the presence of a C-terminal transmembrane domain, this fluorescence is most likely associated with membrane compartments beyond the resolution of light microscopy, and the pattern observed is consistent with these being transport vesicles. PiVAMP726 possesses the RSQAQD motif proposed to be indicative of plasma-membrane/endosome localization (Uemura et al., 2004) and colocalizes with the endocytic marker FM 4-64 in the inverted apical cone within minutes of dye application, which suggests that PiVAMP726 localized to vesicles traditionally regarded as endocytic in nature. In contrast to the results of Uemura et al. (2004), little if any plasma-membrane localization was observed in this study. This may reflect differences in physiology between leaf protoplasts and pollen tubes, endocytosis being considerably more active in the latter cell type.

High levels of YFP-PiVAMP726 overexpression resulted in the formation of novel membrane compartments readily discernable by light microscopy to which the fusion protein localized. This study hypothesized that this phenotype was a result of increased membrane fusion caused by elevated levels of PiVAMP726. Expressing the SNARE domain alone (YFP-PiVAMP726/SD) supported this hypothesis, as this truncated, constitutively active protein was more potent at generating the novel compartments than the wild-type protein. FM-64 also labelled these compartments, which was consistent with the interpretation that they are formed at least in part from vesicles of an endocytic origin.

The N-terminal LD has been reported to be important in encoding localization signals in both animal and plant VAMP7s (Martinez-Arca et al., 2000; Uemura et al., 2005). In this study however, removal of the LD had no discernable effect on localization. Although YFP-PiVAMP726/SD (SNARE domain alone) was more potent in generating the novel membrane compartments, its localization was not distinguishable from that of the full-length fusion protein (but subtle differences such as localization to a different set of vesicles cannot be ruled out). Further, the YFP-PiVAMP726/SD fusion protein was visible throughout the cytoplasm. Both results are consistent with the SNARE domain possessing all motifs necessary for localization and the longin domain not having a role in this function. Interestingly, this result does not contradict published data. Although the broad statement that longin domains encode the targeting signals of VAMP7s is frequently made, close examination of the literature reveals that this is only the case for family members that localize to compartments other than endosomes. The combined available published data suggest that in the absence of a targeting motif in the longin domain, the endosome is the default localization of these proteins (Kloeper et al., 2007). As a result, for endosomal VAMP7s such as PiVAMP726, the longin domain apparently does not play a significant role in protein localization, which is consistent with the data presented.

Separation and expression of the functional domains of PiVAMP726 lead to phenotypes consistent with their previously reported activities in regard to mediating membrane fusion (Martinez-Arca, 2000): i.e. the C-terminal SD being constitutively active and the LD possessing a negative regulatory function. Expression of the SNARE motif-containing C-terminus of PiVAMP726 alone produced a more severe phenotype than the full-length protein (increased growth inhibition and the occurrence of larger membrane compartments within the tip). Expression of the LD alone, which was hypothesized to have an inhibitory effect on SNARE complex formation, was only mildly inhibitory to tube growth, but was able to titrate the inhibitory effects of overexpressing the SNARE domain alone.

As far as is known, human VAMP7 is the only member of the VAMP7 class that has been investigated in regard to tip growth. Although the antagonistic roles of the LD and SD of PiVAMP726 in regard to membrane fusion are consistent with those reported for human VAMP7, the results presented here suggest significant differences in their
additional roles and indeed in the biological functions of the two proteins. First, the LD of PiVAMP726 does not appear to play a significant role in subcellular localization, and second, the effects of overexpressing the full-length and truncated proteins upon tip growth, are very different. Overexpression of human VAMP7 has no effect upon tip growth. In contrast, high expression levels of PiVAMP726 inhibited tube extension and promoted the formation of membrane sacs in the tube tips. The LD of human VAMP7 severely inhibits neurite extension, whereas the LD of PiVAMP726 only has a mild inhibitory effect upon pollen tube growth. Most notably, expression of the C-terminal SD of human VAMP7 promotes neurite extension, whereas in the case of PiVAMP726, expressing this domain alone severely inhibits pollen tube growth and leads to the formation of large membrane sacs within pollen tubes. These differences are most likely a result of PiVAMP726 and human VAMP7 localizing to different subsets of vesicles and hence mediating different membrane fusion events at the site of polar growth. Human VAMP7 regulates fusion of exocytic vesicle with the plasma membrane, whereas the present results suggest that PiVAMP726 regulates fusion between vesicles within the cytosol.

