***Arabidopsis* AtVPS15 is essential for pollen development and germination through modulating phosphatidylinositol 3-phosphate formation**

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Abstract *Arabidopsis thaliana* phosphatidylinositol 3-kinase (AtVPS34) functions in the development and germination of pollen by catalyzing the biosynthesis of phosphatidylinositol 3-phosphate (PI3P). In yeast, Vps15p is required for the membrane targeting and activity of Vps34. The expression of *Arabidopsis thaliana* VPS15 (*AtVPS15*), an ortholog of yeast *Vps15*, is mainly detected in pollen grains and pollen tubes. To determine its role in pollen development and pollen tube growth, we attempted to isolate the T-DNA insertion mutants of *AtVPS15*; however, homozygous lines of *atvps15* were not obtained from the progeny of *atvps15/+* heterozygotes. Genetic analysis revealed that the abnormal segregation is due to the failure of transmission of the *atvps15* allele through pollen. Most pollen grains from the *atvps15/+* genotype are viable, with normal exine structure and nuclei, but some mature pollen grains are characterized with unusual large vacuoles that are not observed in pollen grains from the wild *AtVPS15* genotype. The germination ratio of pollen from the *atvps15/+* genotype is about half when compared to that from the wild *AtVPS15* genotype. When supplied with PI3P, in vitro pollen germination of the *atvps15/+* genotype is greatly improved. Presumably,

AtVPS15 functions in pollen development and germination by regulating PI3P biosynthesis in *Arabidopsis*.

Keywords AtVPS15 · Pollen germination · Vacuole · Phosphatidylinositol 3-phosphate · *Arabidopsis*

Introduction

In higher plants, pollen grains hydrate, germinate, and protrude pollen tubes on the compatible stigma which then grow into the style towards the ovules. The two sperm cells move in the growing pollen tube to enter the female gametophyte for completion of the process of double fertilization. Failure of pollen germination or pollen tube formation will abort fertilization and further seed development. Signaling molecules and genes required for pollen germination and pollen tube growth have been identified in plants (Hepler et al. 2001; Johnson and Preuss 2002; McCormick 2004; Ge et al. 2010). Phospholipids, comprising about 1% of membrane lipids, play critical roles in regulating plant development and environment response (Xue et al. 2009). Phosphatidylinositol 3-phosphate (PI3P) is one type of phospholipid involved in receptor signaling and membrane/vesicle trafficking (Petiot et al. 2003; Roth 2004). In plant cells, PI3P is synthesized in the trans-Golgi/endosome network and transported to the vacuole through the prevacuolar compartment (Kim et al. 2001). As an essential component of plant growth, PI3P signaling functions in diverse physiological processes such as pollen development, germination, and pollen tube growth (Lee et al. 2008a; Xue et al. 2009; Zhang and McCormick 2010).

Yeast Vps34 is a phosphatidylinositol 3-kinase (PI3K) catalyzing phosphoinositide to PI3P (Herman and Emr

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1990). *Arabidopsis* AtVPS34, a class III PI3K, is an ortholog of yeast Vps34 and is involved in the PI3P synthesis. Knockdown of AtVPS34 results in an embryo lethal phenotype in transgenic *Arabidopsis* (Welters et al. 1994). In root cells, the mutant of *pi3K/AtVPS34* shows reduced endocytosis and reactive oxygen species production under salt stress (Leshem et al. 2007). Pollen grains of the T-DNA insertion mutants of *AtVPS34* are sterile and are characterized by abnormal large vacuoles and a reduced number of nuclei (Lee et al. 2008b). In yeast, Vps34 forms a class III PI3K complex with Vps15p and Vps30 (ATG6) (Kihara et al. 2001). Yeast Vps15p is a Ser/Thr protein kinase containing HEAT repeats within its central region and a series of WD-40 domains within its C-terminus (Stack et al. 1993). Vps15p is required for the membrane targeting and activity of Vps34 (Yan and Backer 2007). The *Arabidopsis* AtVPS15 (At4g29380) was identified as the putative ortholog of yeast Vps15 (Mueller-Roeber and Pical 2002); however, its function in *Arabidopsis* development is unknown. In this study, we found that AtVPS15 was mainly detected in anthers. The pollen of the AtVPS15 mutants is defective in vacuolar morphology and pollen germination, but an exogenous supply of PI3P could restore pollen germination of mutants. Apparently, AtVPS15 regulates the formation of PI3P and plays an essential role during the development and germination of pollen grains.

