the plant journal

SEB Society for Experimental Biology

The Plant Journal (2011) **68**, 1081–1092 doi: 10.1111/j.1365-313X.2011.04761.x

Overexpression of *Arabidopsis thaliana PTEN* caused accumulation of autophagic bodies in pollen tubes by disrupting phosphatidylinositol 3-phosphate dynamics

Yan Zhang[1,2,3,*], Sha Li[1], Liang-Zi Zhou[1], Emily Fox[2,3], James Pao[2,3], Wei Sun[1], Chao Zhou[1] and Sheila McCormick[2,3,*]

[1]*State Key Laboratory of Crop Biology, College of Life Sciences, Shandong Agricultural University, Tai'an 271018, China,*

[2]*Plant Gene Expression Center, United States Department of Agriculture/Agricultural Research Service, University of California at Berkeley, 800 Buchanan St., Albany, CA 94710, USA, and*

[3]*Department of Plant and Microbial Biology, University of California at Berkeley, 800 Buchanan St., Albany, CA 94710, USA*

Received 31 March 2011; revised 4 July 2011; accepted 22 August 2011; published online 10 October 2011.

[*]For correspondence (fax +86-538-8248696; e-mails yzhang@sdau.edu.cn or sheilamc@berkeley.edu).

SUMMARY

Autophagy is a pathway in eukaryotes by which nutrient remobilization occurs through bulk protein and organelle turnover. Autophagy not only aides cells in coping with harsh environments but also plays a key role in many physiological processes that include pollen germination and tube growth. Most autophagic components are conserved among eukaryotes, but phylum-specific molecular components also exist. We show here that *Arabidopsis thaliana* PTEN, a protein and lipid dual phosphatase homologous to animal PTENs (phosphatase and tensin homologs deleted on chromosome 10), regulates autophagy in pollen tubes by disrupting the dynamics of phosphatidylinositol 3-phosphate (PI3P). The pollen-specific PTEN bound PI3P *in vitro* and was localized at PI3P-positive vesicles. Overexpression of *PTEN* caused accumulation of autophagic bodies and resulted in gametophytic male sterility. Such an overexpression effect was dependent upon its lipid phosphatase activity and was inhibited by exogenous PI3P or by expression of a class III phosphatidylinositol 3-kinase (PI3K) that produced PI3P. Overexpression of *PTEN* disrupted the dynamics of autophagosomes and a subpopulation of endosomes, as shown by altered localization patterns of respective fluorescent markers. Treatment with wortmannin, an inhibitor of class III PI3K, mimicked the effects by *PTEN* overexpression, which implied a critical role for PI3P dynamics in these processes. Despite sharing evolutionarily conserved catalytic domains, plant PTENs contain regulatory sequences that are distinct from those of animal PTENs, which might underlie their differing membrane association and thereby function. Our results show that PTEN regulates autophagy through phylum-specific molecular mechanisms.

Keywords: autophagy, phosphatidylinositol 3-phosphate, phosphatase, endosome, N-myristoylation, tobacco.

INTRODUCTION

Autophagy is an evolutionarily conserved cellular process in eukaryotes by which nutrient remobilization occurs through bulk protein and organelle turnover (Levine and Klionsky, 2004; Thompson and Vierstra, 2005; Bassham *et al.*, 2006; Nakatogawa *et al.*, 2009). Macroautophagy, the major type of autophagy and hereafter referred to as 'autophagy', starts with the formation of spherical double membrane structures, termed autophagosomes, which enclose damaged proteins and organelles together with cytoplasm (Levine and Klionsky, 2004; Thompson and Vierstra, 2005; Bassham *et al.*, 2006; Nakatogawa *et al.*, 2009). Autophagosomes converge with endocytic vesicles, likely at prevacuolar compartments/multivesicular bodies (PVC/MVB), while moving toward lytic compartments, either lysosomes or vacuoles (Robinson *et al.*, 1998). The outer membrane of autophagosomes fuses with membranes of lysosomes/vacuoles, thereby delivering single-membrane structures, the autophagic bodies, into lysosomal or vacuolar lumens for degradation (Levine and Klionsky, 2004; Nakatogawa *et al.*, 2009).

Autophagy not only aides cells in coping with harsh environments, such as nutrient starvation and oxidative stress, but also may play key roles in many physiological processes (Thompson and Vierstra, 2005; Nakatogawa *et al.*, 2009), which include pollen germination in plants. Pollen germination and tube growth are critical steps to achieve

double fertilization in flowering plants (Lord and Russell, 2002). After landing on compatible stigmatic cells, pollen grains germinate and extend a tube, which grows by tip growth to penetrate different tissues within a pistil, reaching precisely the opening to the embryo sac and finally releasing sperm to fertilize the egg and the central cell (Lord and Russell, 2002). This complex communication process involves multiple cellular activities (Cheung and Wu, 2008). Recent studies indicated that autophagy also may play a role in this process. For example, mutations in *ATG6/Beclin1/Vps30*, an *Arabidopsis thaliana* autophagy-related gene (*ATG*) (Liu *et al.*, 2005; Fujiki *et al.*, 2007; Qin *et al.*, 2007; Harrison-Lowe and Olsen, 2008) as well as in *Vacuolar Sorting Protein 34* (*VPS34*), a gene that encodes class III phosphatidylinositol 3-kinase (PI3K) (Lee *et al.*, 2008), completely abolish pollen germination, which results in defective male transmission. Mutations in two other *ATG* genes, *ATG9* and *ATG4*, reduced fertility (Hanaoka *et al.*, 2002; Yoshimoto *et al.*, 2004). A mutation in *VACUOLARLESS1* (*VL1*), which caused accumulation of autophagic bodies, also reduced male transmission (Hicks *et al.*, 2004). Because these ATGs also regulate vacuolar sorting, a critical role of properly regulated autophagy in pollen germination and tube growth *in vivo* cannot be ascertained, but it is certainly an interesting possibility.

Most autophagic components are conserved in all eukaryotes. *ATGs* were first identified in yeast (Nakatogawa *et al.*, 2009) and later in mammals and plants (Hanaoka *et al.*, 2002; Thompson and Vierstra, 2005; Yorimitsu and Klionsky, 2005; Nakatogawa *et al.*, 2009). In addition, VPS34 sequence and function is also conserved among eukaryotes (Welters *et al.*, 1994; Meijer and Munnik, 2003; Leshem *et al.*, 2007; Lee *et al.*, 2008). VPS34 phosphorylates the D3 position of the inositol ring in phosphatidylinositol (PI) to generate phosphatidylinositol 3-phosphate (PI3P) (Meijer and Munnik, 2003). PI3P is abundant at endosomes and on vacuolar membranes under physiological conditions (Kim *et al.*, 2001; Vermeer *et al.*, 2006; Obara *et al.*, 2008) and is massively transported into vacuoles under starvation conditions (Kim *et al.*, 2001; Obara *et al.*, 2008). PI3P is not only a component of autophagosomes (Wurmser and Emr, 1998; Obara *et al.*, 2008), but it also regulates endosome/autophagosome to vacuole trafficking in yeast (Wurmser and Emr, 1998), and promotes the formation of autophagosomes by recruiting ATGs (Yorimitsu and Klionsky, 2005; Nakatogawa *et al.*, 2009).

However, phylum-specific autophagic components also exist. For example, orthologs for some yeast ATGs have not been found in plants (Hanaoka *et al.*, 2002; Yorimitsu and Klionsky, 2005) or mammals (Yorimitsu and Klionsky, 2005). In addition to PI3P, animals use phosphatidylinositol 3,4,5-trisphosphate (PIP_3) signaling in autophagy. PIP_3 is generated from phosphatidylinositol 4,5-bisphosphate (PIP_2) by class I PI3Ks. Conversion from PIP_3 to PIP_2 is catalyzed by PTEN (Leslie and Downes, 2004), a protein and lipid dual phosphatase that dephosphorylates phosphotyrosines in proteins and removes phosphates at the D3 position in PI. Conversion of PIP_3 to PIP_2 by PTEN caused inhibition of AKT/PKB signaling downstream of PIP_3, leading to activated autophagy (Arico *et al.*, 2001; Ueno *et al.*, 2008). As PIP_3 and class I PI3Ks are absent in yeast and plants (Meijer and Munnik, 2003), it seemed likely that plants exhibit phylum-specific PI signaling.