An important question in assessing the function of PiVAMP726 relates to the identity of the membrane compartments between which it mediates fusion. Given the localization observed, it is reasonable to assume that at least one of these compartments is endosomal in nature, and indeed FM 4-64 was determined to be present in the novel membrane sacs induced by PiVAMP726 overexpression. These novel compartments could potentially result from homotypic fusion or fusion of endocytic vesicles to a second compartment. Traditionally, endocytosis is viewed as a sink, a process whereby internalized nutrients or membrane receptors are degraded. It is now clear, however, that endosomes may be recycled back to the plasma membrane and that extensive exchange can occur between endosomes and vesicles from the secretory pathway (Di Fiore and De Camilli, 2001; Sutter et al., 2007; Bassham and Blatt, 2008; Viotti et al., 2010). It has been suggested that the endoplasmic reticulum, Golgi apparatus, TGN, prevacuolar compartments, lytic compartments, and endosomes should perhaps all be regarded as components of endocytic pathways (Geldner, 2004). In view of these developments, the possibility of whether a secreted protein marker (invertase-CFP) localized to the membrane compartment induced by the truncated constitutively active PiVAMP726 was assessed. This compartment did indeed contain this secreted protein marker in addition to an endosomal marker, suggesting that at least when overexpressed, PiVAMP726 mediates membrane fusion between membrane compartments traditionally regarded as endocytic and an exocytic compartment.

This result supports the possibility of the existence of a pathway that integrates events that traditionally regarded as being distinct and is consistent with a ‘rapid recycling pathway’ proposed to be operating at the tip of pollen tubes (Samaj et al., 2006). Although the clear zone at the apex of tip-growing cells has been proposed to be filled predominately with distinct secretory and endocytic vesicles, 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 span. The putative early endosome may also reside in the clear zone, and endocytic vesicles may fuse into early endosomes and presumably also bud off from them. Besides being involved in recycling, the early endosome or TGN apparently possesses an inherent secretory nature, suggesting a close relationship between secretory and endocytic pathways. The ‘rapid recycling pathway’ of endocytosis has often been evoked to explain this phenomenon, although extensive material exchange between secretory and endocytic vesicles might also account for this phenomenon (Monteiro et al., 2005).

Pharmacological support for rapid recycling has come from studies using the secretion inhibitor brefeldin A (BFA) (Parton et al., 2001, 2003; Hörmanseder et al., 2005). BFA targets guanine exchange factors for ARF GTPases: its presence completely inhibits exocytosis and leads to the formation of BFA-induced membrane aggregations (BIAs). Interestingly in plant cells, BIA compartments are able to receive new endocytic material over time through an actin-dependent pathway (Parton et al., 2003; Hörmanseder et al., 2005), suggesting the possibility of membrane fusion occurring between early endocytic and late exocytic compartments. These observations have lead to the proposal that the TGN in tip-growing cells represents a ‘tip-localized vesicular compartment integrating targeted secretion and endocytosis within the growing tip’ (Samaj et al., 2006). One aspect of this model is that endocytic and exocytic vesicles fuse within the TGN and the product of this fusion is employed directly in growth by fusion with the plasma membrane at the growing tip. The novel compartments formed as a result of PiVAMP726 overexpression are reminiscent of BIAs, and overall the data presented here suggest that PiVAMP726 may be a component of the machinery involved in the integration of endo- and exocytic processes.

There is evidence for the existence of multiple (including recycling) endocytic pathways in plant cells. Endosidin1, an inhibitor of endocytosis, was found to disrupt this process in a selective way in roots. PIN2, AUX1, and BR1 were found to localize to endosidin1-induced membrane compartments while PIN1 and PIN7 remained unaffected (Robert et al., 2008). This indicates that at least two potential pathways recycle plasma-membrane proteins: endosidin1 blocks an early, presumably faster, recycling pathway while a second later pathway was insensitive to this inhibitor. The data presented in this manuscript is consistent with the hypothesis that PiVAMP726 might function in such an early ‘rapid recycling’ endocytic pathway in pollen tubes. In particular, the localization of a secretory enzyme, invertase, to PiVAMP726/SD-induced membrane compartments supports the idea that PiVAMP726 could potentially mediate fusion between vesicles traditionally regarded as being components of endocytic and secretory pathways.
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