The Arabidopsis Phosphatidylinositol 3-Kinase Is Important for Pollen Development[1][W][OA]

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Phosphatidylinositol 3-kinase has been reported to be important for normal plant growth. To characterize the role of the enzyme further, we attempted to isolate Arabidopsis (*Arabidopsis thaliana*) plants that do not express the gene, but we could not recover homozygous mutant plants. The progeny of *VPS34/vps34* heterozygous plants, harboring a T-DNA insertion, showed a segregation ratio of 1:1:0 for wild-type, heterozygous, and homozygous mutant plants, indicating a gametophytic defect. Genetic transmission analysis showed that the abnormal segregation ratio was due to failure to transmit the mutant allele through the male gametophyte. Microscopic observation revealed that 2-fold higher proportions of pollen grains in heterozygous plants than wild-type plants were dead or showed reduced numbers of nuclei. Many mature pollen grains from the heterozygous plants contained large vacuoles even until the mature pollen stage, whereas pollen from wild-type plants contained many small vacuoles beginning from the vacuolated pollen stage, which indicated that vacuoles in many of the heterozygous mutant pollen did not undergo normal fission after the first mitotic division. Taken together, our results suggest that phosphatidylinositol 3-kinase is essential for vacuole reorganization and nuclear division during pollen development.

Phosphatidylinositol 3-phosphate [PtdIns(3)P] is a phosphoinositide that exists only at very low levels in plant cells (Brearley and Hanke, 1992; Meijer and Munnik, 2003). PtdIns(3)P is essential for normal plant growth (Welters et al., 1994) and has been implicated in diverse physiological functions, including root nodule formation (Hong and Verma, 1994), auxin-induced production of reactive oxygen species and root gravitropism (Joo et al., 2005), root hair curling and *Rhizobium* infection in *Medicago truncatula* (Peleg-Grossman et al., 2007), increased plasma membrane endocytosis and the intracellular production of reactive oxygen species in salt tolerance response (Leshem et al., 2007), stomatal closing movement (Jung et al., 2002; Park et al., 2003), and root hair elongation (Lee et al., 2008). Cellular and biochemical mechanisms of action of PtdIns(3)P are the focus of intense investigation. In yeast, PtdIns(3)P is essential for vesicle-mediated delivery of vacuolar enzymes (Stack and Emr, 1994), and a similar role of PtdIns(3)P in vesicle trafficking has been reported in plant and animal cells as well (Brown et al., 1995; Davidson 1995; Matsuoka et al., 1995). In animal cells, inhibition of PtdIns(3)P synthesis by pharmacological agents impairs the targeting of procathepsin D from the trans-Golgi network to the lysosomal compartment (Brown et al., 1995; Davidson, 1995). In plants as well, overexpression of PtdIns(3)P-binding protein in Arabidopsis (*Arabidopsis thaliana*) protoplasts inhibits trafficking of the vacuolar protein sporamin (Kim et al., 2001). A similar failure to deliver vacuolar proteins in tobacco (*Nicotiana tabacum*) suspension cells treated with pharmacological agents that interfere with PtdIns(3)P synthesis was reported (Matsuoka et al., 1995). Moreover, PtdIns(3)P inhibitors cause swelling or vacuolation of the prevacuolar compartment (Tse et al., 2004) and block retrograde transport of vacuolar sorting receptors to the trans-Golgi network (daSilva et al., 2005; Oliviusson et al., 2006). However, trafficking to the vacuole alone may not be sufficient to explain the diverse and profound effects of PtdIns(3)P on growth and physiology of

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plants, and further studies at the cell and biochemical levels are necessary to elucidate the mechanisms of action of the lipid.

The enzyme that produces PtdIns(3)P is phosphatidylinositol 3-kinase (PI3K), which phosphorylates the D-3 position of phosphoinositides. Three classes of PI3K have been defined based on sequence homology and their selective in vitro substrate specificity (Wymann and Pirola, 1998). However, in plants, genes that encode class I or II PI3K are missing, and only the class III PI3K, which makes PtdIns(3)P from PtdIns, has been identified (Hong and Verma, 1994; Welters et al., 1994). The prototype for this type of enzyme, Vps34p, was first identified in *Saccharomyces cerevisiae*, in which it is required for the delivery of soluble proteins to the vacuole (Herman et al., 1992; Schu et al., 1993). Subsequently, a human homolog was identified, and Vps34p-related PI3Ks are now known to exist in a range of other eukaryotes, including *Dictyostelium* (Zhou et al., 1995), *Drosophila* (Linassier et al., 1997), and plants (Hong and Verma, 1994; Welters et al., 1994).

In plants, PI3K is encoded by a single-copy gene, *AtVPS34*. It is essential for normal growth, as demonstrated by the expression of *AtVPS34* antisense constructs, which leads to second-generation transformed plants with very severe defects in growth and development (Welters et al., 1994). Plant PI3K is associated with active nuclear and nucleolar transcription sites (Bunney et al., 2000), which led to the suggestion that it plays a role in active transcription. Together with the fact that there is no other enzyme that can produce PtdIns(3)P, a lipid with many physiological effects in plants, these reports indicate that Vps34p-like PI3K has much broader functions in plants than in animals or budding yeast. However, the severe developmental defect makes mutant analysis difficult, and studies of Vps34p-mediated processes in specific tissues have been lacking.

This study began with our attempt to isolate homozygous T-DNA insertional knockout-PI3K Arabidopsis plants. We discovered that the self-fertilized heterozygous plants produced progeny that segregated 1:1 for wild-type and heterozygous plants, and no homozygous mutant plants were recovered. Reciprocal crosses between heterozygous and wild-type plants revealed a reduction in transmission of the T-DNA insertion allele through the male gametophyte. Cell biological observations confirmed that some male gametophytes of the heterozygous mutant plants had defects that indicated an important role for PI3K during male gametophyte development.