We report here that overexpression of Arabidopsis PTEN in tobacco caused accumulation of autophagic bodies by disrupting PI3P dynamics in pollen tubes; the same construct introduced in Arabidopsis resulted in gametophytic male sterility. We show that PTEN bound specifically to PI3P *in vitro* and reduced PI3P levels *in vivo*. Decreased PI3P interfered with membrane trafficking from PVC/MVB to vacuoles but did not interfere with trafficking between endosomes and the plasma membrane, suggesting that autophagosomes converge with endocytic trafficking at PVC/MVB. Plant PTENs contain conserved catalytic domains but regulatory domains distinct from their animal counterparts. We show that the plant-specific regulatory domains contribute to the different substrate specificities and membrane localizations. Our results indicated that autophagy in pollen is regulated by evolutionarily conserved components through phylum-specific molecular mechanisms.

RESULTS

Accumulation of autophagic bodies in pollen tubes by overexpression of Arabidopsis *PTEN*

PTEN is a pollen-specific gene, with highest expression in mature pollen (Gupta *et al.*, 2002), which suggested that PTEN might function during germination and tube growth. However *PTEN* RNAi lines exhibited pollen abortion before pollen maturation (Gupta *et al.*, 2002), so it is unknown if PTEN plays a later role. We obtained two *PTEN* T-DNA insertion lines, one in the promoter and the other in the 3′UTR, but neither affected *PTEN* mRNA levels (Figure S1). We therefore took a gain-of-function approach to find out if PTEN played roles in pollen tube growth, by transient expression of yellow fluorescent protein (YFP)-fused PTEN from the strong pollen-specific promoter Pro_{LAT52} (Twell *et al.*, 1991). As a control, YFP alone was overexpressed using the same promoter. YFP overexpression did not interfere with normal tube growth or morphology of tobacco pollen (Figure 1a,j,k). Tubes that overexpressed *PTEN–YFP* at early growth stages (<3 h of incubation) grew normally (Figure 1b,j,k). In addition to the cytoplasmic localization, the fluorescence signal was detected at numerous punctate vesicles as well as at the lateral plasma membrane, starting about 6–15 μm from the apex (Figure 1b and Movie S1). However, after 4 h of incubation all tubes that overexpressed *PTEN–YFP* showed an expanded subapical region and were

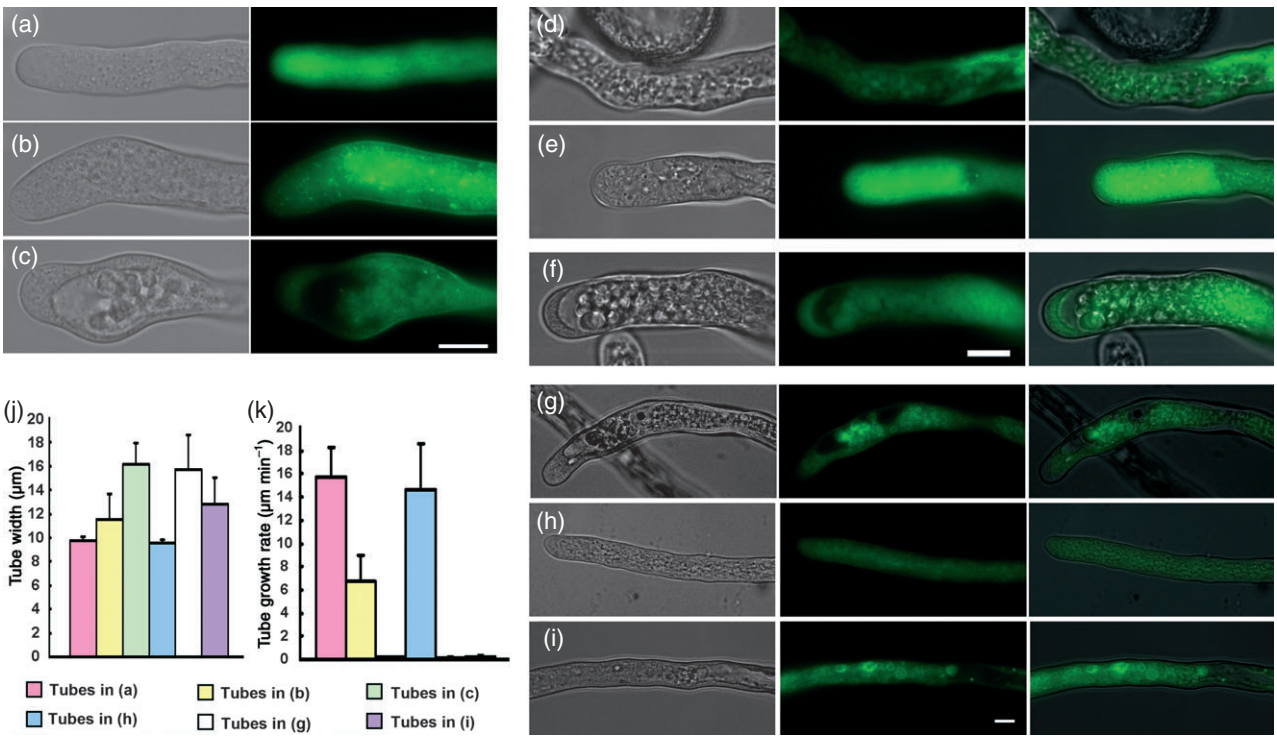

Figure 1. Overexpression of Arabidopsis *PTEN* caused accumulation of autophagic bodies in pollen tubes.
(a) A tobacco pollen tube expressing yellow fluorescent protein (YFP).
(b) A tobacco pollen tube expressing PTEN–YFP at an early growth stage (2–3 h of incubation).
(c) A tobacco pollen tube expressing PTEN–YFP (4 h of incubation).
(d) A Lysotracker Red-treated tobacco pollen tube expressing PTEN–YFP (4 h of incubation).
(e) A Lysotracker Red-treated tobacco pollen tube expressing YFP (4 h of incubation).
(f) A tobacco pollen tube co-expressing PTEN–YFP and CFP (5 h of incubation).
(g) A tobacco pollen tube co-expressing PTEN–YFP and α-TIP–CFP (4 h of incubation).
(h) A tobacco pollen tube co-expressing YFP and α-TIP–CFP (4 h of incubation).
(i) A tobacco pollen tube co-expressing YFP and α-TIP–CFP that was treated with 10 μM E64d (4 h of incubation).
(j) Width (μm) of transformed tobacco pollen tubes. The widest region was measured.
(k) Growth rate (μm min^{-1}) of transformed pollen tubes. Images in (a) to (c) are from the YFP channel, false-colored green. Images in (d) and (e) were with the red fluorescent protein (RFP) channel, false-colored green. Images in (f) to (i) are from the CFP channel. All scale bars = 10 μm. Scale bar in (c) is also for (a) and (b); in (f) is also for (d) and (e); and in (i) is also for (g) and (h).

vacuolated at the apex (Figure 1c,j,k). These pollen tubes contained a huge vacuole penetrating the apical clear zone, in which bulbous entities ranging from 1 to 6 μm diameter rolled around (Movie S2). In all transformed tubes, the YFP signal at punctate vesicles disappeared mostly into the aggregates within the vacuole, although its presence at the lateral plasma membrane was still detectable (Figure 1c).