Results

AtVPS15 appears to be an ortholog of yeast Vps15p and human hVps15

Arabidopsis AtVPS15 (At4g29380) contains 11 exons (Fig. 1a). The AtVPS15 predicted protein shares 22 and 27% identity overall with Vps15p of *Saccharomyces cerevisiae* and hVps15 of *Homo sapiens*, respectively (Herman et al. 1991a; Panaretou et al. 1997). The percent identity is much greater within conserved regions throughout the protein (Online Resource 1). Similar to Vps15p and hVps15, the N-terminus of AtVPS15 also contains the putative catalytic PKc domain of the protein kinase C (Fig. 1b). The lysine (K54, K149) and aspartic acid (D147) residues in the N-terminus of Vps15p are important for its enzymatic activity and vacuole localization (Herman et al. 1991b). These residues are strictly conserved in human hVps15. Similarly, the AtVPS15 protein is conserved also for these amino acid residues (K54, D149, and K151) (Online Resource 1). The N-terminus of Vps15p, namely (Met1)–Gly2–Ala3–Gln4–Leu5–Ser6–Leu7, could be myristoylated, where the glycine residue is required for this process (Herman et al. 1991a,

b). This myristoylation site is also conserved in AtVPS15 (Online Resource 1). In addition, Vps15p contains HEAT repeats, which interact with Vps34, at the central region, and a series of C-terminus WD-40 domains. There are two HEAT repeats and three WD-40 domains at the central and C terminus of the AtVPS15 protein (Fig. 1b and Online Resource 1). AtVPS15 protein is highly conserved in eukaryotes, including higher plants such as *Populus trichocarpa*, and *Vitis vinifera*, as well as with some lower plants (Fig. 1c).

Transcription of AtVPS15 and subcellular localization of AtVPS15

The qRT-PCR was employed to determine the expression pattern of AtVPS15 in *Arabidopsis*. AtVPS15 was abundantly expressed in anther tissues but low in other tissues and organs (Fig. 2a). We further investigated the AtVPS15 expression in anthers, pollen grains, and pollen tubes using in situ hybridization. The AtVPS15 cDNA was barely detected in microsporocytes of anthers at stage 6; however, its accumulation was high in tetrads at stage 7, microspores, mature pollen grains, and tapetum cells (Fig. 2b). AtVPS15 transcript also was detectable in the growing pollen tubes on the stigma (Fig. 2b). These results indicate that AtVPS15 may function in pollen development and pollen tube growth. The YFP-AtVPS15 fusion protein was used to localize the AtVPS15 protein in the tobacco pollen tubes. The fluorescent signal of YFP-AtVPS15 (from Lat52::YFP-AtVPS15) was distributed uniformly in the cytoplasm, which was similar to that of the empty-vector control (Lat52::YFP) (Fig. 2c).

The *atvps15* mutation reduces male gametophyte transmission

To study the function of AtVPS15 in *Arabidopsis* development, we used two T-DNA insertion lines of AtVPS15, GABI_015F04 (insertion in the 8th exon) and SALK_004719 (insertion in the 4th intron). The T-DNA insertions were confirmed by PCR analysis using the AtVPS15-specific primers and T-DNA-specific primers (Fig. 1a). Only plants heterozygous for the AtVPS15 mutation were identified after screening 40 and 35 seedlings of GABI_015F04 and SALK_004719, respectively. The heterozygous lines of GABI_015F04 and SALK_004719 were designated as *atvps15-1/+* and *atvps15-2/+*, respectively. Segregation analysis of the selfed progeny revealed that the ratios of wild-type/heterozygous plants from the *atvps15-1/+* and *atvps15-2/+* genotypes were 1.16:1 and 1.22:1, respectively (Table 1). No homozygous progeny were found for the AtVPS15 mutations. Therefore, the two independent T-DNA insertions in AtVPS15 lead to