RESULTS

The Progeny of *VPS34/vps34* Heterozygous Mutant Plants, Harboring a T-DNA Insertion Allele, Show Distorted Segregation Ratios

To identify Arabidopsis T-DNA insertion mutants at the *VPS34* locus (At1g60490), we screened the Salk Institute collections using PCR-based genotyping and identified one line, SALK_007281 or *vps34-1*, which contained T-DNA at position +2,388 relative to the ATG of *VPS34* (Fig. 1). This line of *VPS34/vps34-1* mutant was sensitive to kanamycin although it harbored T-DNA, which necessitated PCR-based analyses of the genotypes. To identify the homozygous knockout mutant plants, hundreds of seedlings grown from the SALK_007281 seeds and their progeny were harvested and DNA was extracted. In PCR-based genotyping with combinations of different gene-specific primers and the T-DNA border primer, no homozygous mutant plants could be identified. A second T-DNA insertion allele (*vps34-2*; GABI_418H02), which contained a T-DNA at the fifth exon, also did not produce homozygous mutant plants when self-fertilized. Since there were no available homozygous mutant plants, we sought to determine the genetic basis for this phenomenon. Segregation ratios for the self-fertilized progeny of *VPS34/vps34-1* and *VPS34/vps34-2* were determined. PCR analysis of offspring from self-pollinated *VPS34/vps34* plants revealed the ratio for heterozygous and wild-type plants to be 0.83:1 and 1.02:1 in *VPS34/vps34-1* and *VPS34/vps34-2*, respectively (Table I). The segregation ratios for both lines were not significantly different from 1:1, instead of the expected 3:1 ratio, suggesting a gametophytic defect.

VPS34/vps34 Mutants Show Reduced Male Transmission

To determine which gametophyte caused the abnormal segregation ratio, we tested male and female transmission efficiency (TE) by performing reciprocal crosses between heterozygous and wild-type plants and analyzed the F1 progeny by PCR-based genotyping. With the *vps34-1* allele, TE was reduced predominantly through the male and was not significantly reduced in the female (Table II, top). To verify that one-sided TE was not due to self-pollination, crosses were performed between both alleles of *VPS34/vps34* heterozygous plants and homozygous *gl1* mutant plants deficient in *GLABROUS1* (*GL1*). *GL1* is essential for the

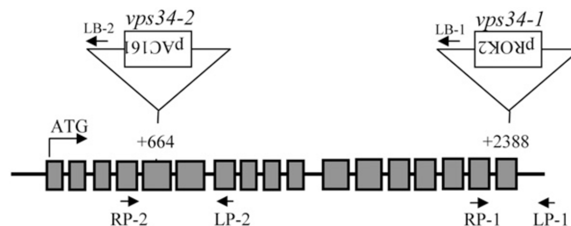

Figure 1. Identification and molecular characterization of *VPS34/vps34-1* and *VPS34/vps34-2*. Exon and intron structure of the Arabidopsis *VPS34* gene, showing the locations of insertion sites in T-DNA mutants. Boxes indicate exons. LB, Left border of the T-DNA; ROK2 and AC161, T-DNA present in the mutant lines provided by the Salk Institute and GABI, respectively; RP and LP, gene-specific primers with which the precise T-DNA integration sites were identified.

Table I. *Segregation analysis of VPS34/vps34-1 and VPS34/vps34-2*

The inheritance of *vps34* was analyzed using PCR-based genotyping as described in "Materials and Methods." The χ^2 test was used to compare the observed ratios with a predicted ratio of 1:1.

Cross	PCR^+	PCR^-	Ratio	χ^2
VPS34/vps34-1 self-fertilized	179	215	0.83:1	3.3 ($P < 0.05$)
VPS34/vps34-2 self-fertilized	94	92	1.02:1	0.02 ($P < 0.05$)

initiation of trichome development, and *gl1/gl1* plants are devoid of trichomes, whereas *GL1/gl1* plants have normal trichome numbers and morphology (Marks and Feldmann, 1989; Esch et al., 1994). The presence of normal trichomes on all F1 progeny from the crosses and the presence of plants without trichomes among F2 progeny indicated that the reciprocal crosses were successful (data not shown). The analyses of F1 progeny of the crosses of *VPS34/vps34-1* and *gl1/gl1* (Table II, middle) and *VPS34/vps34-2* and *gl1/gl1* (Table II, bottom) confirmed the results from crosses of *VPS34/vps34-1* and wild-type plants (Table II, top). When crossed with a *gl1/gl1* female, the TE of *vps34-1* from the male parent was only 1.6% and that of *vps34-2* was 0%. Thus, we conclude that male gametophytes with a *vps34* allele almost always fail to produce progeny.

Expression of the *VPS34* Gene in Arabidopsis

To determine the site of *VPS34* expression during flower development, we generated transgenic Arabidopsis plants harboring the *VPS34* promoter::GUS reporter construct. We cloned the upstream promoter region of *VPS34* (−1,391 to +125 in relation to ATG) and generated a translational fusion with the *GUS* coding sequence in the *pBI121* vector. This construct was introduced into Arabidopsis, and the transformants were analyzed for *GUS* expression. Seven independent lines were tested for GUS activity, and all of them showed similar expression patterns. GUS staining was observed in almost all vegetative organs and reproductive organs, including leaves, roots, stems, floral buds, and open flowers (Fig. 2, C and D). The expression pattern was confirmed by RT-PCR analysis, which showed that transcript for *VPS34* was present in seedlings, leaves, roots, stems, green buds, open flowers, and pollen (Supplemental Fig. S1). *VPS34::GUS* was detected in flowers of all different developmental stages from early flower bud to mature flower (Fig. 2D). In flower buds, *VPS34::GUS* was detected in pollen mother cells (Fig. 2E) and immature microspores (Fig. 2F), and in open flower, it was highly expressed in mature pollen (Fig. 2G). *VPS34::GUS* was also expressed in the carpels, stigmas, and sepals (Fig. 2G).

Loss of VPS34 Function Disrupts Pollen Development

The reduction in TE via the male germ line in *VPS34/vps34-1* plants suggests a role for VPS34 in pollen development and/or function. Biochemical comparison of PI3K or its product PtdIns(3)P levels between wild-type and mutant pollen grains is not likely to yield unequivocal results with currently available approaches, since homozygous plants are not possible to obtain, and also because the PtdIns(3)P level is very low in plants. Instead, we used cell biological methods to examine changes in the mutant pollen grains. To examine defects in *VPS34/vps34* plants, cell viability was tested with propidium iodide, which is excluded from live cells but stains dead cells (Huang et al., 1986), and with fluorescein diacetate (FDA), which stains live cells only (Heslop-Harrison and Heslop-Harrison, 1970). When pollen grains were double stained with FDA and propidium iodide, nearly all of the pollen from wild-type plants and some of the pollen grains from *VPS34/vps34* plants showed green fluorescence with FDA (Fig. 3, B and D). Substantially more pollen grains from mutant plants, compared with wild-type plants, showed red fluorescence (Fig. 3D). Quantitative analyses revealed that the percentage of live pollen grains from heterozygous mutant plants was lower than that of wild-type plants ($P < 0.01$; Fig. 3E): the percentage of live pollen grains was 82% ± 1%, 68% ± 2%, and 65% ± 2% in wild-type, *VPS34/vps34-1*, and *VPS34/vps34-2* plants, respectively.