The bulbous entities in pollen tubes that overexpressed *PTEN* resembled autophagic bodies found in vacuoles of nutrient-deficient yeast (Levine and Klionsky, 2004), i.e. the inner membrane of autophagosomes after its outer membrane fused with the vacuole membrane. We therefore used several approaches to determine whether these entities were autophagic bodies. First, we introduced Lysotracker Red into pollen tubes overexpressing *PTEN*. Lysotracker Red is a fluorescent dye that labels acidic compartments, specifically autophagic bodies, in various cell types (Moriyasu *et al.*, 2003; Takatsuka *et al.*, 2004; Liu *et al.*, 2005; Inoue *et al.*, 2006). All bulbous entities in *PTEN*-overexpressing pollen tubes were labeled with Lysotracker Red (Figure 1d), whereas non-transformed pollen tubes only showed a few small vesicles (Figure 1e). Second, we co-expressed cyan fluorescent protein (CFP) with PTEN–YFP to test whether CFP, a long-lived stable protein, would be sequestered in these entities, a feature of autophagic bodies. The CFP signal was indeed present in these vesicles within vacuoles when co-expressed with *PTEN* (Figure 1f) but was present in the cytoplasm when co-expressed with YFP (data not shown). Third, we co-expressed *PTEN* with α-Tonoplast Intrinsic Protein–CFP (α-TIP–CFP). α-TIP is the only plant protein shown to be present at the membrane of autophagic bodies (Moriyasu *et al.*, 2003). α-TIP–CFP was detected as rings that overlaid perfectly with the surface of autophagic bodies when co-expressed with PTEN–YFP (Figure 1g), but only labeled a few small vesicles and the cytoplasm when co-expressed with YFP (Figure 1h). Fourth, we treated pollen

tubes co-expressing YFP and α-TIP–CFP with E-64d. E-64d is a membrane-permeable cysteine protease inhibitor, whose application causes accumulation of autophagic bodies (Moriyasu *et al.*, 2003; Takatsuka *et al.*, 2004; Yoshimoto *et al.*, 2004; Inoue *et al.*, 2006; Xiong *et al.*, 2007). E-64d (10 μM) caused accumulation of numerous entities whose membranes showed the CFP signal (Figure 1i) in otherwise normal pollen tubes expressing α-TIP–CFP and YFP (Figure 1h). These several lines of evidence showed that overexpression of *PTEN* resulted in accumulation of autophagic bodies in pollen tubes.

Accumulation of autophagic bodies depends on the lipid phosphatase activity of PTEN

Arabidopsis *PTEN* encodes a protein and lipid dual phosphatase whose catalytic activity was demonstrated *in vitro* (Gupta *et al.*, 2002). To determine if the overexpression effect of *PTEN* was due to dephosphorylation of its substrates, we expressed $PTEN_{C152S}$ and $PTEN_{G157E}$ mutants (Figure 2a) in pollen tubes. Because Arabidopsis PTEN shares highly conserved catalytic domain with animal PTENs (Gupta *et al.*, 2002), mutation of Cys152 to Ser would abolish both lipid and protein phosphatase activities of PTEN (Gupta *et al.*, 2002), while mutation of G157E should abolish only lipid phosphatase activity, based on studies in animals (Arico *et al.*, 2001; Vazquez *et al.*, 2006). Under the same expression and growth conditions, overexpression of either *$PTEN_{C152S}$–YFP* (Figure 2b) or *$PTEN_{G157E}$–YFP* (Figure 2c) did not affect growth and morphology of pollen tubes (Figure 2d). Both mutant variants of PTEN were found at the lateral plasma membrane and at punctate vesicles (Figure 2b,c), similar to that of wild-type PTEN before accumulation of autophagic bodies (Figure 1b). These results indicated that accumulation of autophagic bodies in *PTEN*-overexpressing tubes relied on the lipid phosphatase activity of PTEN.

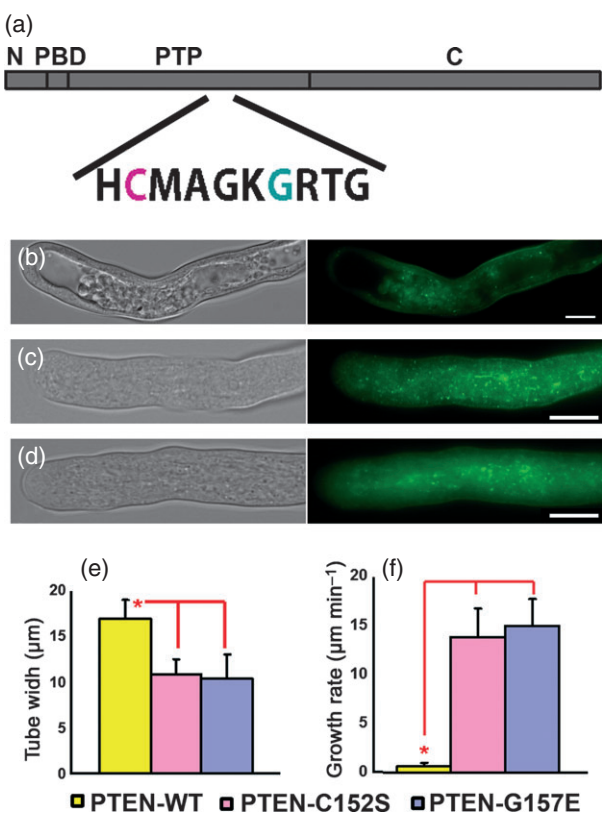

Figure 2. Accumulation of autophagic bodies depends on the lipid phosphatase activity of PTEN.
(a) Schematic illustration of the domain organization of Arabidopsis PTEN. N, N-terminal leader sequence; PBD, PIP_2-binding domain; PTP, Protein tyrosine phosphatase domain; C, C-terminal sequence. The expansion of the PTP domain shows the conserved catalytic sites.
(b) A tobacco pollen tube expressing PTEN–YFP.
(c) A tobacco pollen tube expressing $PTEN_{C152S}$–YFP.
(d) A tobacco pollen tube expressing $PTEN_{G157E}$–YFP.
(e) Tube width (μm) of transformed tobacco pollen tubes. *Significant difference ($P < 0.01$).
(f) Growth rate (μm min^{-1}) of transformed tobacco pollen tubes. *Significant difference ($P < 0.01$). All pollen tubes shown were incubated for more than 4 h. Scale bars = 10 μm.

Arabidopsis PTEN bound to PI3P *in vitro* and localized at PI3P-positive vesicles

To determine which phosphoinositols could account for the accumulation of autophagic bodies in pollen tubes overexpressing *PTEN*, we first tested the *in vitro* binding affinity of PTEN. PTEN–glutathione S-transferase (GST) protein was incubated with a phosphoinositide (PIP) strip on which 14

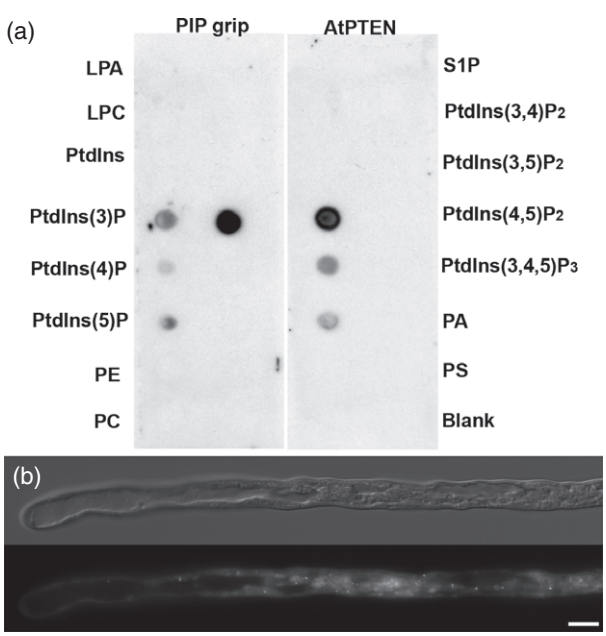

Figure 3. Arabidopsis PTEN bound to PI3P *in vitro* and localized at PI3P-positive vesicles.
(a) Binding of PTEN and PIP grip to PIP strips.
(b) A tobacco pollen tube expressing $PTEN_{G157E}$–YFP, treated with 5 μM wortmannin.

different phospholipids were spotted. GST alone was used as a control. Because animal PTENs bind specifically to phosphatidylinositol 4,5-biphosphate (PIP_2) through a PIP_2-binding domain (PBD) that is conserved in plant PTENs, we also assayed PIP grip, a commercially available PIP_2-binding domain. PTEN showed a strong signal with PI3P (Figure 3a), and a much weaker signal with PI4P and PI5P. PIP grip showed a strong signal with PIP_2 and a much weaker signal with PI3P, PI4P and PI5P. GST alone did not bind to any of the 14 phospholipids (data not shown). These results showed that Arabidopsis PTEN preferentially bound to PI3P *in vitro.*