Fig. 1 Molecular characterization of *AtVPS15* and identification of *atvps15-1/+* and *atvps15-2/+* insertion lines. **a** Schematic representation of *AtVPS15* and *atvps15-1/+* and *atvps15-2/+*. Exons of *AtVPS15* are represented by black boxes and introns by lines. The locations of the T-DNA insertion in the mutant lines are indicated with triangles. *LBs* primers for left border of the T-DNA, *RP*s and *LP*s gene-specific primers at downstream and upstream locations, respectively. **b** Predicated domains of the *AtVPS15*, yeast *Vps15p* (*Saccharomyces cerevisiae*, Genbank No. AAA35214), and human *hVps15* (*Homo sapiens*, Genbank No. NP055417) proteins. **c** Phylogenetic analysis of *AtVPS15* with its orthologs of eukaryotic proteins

segregation distortion indicating a gametophytic defect. Reciprocal crosses between wild-type plants and heterozygous mutants were carried out to test gametophytic transmission efficiency. About 88.0% and 89.2% of the mutant allele was transmitted via the female gametophytes of *atvps15-1/+* and *atvps15-2/+*, respectively, but no

Fig. 2 Expression pattern of *AtVPS15* and subcellular localization of *AtVPS15*. **a** Analysis of the relative expression level of *AtVPS15* in different organs of *Arabidopsis* by qRT-PCR. The experiment was repeated three times; error bars represent the standard errors of the means. **b** RNA in situ hybridization analysis for *AtVPS15* transcription in microsporocytes of anthers at stage 6 (*b1*), tetrads at stage 7 (*b2*), the microspores (*b3*), the tapetum cells (*b2* and *b3*), the mature pollen grains (*b4*), and the pollen tubes (*b6*) of *Arabidopsis*. *b5* and *b7* are the sense probe controls. Arrows indicate the pollen tubes on the surface of stigma. **c** Transient transfection of *Lat52::YFP-AtVPS15* and *Lat52::YFP* constructs in tobacco pollen tube. Bars 50 μ m in *b1*–*b5*, 20 μ m in *b6* and *b7*, and 10 μ m in *c*

transmission was observed via the male gametophytes (Table 2). Clearly, *AtVPS15* is necessary for the development of the fertile male gametophyte.

Pollen grains carrying the *atvps15* mutation are defective for pollen germination

The mature pollen viability was examined for heterozygous mutants of *atvps15-1/+* and *atvps15-2/+* using the

Table 1 Segregation analysis of selfed progeny of *atvps15/+* mutants

Genotype ^a	<i>AtVPS15/+</i>	<i>atvps15/+</i>	<i>atvps15/-</i>	Ratio
<i>atvps15-1/+</i> self-fertilized	158	138	0	1.16:1:0
<i>atvps15-2/+</i> self-fertilized	70	57	0	1.22:1:0

^a The genotype of selfed progeny of *atvps15/+* was analyzed by a PCR-based method as described in “Materials and Methods”

Table 2 Analysis of the genetic transmission of *atvps15* alleles

Genotype (Female × Male)	<i>atvps15/+</i>	<i>AtVPS15/+</i>	TE (%)
<i>atvps15-1/+</i> × Col-0	73	83	88.0 ^a
WT × <i>atvps15-1/+</i>	0	101	0
<i>atvps15-2/+</i> × WT	58	65	89.2
WT × <i>atvps15-2/+</i>	0	189	0

^a The genetic transmission of *atvps15* was analyzed by genotyping F1 progeny of the denoted crosses. The transmission efficient (TE) = (*atvps15/+*)/(*AtVPS15/+*) × 100% was calculated according to Howden et al. (1998)

Alexander’s staining procedure (Alexander 1969). All mature pollen grains of *atvps15/+* heterozygous mutants were red after staining (Fig. 3a), indicating that they were still viable up to this stage. In addition, the propidium iodide dyeing and scanning electron microscopy analysis revealed that the pollen exines of *atvps15-1/+* and *atvps15-2/+* lines were normal as that of the wild-type plants (Online Resource 2). Therefore, we decided to check whether pollen nuclear division is defective using DAPI labeling. Most pollen grains had three nuclei, and there was no significant difference in numbers of nuclei among the wild-type and heterozygous mutants (Fig. 3b, c). Taken together, the *atvps15* mutation did not cause detectable abnormality of the pollen grains. We further examined the germination of mature pollen from wild-type plants, *atvps15-1/+* line, and *atvps15-2/+* line. About 90.3% of the pollen grains from wild type could germinate after 4 h on pollen germination medium. In contrast, the germination ratios were 44.5% and 44.7% of *atvps15-1/+* and *atvps15-2/+* lines, respectively (Fig. 3d, e). These data implied that pollen grains carrying the *atvps15* mutation could not germinate and were responsible for the reduced male transmission.