Next, the number of nuclei in pollen grains was observed to examine whether mutant plants have problems with pollen nuclear divisions. Pollen grains were collected from mature anthers, and the number of nuclei was visualized with 4′,6-diamidino-2-phenylindole (DAPI). The result revealed that 95% ± 1% of the pollen from wild-type plants was trinucleate, whereas 82% ± 1% and 86% ± 1% of the pollen from *VPS34/vps34-1* and *VPS34/vps34-2* plants, respectively, were trinucleate (Fig. 3I). Among the pollen population with less than three nuclei in heterozygous mutants, the largest proportion had two nuclei (Supplemental Table S1). The significantly reduced percentage of pollen with less than three nuclei from *VPS34/vps34* plants ($P < 0.01$) suggests that pollen carrying the mutant allele often failed in nuclear division and could not reach the final stages of development.

Pollen Grains from *VPS34/vps34* Plants Are Defective in Vacuolar Reorganization following the First Mitosis

Since PtdIns(3)P is important for trafficking of vacuolar proteins in somatic plant cells (Matsuoka et al., 1995; Kim et al., 2001; Oliviusson et al., 2006), and because dynamic changes in vacuolar morphology is a prominent feature during pollen development (Yamamoto et al., 2003), we were curious whether the

Table II. *Analysis of the genetic transmission of VPS34/vps34-1 and VPS34/vps34-2*

The inheritance of *vps34* was analyzed by genotyping F1 progeny of the denoted crosses. The TE was calculated according to Howden et al. (1998): TE (%) = (observed PCR$^+$/observed PCR$^-$) × 100. Col0, Ecotype Columbia wild type.

Genotype (Female × Male)	PCR$^+$	PCR$^-$	TE (%)
VPS34/vps34-1 × Col0	27	26	103.9
Col0 × *VPS34/vps34-1*	0	48	0.0
VPS34/vps34-1 × *gl/gl*	52	52	100.0
gl/gl × *VPS34/vps34-1*	2	128	1.6
VPS34/vps34-2 × *gl/gl*	90	74	121.6
gl/gl × *VPS34/vps34-2*	0	106	0.0

absence of PI3K expression results in altered vacuolar morphology of pollen grains. Neutral red, a dye that primarily accumulates in vacuoles and vesicles (Mahlberg, 1972), was used to test this possibility. Wild-type pollen grains showed red staining in numerous membrane-bound structures containing fine fibrillar substances (Fig. 4A), as described previously by Yamamoto et al. (2003). Although some pollen grains of the *VPS34/vps34* plants showed vacuoles similar to those of wild-type plants, others contained large vacuoles that are rarely found in wild-type pollen grains (Fig. 4B). Quantitative analyses showed that pollen grains from *VPS34/vps34* plants contained higher percentages of these abnormal vacuoles (28% ± 3% and 23% ± 3% for *VPS34/vps34-1* and *VPS34/vps34-2* plants, respectively) compared with wild-type plants (3% ± 1%; $P < 0.01$; Fig. 4C).

To identify the exact stage of pollen development when vacuoles become aberrant, we sectioned and observed anthers from wild-type and *VPS34/vps34-1* plants with a light microscope. Anthers were separated into four different developmental stages, including tetrad of microspores, vacuolate pollen, binucleate pollen after the first mitotic division, and trinucleate pollen. We found that pollen from wild-type and mutant plants did not show any significant differences at the tetrad stage (Fig. 5, A, E, and I) or at the vacuolated pollen stage (Fig. 5, B, F, and J). Neither was there a clear difference between wild-type and mutant pollen when the large vacuoles started to fragment into smaller ones following the first mitotic division, which gives rise to a vegetative cell and a generative cell (Fig. 5, C, G, and K). However, after the second mitotic division, which gives rise to two sperm cells, pollen grains from *VPS34/vps34-1* and *VPS34/vps34-2* plants were often distinctly different from those of wild-type plants. In wild-type pollen, the remaining large vacuoles underwent further fragmentation to form numerous small dispersed vacuoles (Fig. 5D), whereas many pollen of mutant plants still contained large and abnormally shaped vacuoles (Fig. 5, H and I, arrowheads). This abnormal vacuolar phenotype was found in 33.8% ± 4.1% (n = 998 pollen grains from eight flower buds) and 36.3% ± 2.6% (n = 1,225 pollen grains from 11 flower buds) of pollen from *VPS34/vps34-1* and *VPS34/vps34-2* plants, respectively, but in only 4.8% ± 1.6% (n = 704 pollen grains from seven buds) of pollen from wild-type plants.