PTEN was localized at punctate motile vesicles before autophagic bodies accumulated in the vacuole (Figure 1b), and these vesicles appeared similar to vesicles where PI3P had been detected in other Arabidopsis cell types (Vermeer *et al.*, 2006). We therefore suspected that the vesicles on which PTEN localized *in vivo* were PI3P-positive. To test this, we applied wortmannin to pollen tubes expressing $PTEN_{G157E}$–eYFP. Wortmannin inhibits class III PI3K therefore reduces PI3P levels (Meijer and Munnik, 2003). We analyzed previously the effect of wortmannin on endocytic trafficking in Arabidopsis pollen tubes (Zhang *et al.*, 2010). Our results (Zhang and McCormick, 2010) as well as others (Vermeer *et al.*, 2006, 2009) showed that wortmannin treatment caused aggregation of PI3P-positive vesicles. $PTEN_{G157E}$ was used in this experiment because it showed same localization as wild-type PTEN but did not affect pollen tube growth (Figure 2c,d). In animal PTENs, mutations at the glycine did not affect their PI affinity (Gericke *et al.*, 2006). Treating pollen tubes that express $PTEN_{G157E}$–eYFP with wortmannin disrupted the localization pattern of the fluorescent signal (Figure 2c) such that it was mostly detected in bulbous entities in the vacuole (Figure 3b), reminiscent of autophagic bodies caused by *PTEN* overexpression (Figure 1c). A fluorescence signal was also detected at a few punctate vesicles outside of vacuoles (Figure 3b). To show that wortmannin treatment indeed reduced PI3P levels, we used the same wortmannin treatment on pollen tubes that overexpressed 2XFYVE–mRFP. 2XFYVE–mRFP binds specifically to PI3P and its overexpression caused increased PI3P levels (Vermeer *et al.*, 2006) as well as vacuolation in pollen tubes (Helling *et al.*, 2006). Tube vacuolation due to high levels of 2XFYVE–mRFP expression was suppressed by wortmannin treatment (Figure S2), which showed that wortmannin treatment did reduce PI3P levels under our experimental conditions.

PI3P suppressed the overexpression effects of *PTEN*

Because PTEN bound to PI3P rather than to PIP_2 *in vitro* and possibly *in vivo*, we suspected that effects of *PTEN* overexpression might be due to disrupted PI3P dynamics. To test whether this situation was the case, we co-expressed *PTEN* with Arabidopsis *VPS34.* Because VPS34 catalyzes the production of PI3P (Meijer and Munnik, 2003), overexpression of VPS34 should compensate for PI3P whose dynamics were disturbed by *PTEN* overexpression. Indeed, expression of VPS34 suppressed the overexpression phenotype of *PTEN*, which resulted in pollen tubes with normal morphologies and no autophagic body accumulation (Figure 4a,d). Overexpression of VPS34–CFP with YFP did not cause growth defects under the same conditions (Figure 4d). We then tested whether accumulation of autophagic bodies by *PTEN* overexpression would be suppressed by addition of PI3P. PI3P (delivered with histone carrier) indeed rescued the overexpression effect of *PTEN*, which resulted in pollen tubes with normal growth morphologies (Figure 4b,d). In contrast, exogenous PIP_2 did not restore normal growth morphologies of pollen tubes that overexpressed *PTEN* (Figure 4), nor did histone carrier when supplied without any phospholipids (Figure 4c,d).

PTEN localized mostly in autophagic bodies once they accumulated, which suggested that PI3P was delivered into the vacuole. We therefore tested the dynamic localization of 2XFYVE–mRFP during PTEN overexpression. 2XFYVE–mRFP was expressed at a level that did not interfere with pollen tube growth (Figure S3). Pollen tubes that overexpress *PTEN* showed the typical accumulation of autophagic bodies in which the mRFP signal accumulated (Figure S3), which suggested that 2XFYVE was sequestered in autophagic bodies. PTEN itself was also sequestered in autophagic bodies (Figure S3). Only a few punctate vesicles that contained PTEN were detected outside of the vacuole (Figure S3). Together, these experiments showed that overexpression of *PTEN* interfered with PI3P dynamics, probably by increasing the delivery of PI3P into the vacuole.

Plant-specific regulatory sequences determine the sub-cellular localization and functionality of Arabidopsis PTEN

It was intriguing that Arabidopsis PTEN showed *in vitro* affinity to PI3P rather than PIP_2, considering that it has a PIP_2-binding domain (PBD) conserved with animal PTENs. To find out the sub-cellular localization of the PBD of Arabidopsis PTEN, we expressed the fluorescent-fused PBD in pollen tubes. Indeed, the PBD domain alone localized at apical plasma membrane of pollen tubes (Figure 5e). Its strong expression caused pollen tubes to bulge at the tip (Figure 5e inset) probably by protecting endogenous PIP_2 from hydrolysis by phospholipase C (Dowd *et al.*, 2006; Helling *et al.*, 2006). This tip bulging effect was similar to that seen in Arabidopsis pollen tubes supplied with exogenous PIP_2 (Figure 4d). Sequence alignments of plant PTENs showed that there was an N-terminal leader sequence present in all plant PTENs (data not shown), each with a glycine at the 2nd position, potentially subject to N-myristoylation. Because N-myristoylation is a key post-translational modification that confers tight membrane association (Johnson *et al.*, 1994), we asked whether the G2 residue, and by inference N-myr-