The *atvps15* mutation affects vacuolar organization of pollen

The AtVPS34 (PI3K) protein is important for vacuolar organization, and the absence of AtVPS34 results in large vacuoles in pollen after the first mitosis (Lee et al. 2008b). Since Vps34 requires the Vps15p to be fully functional, it was of interest to investigate the vacuolar organization in

pollen grains of the *atvps15* mutants. We sectioned anthers of different developmental stages in wild-type and *atvps15/+* lines. At the early stage of bicellular pollen, one or several large vacuoles were observed in the wild-type, *atvps15-1/+* and *atvps15-2/+* mutants, and no structural difference was discovered in all tested lines (Fig. 4a–c). In the late stage of bicellular pollen, the large vacuoles disappeared, and many small ones were seen in wild-type pollen grains (Fig. 4d); however, the large vacuoles remained in some pollen grains in *atvps15/+* plants (Fig. 4e, f). At the mature tricellular stage of pollen grains, the vacuoles in wild-type pollen became smaller than that at the bicellular stage (Fig. 4g), while large vacuoles with irregular morphology were still present in some pollen grains in the *atvps15/+* anthers (Fig. 4h, i). In comparison, all pollen grains had normal vacuoles in the examined wild-type anthers at the mature tricellular stage, but 13.8% and 22.4% of pollen grains displayed irregular vacuoles in the *atvps15-1/+* and *atvps15-2/+* mutants, respectively (Table 3). Transmission electron microscopy revealed that, in mature pollen grains, the large and irregular vacuoles were present in the mutant plants but not in the wild-type plants (Fig. 4j–o). The large vacuoles contained deposits in their lumen that might be degraded cytoplasm (Fig. 4n, o; Lee et al. 2008; Whitley et al. 2009).

Exogenous PI3P supply restores pollen germination of the *atvps15* mutants

Both *atvps15* and *atvps34* mutants displayed similar phenotypes in vacuolar organization and pollen germination indicating that they are in a common pathway. As reported for yeast (Stack et al. 1993), Vps34 converts the phosphoinositide to PI3P, and Vps15p regulates the Vps34 activity. We hypothesized that the knockout of AtVPS15 impairs ATVPS34 activity and interferes with PI3P synthesis in pollen, which prevents pollen germination. Therefore, the germination of pollen from the *atvps15/+* lines was examined on normal germination media supplemented with PI3P and its carrier (Echelon, USA) and normal germination media supplemented with carrier only. The PIP3 and its carrier form complex that facilitates the transportation of PI3P into cells. The pollen germination of the wild-type and *atvps15/+* lines did not show significant change on medium with carrier only (Figs. 5a, 3e). About 79.4 and 78.9% of pollen grains from *atvps15-1/+* and *atvps15-2/+* lines, respectively, germinated on the PI3P-supplemented media, whereas the germination ratio of wild-type pollen was reduced under the same conditions (Fig. 5b, c). Therefore, the exogenous PI3P supply restores the pollen germination in *atvps15* mutants, and the mutation of AtVPS15 reduces the PI3P level in pollen that prevents the pollen germination in the *atvps15* mutants.