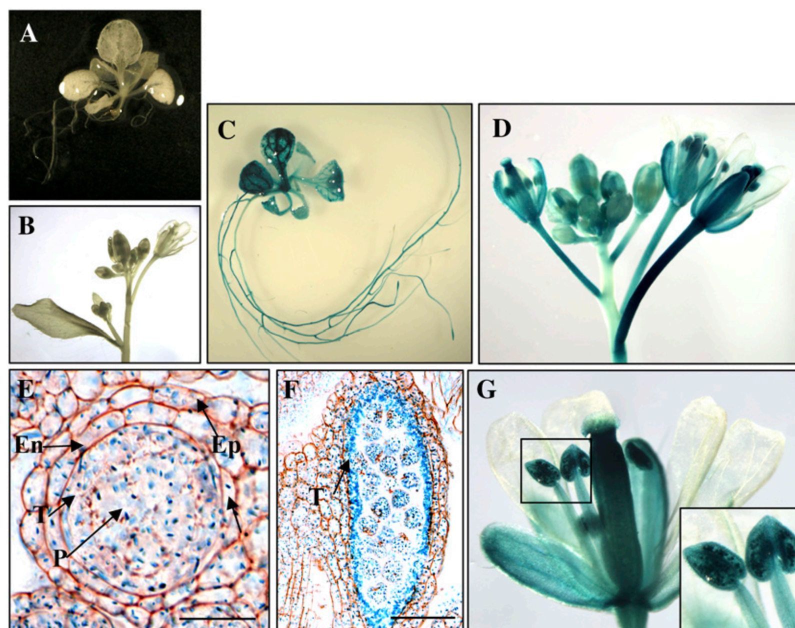

Figure 2. Tissue-specific expression of *VPS34* in Arabidopsis plants. A and B, GUS activity of a control plant lacking the *GUS* reporter gene. C to G, Transgenic plants harboring the *VPS34* promoter::*GUS* reporter construct were generated and analyzed histochemically for GUS activity (indicated by blue) in a 2-week-old plant (C), a flower stalk with a cluster of flower buds and flowers (D), pollen mother cells (E), immature microspores (F), and a mature flower (G). E and F show photomicrographs of 10-μm-thick sections of anthers in transverse and longitudinal directions, respectively. A short-term staining with propidium iodide gives red-brown color to the cell walls. In E, pollen mother cells and surrounding layers of tapetum, endothecium, and epidermis cells show GUS activity. In F, immature microspores and the surrounding cells show GUS activity. En, Endothecium; Ep, epidermis; P, pollen mother cells; T, tapetum. Bars = 20 μm (E) and 50 μm (F).

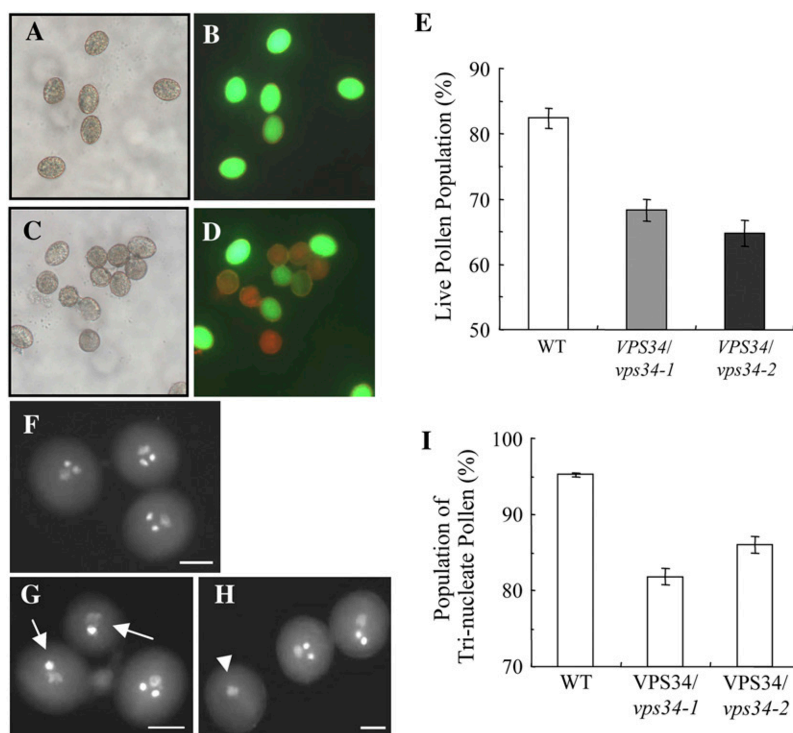

Figure 3. Phenotypic analysis of pollen from wild-type and *VPS34/vps34* heterozygous plants. A to D, Bright-field (A and C) and fluorescence (B and D) images of propidium iodide/FDA double-stained mature pollen grains from wild-type plants (A and B) and *VPS34/vps34-1* plants (C and D). Propidium iodide stains dead cells red, and FDA stains live cells green. E, The proportion of live pollen grains determined by double staining with propidium iodide and FDA. Plotted are the means ± SE from five independent experiments, with three replicates each; n = 3,813 (wild type [WT]), 3,212 (*VPS34/vps34-1*), and 4,125 (*VPS34/vps34-2*). F to H, Images of pollen nuclei detected with DAPI staining of pollen from wild-type plants (F) and *VPS34/vps34-1* plants (G and H). Arrows point to binucleate pollen (G), and the arrowhead indicates uninucleate pollen (H). Bars = 10 μm. I, Pollen population with different numbers of nuclei as determined with DAPI staining. Plotted are the means ± SE from 10 flowers; n = 2,677 (wild type), 2,203 (*VPS34/vps34-1*), and 2,871 (*VPS34/vps34-2*).

Similar differences in the vacuolar shape were also found in pollen grains at the mature stage after dehiscence. The proportion of abnormal pollen was 3.7% ± 1.5% (n = 594 pollen grains from five buds), 34.6% ± 3.6% (n = 827 pollen grains from eight buds), and 37.0% ± 2.7% (n = 943 pollen grains from eight buds) from wild-type, *VPS34/vps34-1*, and *VPS34/vps34-2* plants, respectively. This result suggested that vacuoles of *vps34* pollen become aberrant at the stage of vacuole reorganization following the first mitotic division.

Transmission electron microscopy observations confirmed the results from light microscopy; pollen grains from *VPS34/vps34-1* and wild-type plants at the tetrad stage (Fig. 6, A and E, and I and M) and the vacuolated stage (Fig. 6, B and F, and J and N) were similar, whereas significant differences in the shape of vacuoles at the trinucleate stage were found. At the trinucleate pollen stage, the pollen of *VPS34/vps34-1* often contained large vacuoles with irregular protrusions and/or a rugged appearance (Fig. 6, K and O). This contrasts with the small vacuoles of wild-type pollen, which were round and had a smooth periphery (Fig. 6, C and G). The vacuolar abnormality became even more severe in mature pollen of mutant plants after dehiscence; some pollen grains of *VPS34/vps34-1* plants were partly empty (Fig. 6, L and P) compared with wild-type pollen (Fig. 6, D and H), which contained dense cytoplasm and many organelles, suggesting cytoplasmic degradation in the pollen of mutant plants. At this stage, pollen grains of *VPS34/vps34-1* plants showed diverse and abnormal vacuolar morphology. They had irregular shapes and tended to aggregate (Supplemental Fig. S2A). The large vacuoles had protrusions and contained cytoplasmic fragments and intracellular organelles inside, suggesting that they engulfed the cellular contents (Supplemental Fig. S2). In some severe cases, little cytoplasm remained inside the cells. Mitochondria, endoplasmic reticulum, and Golgi stacks maintained their normal morphology throughout all stages of pollen development, even until most of cytoplasm was inside the vacuoles (Supplemental Fig. S2H).