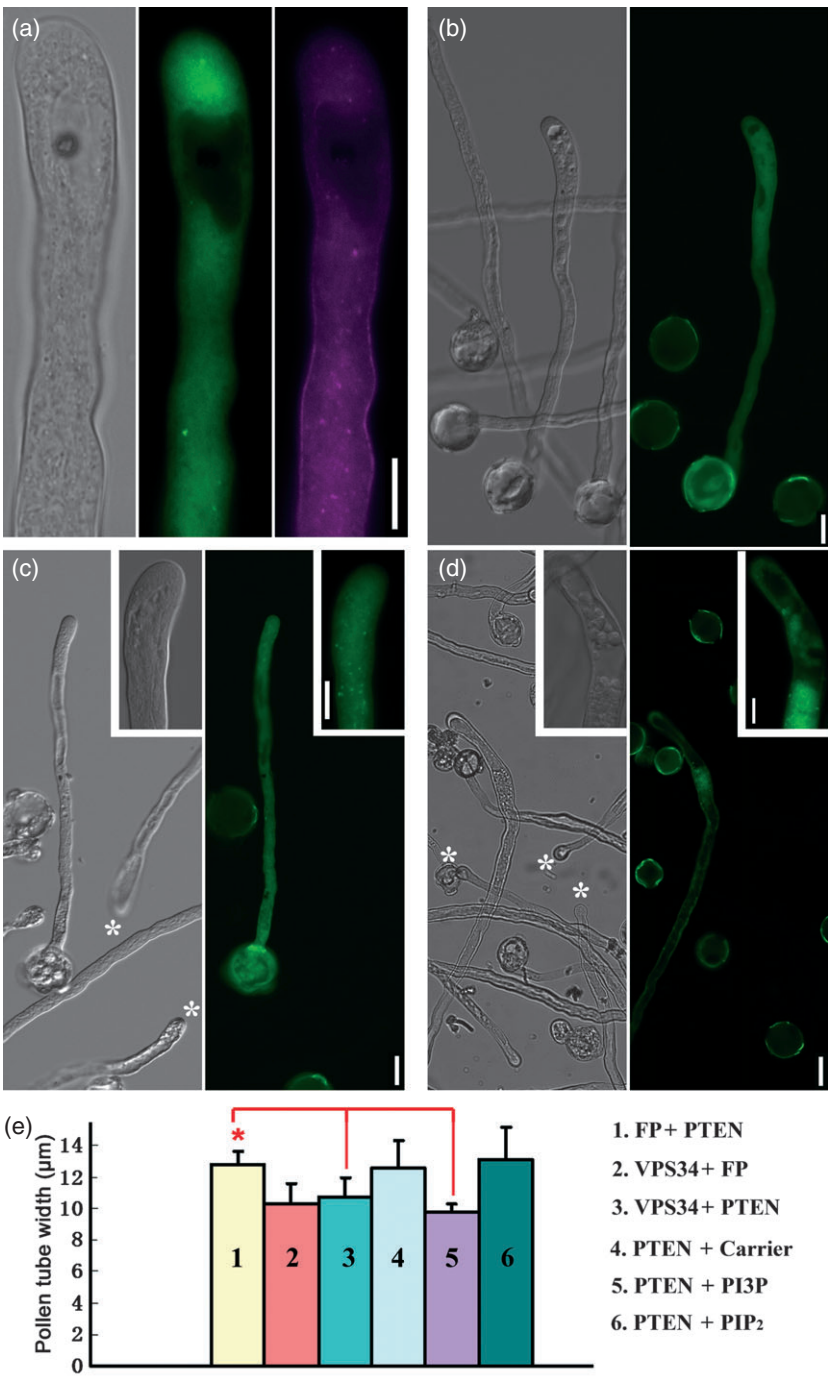

Figure 4. PI3P suppressed the overexpression effects of *PTEN*.
(a) A tobacco pollen tube co-expressing PTEN–YFP (false colored green) with Vps34–CFP (false-colored magenta). (b) A pollen tube expressing PTEN–YFP and treated with histone carrier alone. (c) A tobacco pollen tube expressing PTEN–YFP and treated with a histone carrier–PI3P mix. Asterisks indicate a highly vacuolated, non-transformed pollen tube affected by PI3P treatment. Inset is a close-up of a transformed tube. (d) A tobacco pollen tube expressing PTEN–YFP and treated with a histone carrier–PIP_2 mix. Asterisks indicate non-transformed pollen tubes with bulging tips by PIP_2 treatment. Inset is a close-up of a transformed tube.
(e) Tube width (μm) of transformed pollen tubes. Asterisk indicates statistical significance ($P < 0.01$). Scale bars for (a) and inset of (c) and (d) = 10 μm. Scale bars for (b), (c) and (d) = 20 μm.

istoylation, might affect the sub-cellular localization of Arabidopsis PTEN. Mutation of G2 to alanine ($PTEN_{G2A}$) or deletion of the N-terminal leader sequence ($PTEN\Delta N_{29}$) reduced the membrane association of PTEN at the lateral plasma membrane and more substantially, its localization at punctate vesicles (Figure 5a,b). Pollen tubes that express either of these constructs showed normal growth and morphologies (Figure 5d), whereas under the same conditions *PTEN* overexpression caused massive accumulation of autophagic bodies in the vacuole (Figure 1c).

Plant PTENs also differ from animal PTENs at the C-terminus. Deletion of the C-terminus of Arabidopsis PTEN ($PTEN\Delta C_{212-412}$) resulted in stronger membrane association at the lateral plasma membrane but abolished PTEN localization at punctate vesicles (Figure 5c). Pollen tubes that expressed $PTEN\Delta C_{212-412}$ showed normal growth morpho-

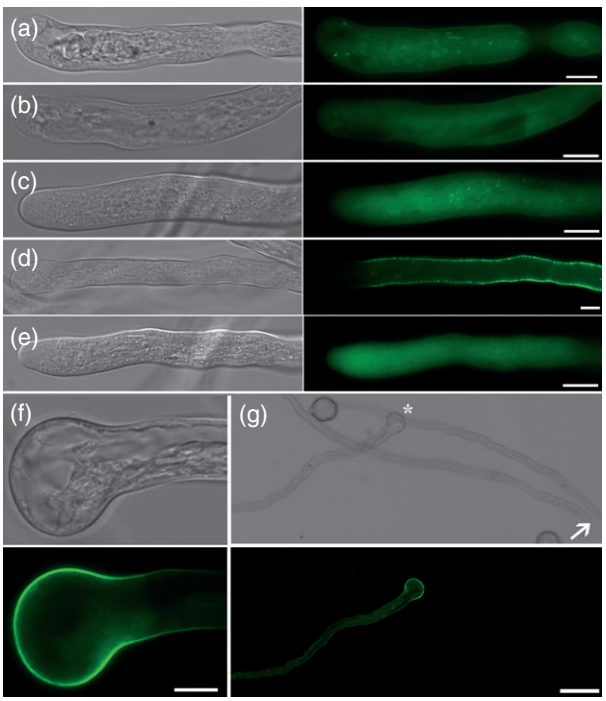

Figure 5. Plant-specific regulatory sequences determine the sub-cellular localization and functionality of Arabidopsis PTEN. Bright-field and fluorescent images are shown side by side.
(a) A tobacco pollen tube expressing PTEN–YFP.
(b) A tobacco pollen tube expressing $PTEN_{G2A}$–YFP.
(c) A tobacco pollen tube expressing $PTEN\Delta N_{29}$–YFP.
(d) A tobacco pollen tube expressing $PTEN\Delta C_{212–412}$–YFP.
(e) A tobacco pollen tube expressing YFP–$PTEN\Delta N_{1–211}$.
(f) Tip of a tobacco pollen tube expressing PTEN-$_{PBD}$–YFP.
(g) A tobacco pollen tube expressing PTEN-$_{PBD}$–YFP and two non-transformed pollen tubes. The arrow indicates the normal tip of a non-transformed tube while the asterisk indicates the bulged tip of the transformed tube. Scale bars: (a–f) = 10 μm; (g) = 50 μm.

logies (Figure 5c). These results indicate that PTEN localization at punctate vesicles, conferred by the plant-specific N-terminal leader sequence and the C-terminus, was critical for its overexpression effect.

PTEN overexpression interfered with endomembrane trafficking to vacuoles

To find out which steps during autophagy were perturbed by *PTEN* overexpression, we co-expressed *PTEN* with different endosomal markers. ARA6 and ARA7 have been used in different plant cells as markers for endosomes and label different endocytic compartments depending on cell type (Geldner, 2004; Zhang *et al.*, 2010). Our previous results indicated that in pollen tubes ARA6 labeled PVC/MVB involved in endocytic trafficking to vacuoles whereas ARA7 labeled early endosomes involved in membrane trafficking to and from the plasma membrane (Zhang *et al.*, 2010). The localization pattern of ARA6 (Figure 6a) was disrupted when co-expressed with PTEN, in that the ARA6 signal was mostly sequestered in autophagic bodies (Figure 6b). However, overexpression of *PTEN* appeared not disturb ARA7-positive endosomes. Similar to the pattern seen when co-expressed with fluorescent protein alone (Figure 6c), a substantial portion of ARA7 signal still localized at small vesicles (Figure 6d).

We also tested the effect of *PTEN* overexpression on the distribution pattern of other membrane markers. RabA4b labels transporting vesicles that show an inverted cone-shaped distribution at the tip of growing pollen tubes (Zhang *et al.*, 2010). The regular distribution pattern of RabA4d (Figure S4) in *PTEN*-overexpressing tubes was disrupted even before overexpression of *PTEN* had caused any morphological changes (Figure S4). However, the distribution pattern of Golgi dynamics, as shown with ERD2 labeling (Cheung and Wu, 2007), was not disturbed by *PTEN* overexpression (Figure S4). These results showed that *PTEN* overexpression disrupted certain steps of vesicle trafficking, possibly between PVC/MVB and vacuole.

To find out whether *PTEN* overexpression interferes with the dynamics of autophagosomes, we took a similar approach and co-expressed *PTEN* with *ATG8*, as ATG8s are specific markers for autophagosomes in plant cells (Yoshimoto *et al.*, 2004; Contento *et al.*, 2005; Xiong *et al.*, 2007; Slavikova *et al.*, 2008). In normal growing pollen tubes, ATG8–CFP was localized at a few motile vesicles (Figure 6e), sometimes as ring-shaped compartments (Figure 6e inset), in addition to the diffuse cytoplasmic localization (Figure 6e). However, co-expression with PTEN caused delivery and accumulation of ATG8-labeled vesicles into vacuolar autophagic bodies (Figure 6f). Similar accumulation was also observed when pollen tubes that expressed ATG8–CFP were treated with wortmannin (Figure S5).