Fig. 3 Pollen viability and germination of *atvps15*/+ lines and wild-type (WT) plants. **a** Alexander's staining to detect the viability of mature pollen. **b** Mature pollen grains stained with DAPI showing that most pollen contained three nuclei. Bars 10 μ m. **c** Statistics of the mature pollen with three nuclei. The experiment was repeated three times; error bars represent the SE of the mean. $n = 325$ (wild-type), 364 (*atvps15-1*/+), and 346 (*atvps15-2*/+). **d** Pollen germination on medium. **e** Statistics of the germination ratio of the pollen on medium. The experiment was repeated three times; error bars represent the standard error of the mean; $n = 1,636$ (wild-type), 1,660 (*atvps15-1*/+), and 1,353 (*atvps15-2*/+)

Discussion

Phosphoinositides function in membrane trafficking and polarized growth of pollen tube (Monteiro et al. 2005; Samaj et al. 2006; Zonia and Munnik 2008; Ischebeck et al. 2008; Sousa et al. 2008). In yeast, Vps15p plays its role by forming a class III PI3K complex with Vps34 and Vps30. Vps34 has lipid kinase activity and functions in PI3P synthesis (Herman and Emr 1990). In *Arabidopsis*, the *atvps34* mutation impairs the production of PI3P and results in failure of pollen germination (Lee et al. 2008b). In yeast, Vps15 is required for membrane targeting and function of Vps34 (Stack et al. 1993). Our results revealed

that AtVPS15 is important for pollen development and germination, and further that an exogenous supply of PI3P improves the pollen germination of the *atvps15* mutants. Due to the close phenotype between the *atvps15* mutants and the *atvps34* mutants, AtVPS15 potentially interacts with AtVPS34 and regulates AtVPS34 activity and PI3P synthesis in vivo, as in yeast (Stack et al. 1993). Additionally, AtAtg6 (AtVPS30) plays roles in pollen germination of *Arabidopsis*. The mutation of *AtAtg6* leads to failure of pollen germination (Fujiki et al. 2007; Qin et al. 2007; Harrison-Lowe and Olsen 2008). PI3K complex is involved in autophagy and vacuolar protein sorting (VPS) in yeast (Yorimitsu and Klionsky 2005). However, whether

Fig. 4 Vacuolarization in bicellular and tricellular pollen grains of *atvps15/+* lines and wild-type (WT) plants. Pollen grains at the early bicellular (a through c), late bicellular (d through f) and mature (g through o) stages of wild-type (left lane), *atvps15-1/+* (middle lane), and *atvps15-2/+* (right lane) plants. a–i are the photos of semi-thick sections; j–o are the photos of ultrathin sections by transmission electron microscopy. m–o are the higher magnifications of the square areas in j–l, respectively. Arrowheads indicate the pollen grains containing abnormal vacuoles of *atvps15/+* lines. Arrows indicate the deposits in irregular vacuoles of *atvps15/+* pollen grains. v vacuole. Bars 20 μ m (a–i), 2 μ m (j–l), and 1 μ m (m–o)

the *Arabidopsis* Atg-regulated autophagy plays a role in pollen germination remains unknown (Fujiki et al. 2007; Qin et al. 2007; Harrison-Lowe and Olsen 2008).

In *Arabidopsis*, PI3K can phosphorylate the phosphatidylinositol at the D-3 position to generate the PI3P, and then the PIKfyve/Fab1 family will phosphorylate the PI3P

Table 3 Population of mature pollen with abnormal vacuoles of *atvps15/+*

Genotype	Abnormal pollen
WT	0% (0/68)
<i>Atvps15-1/+</i>	13.84 ± 0.48% (15/123)*
<i>Atvps15-2/+</i>	22.41 ± 2.51% (17/94)*

The mature pollen grains with abnormal vacuoles were observed and counted from the semi-thin sections as described in “Materials and Methods”. Actual numbers of pollen grains counted are indicated in parentheses (Abnormal pollen/total count). The results from three independent experiments were combined for statistical analysis (Student's t test, * $P < 0.01$)

at the D-5 position to produce the phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂) (Cantley 2002). *Arabidopsis* FAB1A and FAB1B are orthologs of the PIKfyve/Fab1 family proteins. The *Arabidopsis fab1a/fab1b* mutants are defective in pollen development and germination, which may be caused by the decrease of the PI(3,5)P₂ level in