VPS34/vps34 Plants Are Defective in Pollen Germination and in Pollen Tube Growth

Nearly 70% of the pollen grains from *VPS34/vps34* plants survived (Fig. 3E). Although half of the pollen grains from *VPS34/vps34* plants are expected to be genotypically normal, the remaining viable pollen grains may carry a *vps34* allele. This is in contrast to the genetic data, which showed that male gametophytes with the *vps34* allele produced scarcely any progeny. This discrepancy, together with the presence of large abnormal vacuoles in some pollen grains of *VPS34/vps34* plants, led us to suspect that a subpopulation of the viable pollen grains may have defects in the subsequent steps: germination, pollen tube elongation, and fertilization. To test whether the loss of *VPS34* leads to defects in the steps following pollen maturation, we analyzed pollen germination and tube length in wild-type and *VPS34/vps34* plants.

Mutant plants showed abnormality in a pollen germination assay in vitro. Pollen grains were transferred to agar medium by gently brushing the inverted

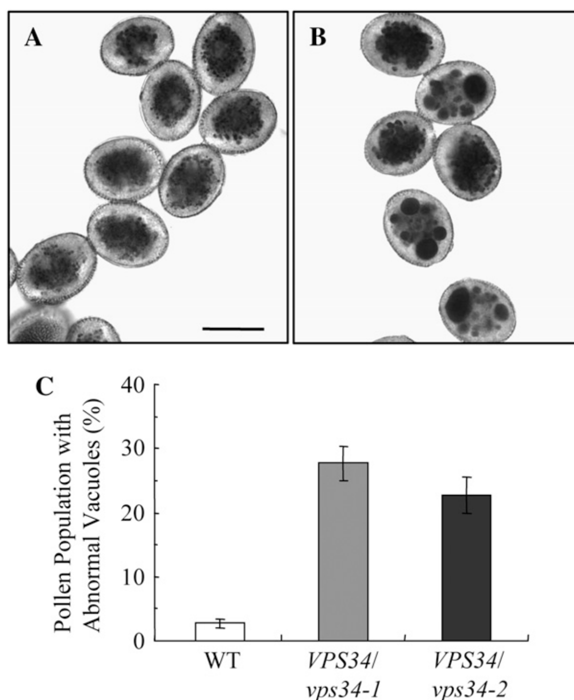

Figure 4. Staining of vacuoles in wild-type and *VPS34/vps34* pollen. A and B, Neutral red-stained mature pollen from wild-type plants (A) and *VPS34/vps34-1* plants (B). Neutral red accumulates in vacuoles and appears red when viewed with white light. Bar in A = 10 μm. C, Pollen population (%) with abnormal vacuoles as determined with neutral red staining. Plotted are the means ± SE from three independent experiments, each with three replicates; n = 1,686 (wild type), 1,929 (*VPS34/vps34-1*), and 1,647 (*VPS34/vps34-2*).

flower across the surface and incubated at 28°C for 4 h, and germination rate was analyzed. The percentage of germinated pollen was lower in mutants than in the wild type: 33% ± 1% and 34% ± 1% of pollen grains in *VPS34/vps34-1* and *VPS34/vps34-2*, respectively, compared with 55% ± 1% in the wild type ($P < 0.01$; Fig. 7A).

For the germinated pollen, pollen tube length was measured. Pollen tubes from *VPS34/vps34* plants were significantly ($P < 0.01$) shorter than those of wild-type plants: 353.1 ± 3.6 μm, 253.6 ± 5.3 μm, and 266.1 ± 9.1 μm for wild-type, *VPS34/vps34-1*, and *VPS34/vps34-2* plants, respectively. This difference was clearer when the percentage of pollen tubes shorter than 100 μm was analyzed: 30% ± 3% and 22% ± 4% of pollen in *VPS34/vps34-1* and *VPS34/vps34-2*, respectively, could not grow a tube longer than 100 μm, whereas only 7% ± 3% in the wild type could not (Fig. 7B). The pollen tube morphology, however, was normal in the sense that no major difference was apparent in the heterozygous knockout mutants (data not shown). This result suggests that many *vps34* pollen could not reach the ovary for fertilization.

VPS34/vps34 Plants Have Reduced Seed Set

Even after germination and growth of pollen tubes, the genotype of male gametophytes might affect seed development at the fertilization and embryogenesis stages that occur in the ovary. In the mature siliques of *VPS34/vps34* plants, aborted seeds were seldom detected, but the seed set was slightly sparser than that in the wild type (Fig. 8). When seed sets of self-fertilized mature siliques were analyzed quantitatively, the percentage of developed seeds (fertilized ovules) was reduced from 98% ± 0.8% in the wild type to 92% ± 1% and 87% ± 2% in *VPS34/vps34-1* and *VPS34/vps34-2*, respectively; as a result, seed number per silique in mutant plants was significantly reduced compared with that of wild-type plants ($P < 0.01$; Table III).

DISCUSSION

In this article, we provide several lines of evidence that PI3K plays a pivotal role during pollen development. In reciprocal cross experiments, scarcely any pollen carrying *vps34* successfully transmitted the mutation, whereas female TE was close to 100% (Table II), suggesting that the expression of *VPS34* in the male gametophyte is important for gene transmission. Defective phenotypes of *VPS34/vps34* plants were observed at multiple stages during the reproductive process, including pollen development, germination, tube elongation, and fertilization. The most seriously affected stage was pollen development. Less than 70% of the pollen grains from *VPS34/vps34* plants survived, and among them many had abnormal vacuoles. Considering that half of the pollen grains from *VPS34/vps34* plants should be genotypically and phenotypically normal, it is likely that most male gametophytes with the *vps34* allele were already defective prior to germination. The defects in the preceding stages most likely abolish the chance for gametes with the *vps34* allele to fertilize eggs and produce a *vps34/vps34* zygote. The absence of aborted seeds in the silique of *VPS34/vps34* plants is consistent with this interpretation. It remains unknown whether PI3K has a role in the embryogenesis steps as well.