PTEN overexpression results in male gametophytic sterility

To find out how overexpression of *PTEN* would influence pollen tube growth in Arabidopsis, we generated transgenic Arabidopsis plants that overexpressed PTEN. Twenty-nine individual transgenic lines that expressed *Pro_{LAT52}:PTEN–YFP* were obtained; five had single copy insertions based on the number of fluorescent pollen grains in each tetrad (*qrt1* background). All showed strong *PTEN* expression as measured by both fluorescence signal and transcript levels (Figure S6a,b). Transgenic Arabidopsis pollen tubes showed vesicle aggregation, although no massive accumulation of autophagic bodies was observed (Figure S6b). PTEN–YFP was present in ring-shaped compartments close to the vacuoles (Figure S6c).

To find out whether *PTEN* overexpression interfered with the function of male gametophytes *in vivo*, we analyzed the transmission efficiency of Arabidopsis pollen overexpressing *PTEN.* We analyzed the selfed progeny of five single copy lines for segregation ratio of the linked Basta resistance gene. Table 1 shows that heterozygous transgenic plants had a 1:1 resistance to sensitive ratio; no homozygous

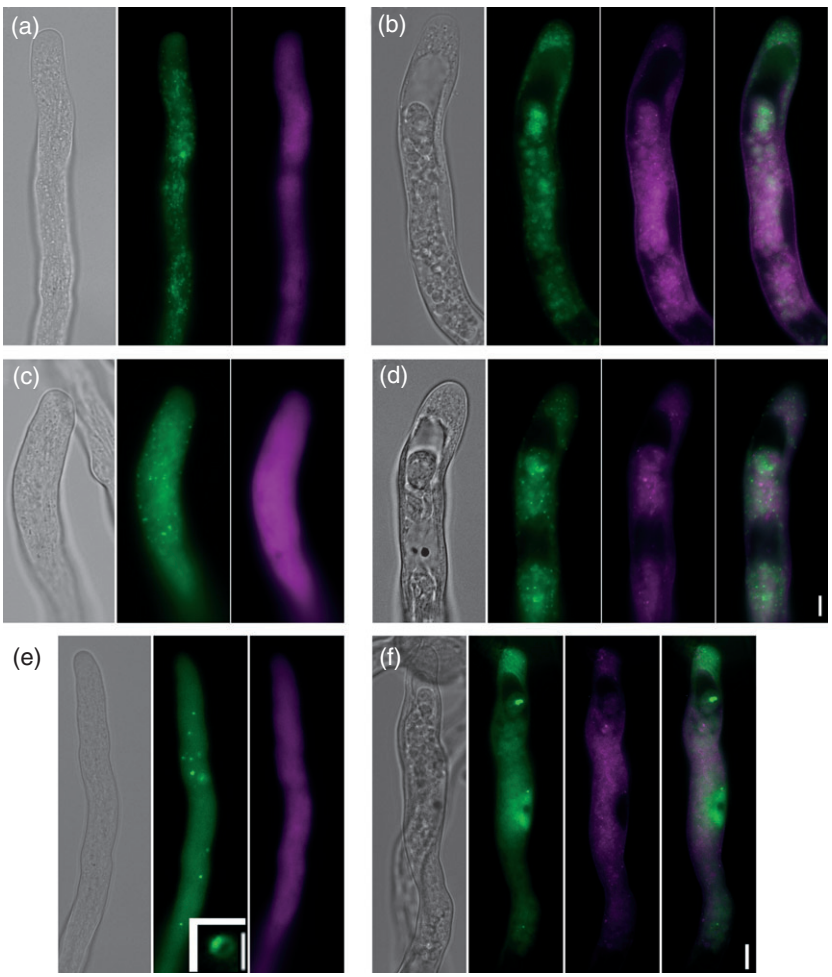

Figure 6. Overexpression of *PTEN* interfered with endomembrane trafficking to vacuoles.
(a) A tobacco pollen tube expressing YFP (false-colored magenta) and ARA6–CFP (false-colored green). (b) A tobacco pollen tube expressing PTEN–YFP and ARA6–CFP. (c) A tobacco pollen tube expressing YFP and CFP–ARA7. (d) A tobacco pollen tube expressing PTEN–YFP and CFP–ARA7. (e) A tobacco pollen tube expressing YFP and CFP–ATG8. Inset shows a ring-shaped endomembrane structure labeled by CFP. (f) A tobacco pollen tube expressing PTEN–YFP and CFP–ATG8. For (a), (c) and (e), the images (from left to right) are bright field, CFP channel and YFP channel. For (b), (d) and (f), the images (from left to right) are bright field, CFP channel, YFP channel and merged. Scale bars = 10 μm. Scale bar for (e) inset = 2 μm.

Table 1 Overexpression of Arabidopsis *PTEN* resulted in defective male transmission

Parent	Segregation of progenies (resistant:sensitive)	
Female × Male	Expected	Observed ratio
PTEN OX #1 ± selfed	1:2:1	1321:1257[a]
PTEN OX #2 ± selfed	1:2:1	2103:1872[a]
PTEN OX #3 ± selfed	1:2:1	1543:1620[a]
PTEN OX #4 ± selfed	1:2:1	1281:1546[a]
PTEN OX #5 ± selfed	1:2:1	1926:1771[a]
PTEN OX #1 ± X WT	1:1	212:235[c]
WT X *PTEN OX #1*±	1:1	0:431[b]
PTEN OX #2 ± X WT	1:1	279:254[c]
WT X *PTEN OX #2*±	1:1	0:3781[b]

[a]Significantly different from a 1:2:1 ratio (γ^2, $P < 0.01$).
[b]Significantly different from a 1:1 ratio (γ^2, $P < 0.01$).
[c]Not significantly different from a 1:1 ratio (γ^2, $P > 0.05$).

insertion lines were obtained. We used heterozygous plants from two of these transgenic lines in reciprocal crosses with *qrt1.* There was no effect on female transmission, as expected for a pollen-specific gene, but overexpression of *PTEN* caused male gametophytic sterility (Table 1).

DISCUSSION

PTEN has been studied extensively for its activity in dephosphorylating PIP_3, thereby regulating chemotaxis, genome stability and autophagy in animal cells (Gericke *et al.*, 2006). Although PIP_3 is a critical signaling component in animal cells, it has not been identified in plants (Munnik *et al.*, 1994). Furthermore, genes that encode class I PI3K, whose catalytic activity converts PIP_2 to PIP_3, are not apparent in plant genomes (Meijer and Munnik, 2003). It was therefore intriguing if or how plant PTENs function in phosphatidylinositol modifications. Arabidopsis PTEN is expressed specifically in mature pollen (Gupta *et al.*, 2002), which suggested a function during polarized growth of pollen tubes, a process that requires fine-tuned PI signals (Zhang and McCormick, 2010). We took a gain-of-function approach to explore whether PTEN played a role in PI signaling in pollen tubes. Our results indicate that Arabidopsis PTEN may regulate autophagy or vacuolar degradation by disrupting PI3P dynamics in pollen tubes.

PTEN regulates autophagy through PI3P dynamics

Several lines of evidence showed that overexpression of *PTEN* caused accumulation of autophagic bodies in pollen tubes. For example, accumulation of stable cytoplasmic proteins is a feature of autophagic bodies. When PTEN–YFP was co-expressed with CFP or pollen was stained with Lysotracker Red, CFP or Lysotracker Red were detected in autophagic bodies but not in the vacuolar lumen. Furthermore, membranes of autophagic bodies were labeled by α-TIP in pollen tubes that overexpressed PTEN or after E64d treatment, whereas α-TIP labeled only a few punctate vesicles in normal pollen tubes (Figure 1).