pollen (Whitley et al. 2009). ATVPS15 may affect the PI(3,5)P₂ synthesis in pollen by regulating ATVPS34 activity and the PI3P level. The similar vacuolar phenotype of *atvps15*, *atvps34* and *fab1a/fab1b* mutants provides an additional line of evidence. Studies in non-plant cells revealed that PI(3,5)P₂ mediates the fission of vacuoles (or its equivalent organelles) by increasing the surface-to-volume ratio of intracellular organelles (Weisman 2003; Sbrissa and Shisheva 2005). Although the *atvps15*, *atvps34*, and *fab1a/fab1b* mutants are similar in pollen germination, they differ in other stages during the pollen development. The *atvps34* and *fab1a/fab1b* mutants associate with reduced pollen viability, but some pollen grains of *fab1a/fab1b* mutant are collapsed; however, *atvps15* pollen had normal viability and morphology. Additionally, the *atvps15* mutants associate with the largest fraction of pollen with normal vacuoles, while the *fab1a/fab1b* mutant has the least fraction of pollen with normal vacuoles. The different levels of PI(3,5)P₂ in these mutants may lead to

Fig. 5 Pollen germination of *atvps15/+* heterozygous and wild-type (WT) plants on media supplemented with exogenous PI3P carrier with and without PI3P. **a** Pollen germination on the medium adding exogenous carrier for PI3P. **b** Pollen germination on the medium adding exogenous PI3P and its carrier. **c** Statistics of pollen germination on the medium containing PI3P and its carrier. The experiment was repeated three times; error bars represent the standard error of the mean. n = 816 (wild-type), 800 (*atvps15-1/+*), and 837 (*atvps15-2/+*) on medium containing carrier and = 810 (wild-type), 874 (*atvps15-1/+*), and 842 (*atvps15-2/+*) on medium containing PI3P and carrier

different phenotypes in pollen development. How ATVPS15, ATVPS34, and FAB1A/FAB1B contribute to the PI(3,5)P₂ accumulation in pollen is likely conditioned by their position in the PI(3,5)P₂ metabolism pathway. FAB1A/FAB1B directly catalyzes the production of PI(3,5)P₂ and the *fab1a/fab1b* mutations cause significant changes in PI(3,5)P₂ level. ATVPS15 may only cause minor change in PI(3,5)P₂ level since the ATVPS15 controls the PI3P accumulation by regulating the ATVPS34 activity. Alternatively, AtVPS15, AtVPS34, and FAB1A/FAB1B may be involved in other pathways that regulate pollen development and germination.

Materials and methods

Plant materials

Two T-DNA insertion lines of *Arabidopsis thaliana* (Col-0 ecotype) of *AtVPS15* (GABI_015F04 and SALK_004719) were obtained from NASC and ABRC, respectively. The genotypes of T-DNA insertion line plants were determined by a PCR-based method. The following primers were used: LB1 (5'-ATATTGACCATCATACTCATTG-3'), LP1 (5'-CTCCGTCTGATCACTCAAAGC-3'), and RP1 (5'-CAATGAACGCAAGCACAGTAC-3') for GABI_015F04 and LB2 (5'-ATTTTGGCCGATTTCCGGAAC-3'), LP2 (5'-GAATGATTTTGCAGCTTCTGG-3'), and RP2 (5'-CAAGAGAATCGATGCGAAAAG-3') for SALK_004719. The wild-type *Arabidopsis* and T-DNA insertion plants were grown at 22°C in a 16/8 h light/dark cycle.

Pollen analysis and microscopy

For pollen staining, mature pollen grains or the anthers were used. The mature anthers were soaked in Alexander stain for 1–2 days to detect pollen viability according to Alexander (1969) under a BX51 microscope (Olympus, www.olympus.com). To observe the exines of pollen grains, we used 10 µg/ml (w/v) propidium iodide (Sigma, www.sigmaaldrich.com) to stain mature pollen grains followed by observation under Zeiss CLSM 510 meta (www.zeiss.com), according to Tian et al. (2006). The nuclei of pollen grains were stained with 1 µg/ml (w/v) DAPI (Sigma, www.sigmaaldrich.com) for 30 min and observed by epifluorescence microscopy (Olympus BX51 microscope). For the sections of anthers and pollen grains, the materials were embedded in Spurr resin and after-fixed with a solution of 4% (w/v) glutaraldehyde and 1% (w/v) OsO₄ in phosphate buffer (pH 7.2). The semi-thick sections were stained with 0.5% (w/v) toluidine blue-O (Sigma, www.sigmaaldrich.com) and observed with an Olympus BX51 microscope.