The broad and significant role of PI3K was first suggested by the results of Welters et al. (1994) showing that plants containing an antisense construct for *VPS34* are severely inhibited in growth and development. Therefore, we expected that some progeny from self-fertilized *VPS34/vps34* plants would show severe reduction in growth or death, but we failed to detect any such phenomenon. This result led us to hypothesize that disruption of *VPS34* causes abortion at a gametophyte stage. This possibility was tested by observing segregation ratios for the progeny from self-fertilized *VPS34/vps34* plants, which showed distorted segregation ratios of 1:1:0 for wild-type, heterozygous, and homozygous plants. This is consistent with a putative gametophyte-lethal effect caused by T-DNA insertion at the *VPS34* gene (Table I). Genetic analyses of T-DNA transmission revealed that TE was reduced predominantly through the male and was not affected through the female (Table II). Consistent with

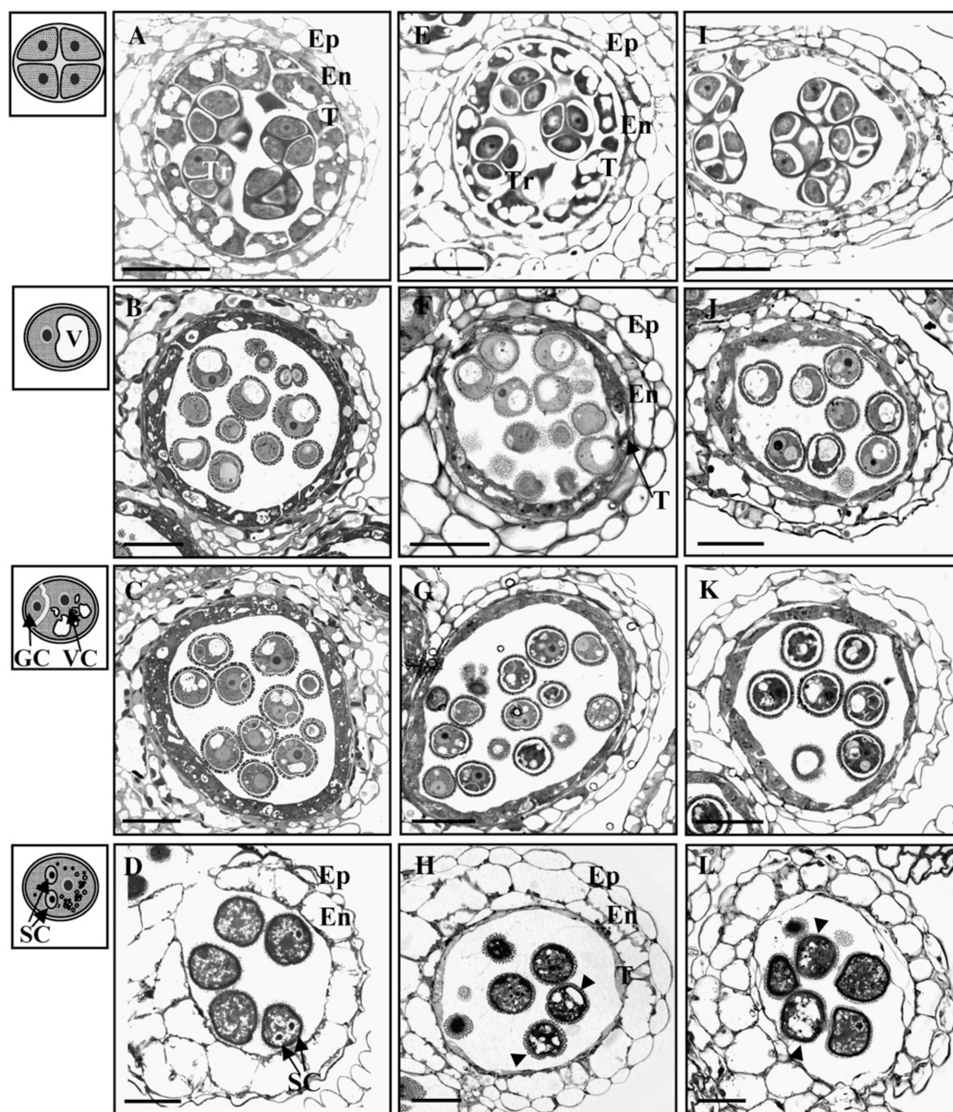

Figure 5. Light microscopic observation of wild-type, *VPS34*/*vps34-1*, and *VPS34*/*vps34-2* pollen development within anthers. Pollen grains of wild-type (A–D), *VPS34*/*vps34-1* (E–H), and *VPS34*/*vps34-2* (I–L) plants at different stages of development. Schematic representations of the normal microspore or pollen at each developmental stage are included as insets at the left of A to D. Shown are the tetrad stage of microspores (A, E, and I), uninucleate vacuolate pollen stage (B, F, and J), binucleate pollen stage after the first mitotic division (C, G, and K), and trinucleate pollen stage (D, H, and L). At the trinucleate pollen stage, most wild-type pollen grains contain many small vacuoles (D), whereas some mutant pollen grains contain large vacuoles indicated by arrowheads (H and L). En, Endothecium; Ep, epidermis; GC, generative cell; SC, sperm cell; T, tapetum; Tr, tetrad; V, vacuole; VC, vegetative cell. Bars = 20 μm.

the TE results, some of the mature pollen grains produced by heterozygous plants showed defective phenotypes during pollen development.

Defective vacuole reorganization was prominent in pollen grains of *VPS34*/*vps34* plants (Fig. 5). In wild-type pollen grains, vacuoles are fragmented after the first mitotic division, whereas in some pollen of mutant plants, large vacuoles remained and were retained even until the mature stage of development (Fig. 6). The appearance of a large vacuole before generative cell formation (first pollen mitosis) and its fission into many small vacuoles are generally observed in pollen of various plant species. In wild-type pollen development, enlarged vacuoles that are presumably created by the fusion of preexisting small vacuoles (Owen and Makaroff, 1995) are subsequently divided by the invagination of the tonoplast, and some vacuoles are exocytosed (Yamamoto et al., 2003). Two possibilities for the abnormal vacuolar dynamics in mutant pollen can be entertained. First, protein targeting to vacuoles, where PtdIns(3)P has been shown to be essential (Herman et al., 1992; Schu et al., 1993), may be defective in the mutant. In yeast, protein transport to the vacuole depends on the Vam7 t-SNARE, and its targeting to the vacuole is dependent on PtdIns(3)P generation (Cheever et al., 2001). The role of PtdIns(3)P in vacuolar sorting has also been reported in plant cells (Matsuoka et al., 1995; Kim et al., 2001; Tse et al., 2004; daSilva et al., 2005; Oliviusson et al., 2006). Therefore, shortage of PtdIns(3)P may lead to failure in the recruitment and targeting of proteins necessary for vacuolar function, thus causing defects in vacuolar dynamics.