PTEN loss-of-function caused strong inhibition of autophagy in mammalian cells (Ueno *et al.*, 2008). Our finding that PTEN may also regulate autophagy in pollen tubes seemingly suggested an evolutionary conservation of PTEN involvement during this process. However, animal PTENs promote autophagy by inhibiting PIP_3-mediated signaling (Arico *et al.*, 2001; Ueno *et al.*, 2008). We showed that Arabidopsis PTEN regulates autophagy through PI3P. Although Arabidopsis PTEN contains highly conserved PBD domain (Gupta *et al.*, 2002), it bound to PI3P rather than PIP_2. Furthermore, that wortmannin disrupted its localization at punctate vesicles suggested that PTEN localization *in vivo* depends on PI3P. The overexpression phenotype of PTEN was suppressed by co-expressing VPS34, whose activity regulates PI3P production. Additionally, delivery of exogenous PI3P but not PIP_2 partially suppressed the overexpression phenotypes of PTEN, as manifested by the absence of autophagic bodies (Figure 4). Animal PTENs can use PI3P as an *in vitro* substrate (Gericke *et al.*, 2006) and plant PTENs share highly conserved catalytic domains with animal PTENs (Gupta *et al.*, 2002), which suggested that Arabidopsis PTEN catalyzes the dephosphorylation of PI3P.

PI3P is critical for autophagy in pollen tubes

Fluorescence-based studies indicated that PI3P localized at punctate vesicles in plants (Kim *et al.*, 2001; Vermeer *et al.*, 2006) and that this localization pattern was sensitive to wortmannin (Kim *et al.*, 2001; Vermeer *et al.*, 2006, 2009). In pollen tubes, wortmannin disrupted the distribution pattern of both ARA6- (Zhang *et al.*, 2010) and ATG8-positive vesicles (Figure S3), which suggested that PI3P is enriched in a subpopulation of endosomes as well as in autophagosomes. A similar localization for PI3P was reported in yeast (Wurmser and Emr, 1998) and animals (Obara *et al.*, 2008; Martin *et al.*, 2011), suggesting evolutionary conservation of its endomembrane distribution.

PI3P is known to be involved in autophagic regulation by recruitment of ATG proteins to membranes and delivery of vacuolar hydrolases for autophagic degradation (Levine and Klionsky, 2004), and was proposed to regulate the convergence of autophagosomes and PVC/MVB in animals (Robinson *et al.*, 1998). The presence of conserved PI-binding domains in plant ATGs (Thompson and Vierstra, 2005) suggested that PI3P may similarly regulate their membrane association. Yeast mutants of VPS34 showed severe defects in hydrolase sorting into vacuoles and therefore contained enlarged vacuoles (Wurmser and Emr, 1998). The plant homolog of yeast *VPS34*, when mutated, caused accumulation of neutral red-labeled granules inside the pollen grains (Lee *et al.*, 2008), which suggested a similar mechanism. It is an interesting possibility that reduction of PI3P levels, either by PTEN overexpression or by wortmannin treatment, caused a lack of vacuolar degradation and therefore accumulation of autophagic bodies.

Transport of PI3P into the vacuole as a component of the autophagosome was also induced by starvation conditions in yeast (Obara *et al.*, 2008). Based on studies in yeast, it was proposed that autophagy, lytic and endocytic pathways converge at the level of prevacuolar compartments (PVC) or multivesicular bodies (MVB) (Robinson *et al.*, 1998). It was shown previously that PI3P was transported via *trans*-Golgi network (TGN) to PVC and finally to the vacuole in plant cells (Kim *et al.*, 2001). We showed that overexpression of PTEN disrupted ARA6-positive endosomes, previously shown to be PVC/MVB in pollen tubes (Zhang *et al.*, 2010), but had little effect on ARA7-positive endosomes, which in pollen tubes are considered early endosomes. These results indicated that PI3P regulates autophagy most likely during the convergence between endocytic and autophagic trafficking.

Distinct features of plant PTENs might determine functional divergence

That Arabidopsis PTEN bound to PI3P was unexpected because the PBD domain is well conserved in PTENs among different species. In addition, the PBD domain of Arabidopsis PTEN localized at apical plasma membrane of pollen tubes (Figure 5) where PIP_2 is enriched (Kost *et al.*, 1999; Dowd *et al.*, 2006; Helling *et al.*, 2006). Such a different binding affinity was most likely due to the presence of plant-specific regulatory sequences. Indeed, plant PTENs contain conserved N-terminal sequences that are absent in animal PTENs. The glycine at the second position is a putative site for N-myristoylation, an irreversible lipid modification that anchors proteins to membranes. As was seen for mutations in the myristoylation site in ARA6 (Ueda *et al.*, 2001), the G2A mutation caused loss of PTEN signal at the plasma membrane along the pollen tube shank in all transformed pollen tubes, among which some still contained a few punctate vesicles. Membrane association of PTEN, especially at the punctate vesicles, was also compromised by deletion of the N-terminal leader sequence. The slight difference between the G2A mutation and the deletion of the N-terminal leader sequence might indicate that PTEN membrane association is regulated in ways still to be understood.

Plant PTENs are also distinguishable from animal PTENs in that no C2 domain is found at their C-termini. The C2 domains in animal PTENs contribute to membrane association (Das *et al.*, 2003) either by binding to transmembrane proteins or through conformational changes or electrostatic repulsion that results from changes in phosphorylation status (Leslie and Downes, 2004). Plant PTENs share conserved C-terminal sequences that have no discernable motifs but are enriched in potential phosphorylation sites. A C-terminally truncated Arabidopsis PTEN was associated more strongly at the lateral plasma membrane but was not detected at punctate vesicles (Figure 5), which suggested that the C-terminus contributed to the association of PTEN at punctate vesicles.

The localization of Arabidopsis PTEN at the lateral plasma membrane is notable; chemotactic cells such as mammalian neutrophils and *Dictyostelium discoideum* exhibit asymmetric membrane localization of PTEN at lateral and trailing plasma membranes and this localization is critical for their chemotactic migration. Mammalian PTEN binds dynamically to the plasma membrane for a few hundred milliseconds (Vazquez *et al.*, 2006). Upon application of chemoattractants, PTEN is dissociated rapidly from the plasma membrane and is re-localized to the side opposite the chemoattractant source (Funamoto *et al.*, 2002; Li *et al.*, 2005). Therefore the membrane association of animal PTENs has to be dynamic to cope with changing gradients of chemoattractants. Perhaps the distinct membrane association of PTEN at the lateral plasma membrane of pollen tubes is adaptive for their default pattern of polarized growth.

Autophagy in polarized growth of pollen tubes

Autophagy was considered previously to be a cellular process involved in adapting to harsh environments, such as nutrient starvation and abiotic stresses. However, more and more studies have indicated that autophagy occurs within the endomembrane trafficking system in various cell types even under normal conditions (Thompson and Vierstra, 2005; Bassham *et al.*, 2006). We observed constitutive autophagy using fluorescent ATG8 fusion proteins in pollen tubes growing *in vitro* (Figure 6). Pollen tube growth was arrested by PTEN overexpression (Figure 6) or by pharmacological treatment (Figure S5), which correlated with a disrupted ATG8 distribution pattern. The reason that *in vitro* germinated Arabidopsis pollen tubes did not exhibit extreme accumulation of autophagic bodies as seen in tobacco pollen tubes might be that pollen tube growth is more rapid and robust in tobacco, so that the overexpression phenotype develops more rapidly and is therefore obvious. Induced autophagy might be more critical for pollen tube growth *in vivo*. The polarized and directional growth of pollen tubes is a critical step to deliver sperm for double fertilization. Because female gametes are deeply embedded in the pistil, pollen tubes need to penetrate different female tissues, a stress-laden process involving production of reactive oxygen species (ROS) (Cheung and Wu, 2008). Overproduction of ROS during *in vivo* pollen tube growth might explain the gametophytic male sterility observed in transgenic Arabidopsis.

EXPERIMENTAL PROCEDURES

Plant materials, growth conditions and plant transformation

Arabidopsis plants were grown in a 4:1:1 mix of Fafard 4P:perlite:vermiculite under an 18-h-light/6-h-dark cycle at 21°C. The SALK_036717C and SALK_142405 T-DNA insertion lines were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University). The Col-0 *quartet1-2* (*qrt1*) mutant was used for pollen tube germination and reciprocal crosses. Tobacco SR1 was grown in a 1:1 mixture of perlite:vermiculite with slow-releasing fertilizer pellets. Tobacco pollen transformation and germination were as described (Kaothien *et al.*, 2005). Stable Arabidopsis transformation was done with the floral dipping method (Clough and Bent, 1998) in the *qrt1* background as described (Zhang *et al.*, 2009). Transgenic plants were selected on Murashige and Skoog medium supplemented with 30 mg mg^{-1} Basta salt (Sigma, http://www.sigmaaldrich.com/). The segregation ratio of progenies from selfed transgenic lines or from reciprocal crosses was determined by resistance or sensitivity to Basta selection.