Pollen germination in vitro

To analyze pollen germination in vitro, the pollen grains at anthesis of wild-type *Arabidopsis* and T-DNA insertion lines were collected and cultured on medium containing 1 mM CaCl₂, 1 mM Ca(NO₃)₂, 1 mM MgSO₄, 0.01% (w/v) H₃BO₃, and 18% (w/v) sucrose solidified with 0.5% (w/v) agar (pH 7.0). To analyze the role of PIP3 in pollen germination, PIP3 (PtdIns(3)P di-C₁₆, P-3016) and its carrier (Carrier 3, P-9C3) (Echelon Biosciences Inc, www.echelon-inc.com) were added into the pollen germination medium according to the manufacturer's recommendation. In medium, the ratio of PIP3 and carrier is 1:1 to form the PIP3–carrier complex at a final concentration of 6.25×10^{-3} mg/ml. The pollen grains were cultured immediately at 28°C, 100% relative humidity, and cool light at 30 mmol/m²s. The germinated pollen grains were counted and images were captured at 4 h after germination.

Quantitative Real-time PCR (qRT-PCR)

The *Arabidopsis* seedlings of 3-week-old (for seedling, root and leaf) and 5-week-old (for stem, flower, anther, pistil and silique) were collected for *AtVPS15* expression analysis. Total RNA was isolated with the Trizol isolation reagent (Invitrogen, www.invitrogen.com) for reverse transcription. qRT-PCR was conducted to determine *AtVPS15* transcript levels with the primer pairs *VPS15*RT-sense (5'-GGAA GTTACCAAGCAATAAGG-3') and *VPS15*RT-antisense (5'-AGAGCGGATACCAGGAAG-3'). *Tubulin* was used as a quantitative control and amplified using the primers, *TUB2*-RT-sense (5'-ATCCGTGAAGAGTACCCAGAT-3') and *TUB2*-RT-antisense (5'-AAGAACCATGCACTCAT CAGC-3'). The line arrangement of the PCR products was established by comparing samples that were run for different numbers of cycles. All experiments were repeated three times.

Transient expression YFP fusion protein in tobacco pollen tubes

Transient expression of YFP-AtVPS15 fusion protein in pollen tubes of tobacco was used to study the intracellular localization of AtVPS15. The full-length cDNA of *AtVPS15* was amplified by PCR using GATEWAY-compatible primers *VPS15*-sense (5'-CACCATGGGAAACA AAATCGCTCGTACGACAC-3') and *VPS15*-antisense (5'-TTACTTCCAGACCTTTATGGCTCCATCT-3') and cloned into the pENTRY/D-TOPO vector (Gateway, Invitrogen, www.invitrogen.com) for sequencing, and then recombined into pAL20 to create Lat52::YFP-AtVPS15 construct. The Lat52::YFP construct was used as the control. Each construct was introduced into tobacco (*Nicotiana*

tabacum) pollen using a pneumatic particle gun (PDS-1000/He; Bio-Rad, www.bio-rad.com), as described by Zhang and McCormick (2007). After 4–8 h cultivation on medium, the pollen grains were visualized under a fluorescence microscope (Olympus BX51).

RNA in situ hybridization

RNA in situ hybridization was performed as previously described by Li et al. (2010). The specific *AtVPS15* probe is a 570-bp fragment amplified in the coding region of *AtVPS15* with the primers VPS15-IF (5'-TCACAAGGGA GGTAGTTTATCG-3') and VPS15-IR (5'-GCTTAGGCA CTGGGACCGAACT-3'). The samples were observed using an Olympus BX51 microscope.

Electron microscopy

For the observation of pollen morphology, mature pollen grains coated with gold palladium were observed under a JSM-6610LV scanning electron microscope (JEOL, Japan) and photographed. Transmission electron microscopy was performed as described by Wang et al. (2009), with modification. The mature anthers were fixed in 4% (v/v) glutaraldehyde and 1% (w/v) osmic acid (Sigma). After washing, the specimens were dehydrated in a graded ethanol series and embedded in Epon 812 resin (SPI-CHEM, USA). Ultrathin sections were stained with acetate uranium and lead citrate. The specimens were observed using a JEM-1200EX transmission electron microscope (JEOL, Japan).

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