Alternatively, according to studies on vacuolar dynamics in yeast, PI3K may regulate vacuole morphology by providing the precursor of phosphatidylinositol 3,5-bisphosphate [PtdIns(3,5)P_2]. PtdIns(3,5)P_2 is generated from PtdIns(3)P by Fab1p, the PtdIns(3)P 5-kinase, and is essential for normal vacuole function and morphology in yeast (Yamamoto et al., 1995). A

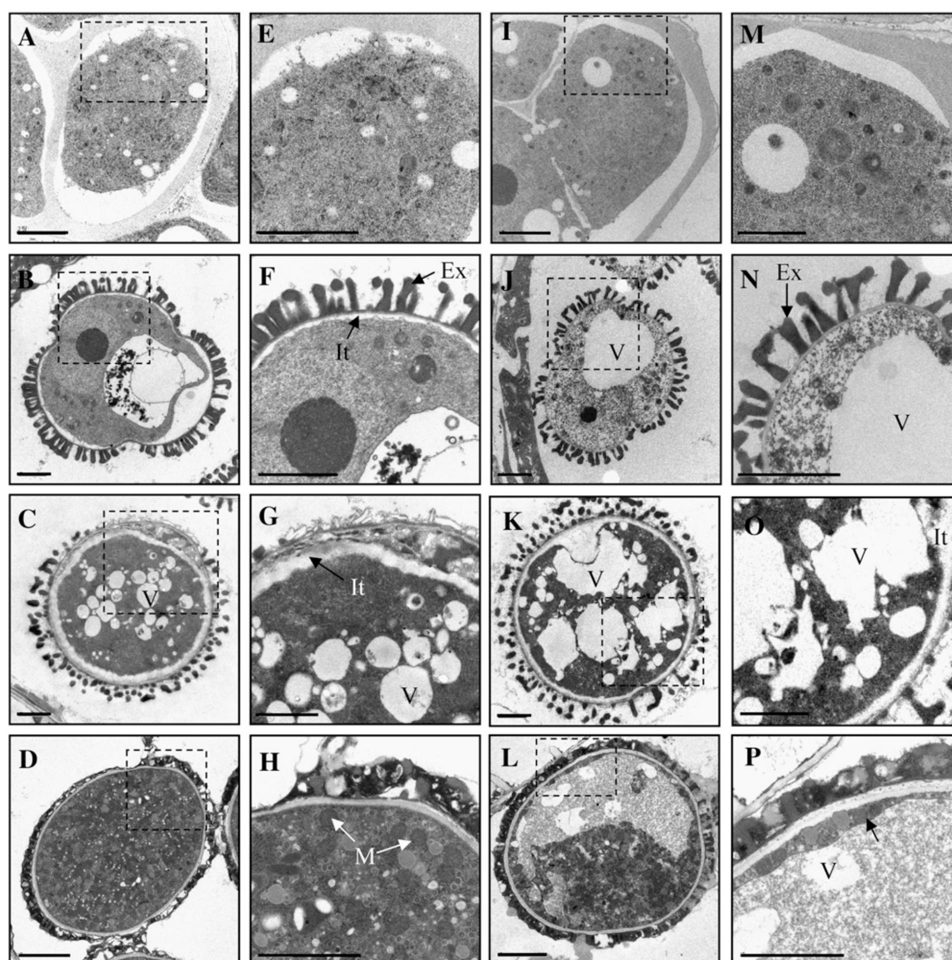

Figure 6. Transmission electron microscopy analysis of pollen from wild-type and *VPS34*/*vps34-1* plants. Microspores and pollen of wild-type (A–H) and mutant (I–P) plants, and magnification of the dotted box area at each stage of pollen from wild-type (E–H) and mutant (M–P) plants. A, E, I, and M, Tetrad of microspores. B, F, J, and N, Uninucleate vacuolate pollen. C, G, K, and O, Trinucleate pollen. Numerous small vacuoles are present in wild-type pollen (C and G), whereas several large vacuoles can be found in *VPS34*/*vps34-1* pollen (K and O). D, H, L, and P, Mature pollen after dehiscence. Pollen from wild-type plants contains many small, dispersed vacuoles (D and H), whereas pollen from *VPS34*/*vps34-1* plants has large vacuoles with abnormal shape (L and P). The arrow in P indicates a cytoplasmic area remaining during the cytoplasmic degradation of the pollen in mutant plants. Ex, Exine; It, intine; M, mitochondria; V, vacuole. Bars = 2 μm.

fab1 null strain entirely devoid of PtdIns(3,5)P_2 displays a dramatically enlarged vacuole. In addition, *fab1* mutants show a reduction of vacuolar acidification (Weisman, 2003), which plays crucial roles in vacuolar fission (Baars et al., 2007) and vacuolar membrane structure (Hope et al., 1989). In Arabidopsis, there are four genes encoding putative Fab1p homologs (Mueller-Roeber and Pical, 2002). Two of them, *Fab1a* (At4g33240) and *Fab1b* (At3g14270), have a PtdIns(3)P-binding FYVE domain at their N termini, and according to the publicly available microarray data, they are highly expressed in pollen (https://www.genevestigator.ethz.ch/; Zimmermann et al., 2004). None of the four putative Fab1 enzymes has yet been characterized in vitro or in vivo. It will be of interest to test whether the putative Fab1 enzymes also function in vacuole dynamics during pollen development.