DNA manipulations

PTEN, *PTENΔN_{29}*, *$PTEN_{G2A}$*, *PTENΔ$C_{212–412}$*, *$PTEN_{PBD}$*, PTENΔ$N_{1–211}$, *VPS34*, *α-TIP*, and *ATG8* were PCR-amplified from cDNA prepared from inflorescences and ligated into a pENTR/D-TOPO vector (Invitrogen) to generate entry vectors using the following primer pairs: *PTEN*: 5′-CACCATGGGTCTCAAGCTCTCACGAG-3′ and 5′- AGAG AGAGAAAGGTCATCGCGG-3′; *PTENΔN_{29}*: 5′- CACCATGAATTCTT ACCTACGTAACTTGGTGTCC-3′ and 5′- AGAGAGAGAAAGGTCAT CGCGG-3′; *$PTEN_{G2A}$*: 5′- CACCATGGCTCTCAAGCTCTCACGAGGG C3′ and 5′-AGAGAGAGAAAGGTCATCGCGG-3′; *PTENΔ$C_{212-412}$* 5′- CACCATGGCTCTCAAGCTCTCACGAGGGC-3′ and 5′- ACT GAACGAAAGTAAATCTGACCAGTAC-3′; *$PTEN_{PBD}$* 5′-CACCATGGG TCTCAAGCTCTCACGAG-3′ and 5′-GTCGAGATCATATCCACCG ATG-3′; PTENΔN_{1-211} 5′-CACCATGAAAAAAGGACCTCCCGAGG-3′ and 5′-AGAGAGAGAAAGGTCATCGCGG-3′; *VPS34*: 5′-CACCA TGGGTGCGAACGAGTTTCG-3′ and 5′-ACGCCAGTATTGAGCCCA TCTG-3′; *α-TIP*: 5′-CACCATGGCAACATCAGCTCGTAG-3′ and 5′-GTAATCTTCAGGGGCCAAGG-3.′ Entry vectors for *$PTEN_{C152S}$* and *$PTEN_{G157E}$* were generated by Phusion™ site-directed mutagenesis (Finnzyme, http://www.finnzymes.com) using the *PTEN* entry vector as template. The entry vector for *ATG8* (G22921) was obtained from ABRC. Expression vectors with YFP or CFP fusions were generated by LR reactions using LR Clonase II (Invitrogen, http://www.invitrogen.com) with previously described pollen-specific Gateway destination vectors (Zhang and McCormick, 2007). The CFP- and YFP-expression vectors were described previously (Zhang and McCormick, 2007). Expression vectors for ARA6–CFP, CFP–ARA7, and RabA4b–CFP were described previously (Zhang *et al.*, 2010).

All PCR (polymerase chain reaction) amplifications used Phusion™ hot start high-fidelity DNA polymerase with the annealing temperature and extension times recommended by the manufacturer (Finnzyme). All entry vectors were sequenced using an ABI 3300 sequencer and sequences were analyzed using Vector NTI (Invitrogen). The QIAquick® PCR purification kit, QIAprep® Spin miniprep kit and Qiagen TIP-100 kit (Qiagen, http://www.qiagen.

com/default.aspx) were used for PCR product recovery, DNA minipreps, and DNA midipreps, respectively.

Pharmacological treatments and fluorescent labeling of pollen tubes

Stock solutions of pharmacological drugs (Sigma) were prepared in dimethyl sulfoxide (DMSO) at the following concentrations: 1 mM for E-64d and 2.4 mM for wortmannin. Dilutions in DMSO were prepared and added to liquid pollen germination medium (PGM). Pharmacological drugs were added to PGM at final concentrations of 10 μM for E-64d and 5 μM for wortmannin. Lysotracker Red (Invitrogen, final concentration of 50 nM) and FM4-64 (Invitrogen, final concentration of 2.5 μM) were prepared according to manufacturers' instructions (Invitrogen). Equivalent volumes of DMSO were added to controls. Pharmacological drugs or fluorescent dyes were added to liquid PGM 2 h after the start of incubation.

Biolistic delivery of expression constructs in tobacco pollen

Transient expression assays in tobacco pollen were as described (Zhang and McCormick, 2007). Images were captured 2–8 h after germination. Every construct was tested three times and around 30 fluorescent tubes from each transformation were scored to ensure consistency of the reported phenotypes. Images and movies shown are representative of ~30 pollen tubes observed for each experiment at various time points between 2–8 h. Growth rates were calculated from the distance a transformed pollen tube grew in 1 min. In each experiment, 20–30 pollen tubes were measured for tube width and growth rate.

Microscopy and image processing

Microscopic imaging was performed using an inverted Axiophot microscope (Zeiss, http://www.zeiss.com) with either bright-field or epifluorescence optics, or with LSM510 laser scanning confocal microscope (Zeiss). Images were captured using a Spot digital camera (Diagnostic Instruments, http://www.diaginc.com/), exported using AxioVision software (Zeiss), and processed using Adobe Photoshop 7.0 (Adobe).

PI Strip binding assay

We used GST-PTEN described in Gupta *et al.* (2002) in the *in vitro* PI-binding assay. The *in vitro* PI-binding assay was according to manufacturer's instruction using PIP strip (Echelon, http://www.echelon.com), which contains 14 different kinds of lipids. PIP grip (Echelon), which binds specifically to PI(4,5)P_2, and GST were used as controls.

PI3P and PIP_2 delivery

Long chain (Di-C_{16}) synthetic PI3P and PIP_2 (Echelon) were prepared freshly at 300 μM in 150 mM sodium chloride, 4 mM potassium chloride and 20 mM HEPES at pH 7.2, and resuspended by bath sonication or vigorous vortexing. A histone carrier–PI3P complex was prepared by incubating 300 μM PI3P with 100 μM freshly prepared histone (Echelon), vortexed vigorously, incubated for 5 min at room temperature and diluted 1:10 with modified Hanks-buffered saline solution immediately before addition to pollen tubes that were growing in liquid PGM. Histone carrier prepared in a similar manner, but without PI3P, was used as the control.

ACKNOWLEDGEMENTS

We appreciate the kind gifts of the Pro_{LAT52}:2XFYVE–mRFP construct (Helling *et al.*, 2006) from Dr Benedikt Kost, of the Pro-LAT52:ERD2–GFP construct (Cheung and Wu, 2007) from Dr Alice Cheung, purified GST-PTEN (Gupta *et al.*, 2002) from Dr Sheng Luan and purified GST from Dr Weimin Ni. T-DNA insertion mutants were obtained from the ABRC (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm). This research was supported by the United States Department of Agriculture Current Research Information System (grant # 5335-21000-030-00D) to SM, by a grant (2007CB947600) from the Ministry of Science and Technology (MOST) of China to YZ, and by start-up funds from Shandong Agricultural University. YZ is supported by the Tai-Shan Scholar program. EF and JP were UC-Berkeley URAP (undergraduate research apprentice program) students.

SUPPORTING INFORMATION

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

Figure S1. Two insertion mutants in Arabidopsis *PTEN* did not affect *PTEN* mRNA levels.

Figure S2. Wortmannin treatment reduces PI3P levels in tobacco pollen tubes.

Figure S3. Tobacco pollen tubes overexpressing PTEN sequester 2XFYVE in autophagic bodies.

Figure S4. PTEN overexpression disrupted RabA4b-labeled transporting vesicles but had no effect on the ERD2-labeled Golgi apparatus.

Figure S5. Wortmannin treatment interfered with ATG8-labeled autophagosome dynamics.

Figure S6. Overexpression of *PTEN* caused pollen tube growth defects in Arabidopsis.

Movie S1. A tobacco pollen tube overexpressing PTEN-YFP at an early growth stage (between 2 and 3 h of incubation).

Movie S2. A pollen tube overexpressing PTEN-YFP after 5 h of incubation.

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