The number of nuclei was reduced in some pollen grains of mutant plants (Fig. 3). Among them, the largest population comprised binucleate pollen, the stage during which vacuoles actively fragment in the wild type. These results suggest that the reduction in the number of nuclei may be a consequence of defective vacuolar dynamics in the mutant pollen. A large vacuole starts to fragment after or concurrent with the first nuclear division, and hardly any large vacuoles are detected following the second nuclear division. A generative cell is produced by highly asymmetric division at the side of a microspore, moves into the center of the cell, and proceeds to divide. A large vacuole at the vacuolated stage occupying part of the cytoplasm may hinder movement of the generative cell to the central region of the microspore. Structural changes of vacuoles in dividing cells have also been reported in meristematic cells (Segui-Simarro and Staehelin, 2006). The large vacuole, which appears during the G2 phase, fragments into many vacuoles of variable sizes and undergoes a major reduction (approximately 50%) in surface area and an even greater reduction in volume (approximately 80%). This structural rearrangement of the vacuolar system may be necessary for the early stages of cytokinesis, in particular during the establishment of the cytokinetic machinery (Segui-Simarro and Staehelin, 2006). Therefore, it is possible that the failure to reabsorb vacuole may have hindered normal cell division in the developing mutant pollen, reducing the number of nuclei.

PI3K may be involved in the regulation of nuclear function directly. In animal cells, class I PI3Ks, which

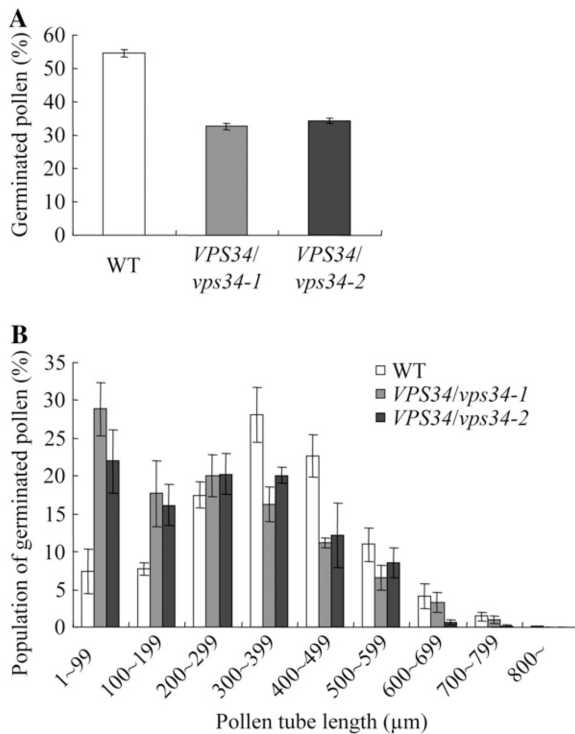

Figure 7. Analysis of pollen germination and tube growth in wild-type and *VPS34/vps34* plants. A, Percentage of pollen germination in vitro for wild-type, *VPS34/vps34-1*, and *VPS34/vps34-2* plants. Plotted are the means ± SE from three independent experiments using 21 flowers from wild-type and *VPS34/vps34-1* and *VPS34/vps34-2* plants. In a single flower, four replicates were tested, and the average value was taken as the germination rate for the flower. In each replicate, over 100 pollen grains were counted. B, Pollen tube growth of germinated pollen for wild-type, *VPS34/vps34-1*, and *VPS34/vps34-2* plants was analyzed after 4 h of incubation on germination medium. Plotted are the means ± SE; n = 1,558 (wild type), 1,268 (*VPS34/vps34-1*), and 354 (*VPS34/vps34-2*).

generate PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3, have been reported as important factors in various events of cell division, such as control of cell cycle entry (Álvarez et al., 2003), regulation of cyclin/Cdk (Olson et al., 1995; Klippel et al., 1998), and progression of G2/M phases (Álvarez et al., 2001). Nuclear PtdIns(3)P has been reported in BHK cells and human fibroblasts by the use of FYVE domain probes (Gillooly et al., 2000), and in HL-60 cells, PtdIns(3)P level increases at G2/M phase of the cell cycle (Visnjic et al., 2003). In plants, PI3K is associated with active nuclear and nucleolar transcription sites (Bunney et al., 2000), and some of the proteins with a FYVE domain have regulator of chromosome condensation 1 tandem repeats, known to be important in nuclear processes (Drøbak and Heras, 2002). Therefore, the control of DNA replication or transcription is a possible mechanism for PI3K regulation of the cell cycle. It is an open question whether the role of PI3K in nuclear division is an indirect effect resulting from defective vacuole dynamics or via a pathway independent from it.

Aside from the defects during development, mutant pollen was also impaired in tube growth. Average pollen tube length was markedly shorter in *VPS34/vps34* than in wild-type plants, and the tube length of mutant plants showed a bimodal distribution, suggesting that a defective group *vps34* was mixed with a normal group *VPS34*. This bimodal distribution was not found in the wild type, which showed the normal distribution (Fig. 7). The pollen tube phenotype could be, at least in part, due to earlier defects during pollen development. However, it is also possible that PI3K activity is required during pollen tube growth per se. During the sexual reproduction of flowering plants, pollen grains germinate on receptive stigmas and produce a tip-growing tube, which rapidly penetrates the style to deliver the male gametes to the ovules. To achieve this, pollen tubes undergo highly polarized, tip-localized expansion, which depends on a complex signaling network that includes ion fluxes and gradients, cytoskeletal reorganization, and membrane trafficking (Hepler et al., 2001). Among the components of the signaling network for tip growth, reactive oxygen species, Ca^{2+}, cytoskeleton, or membrane trafficking have been shown to be affected by PI3K in other plant cell types, including guard cells (Jung et al., 2002; Park et al., 2003; Joo et al., 2005; Leshem et al., 2007; Choi et al., 2008). In particular, it is worth mentioning that a major G-actin-binding protein present in pollen, profilin, was recently shown to bind VPS34/PI3K from bean (*Phaseolus vulgaris*; Aparicio-Fabre et al., 2006) and that PI3K activity in cultured carrot (*Daucus carota*) cells associates with the cytoskeleton (Dove et al., 1994). Therefore, it is possible that the mutant pollen grains are inhibited in tube elongation via a process independent from earlier developmental defects. Suppression of *VPS34* expression specifically during pollen tube growth using an inducible RNA interference system will be helpful to distinguish the roles of PI3K in pollen tube growth from those of pollen development.

Several genes functionally related to PI3K have been reported to function in late stages of pollen development and/or pollen tube growth, and defects in the expression of these genes impair fertility. PTEN1, which has been shown to dephosphorylate PtdIns(3,4,5)P_3 in vitro, is expressed specifically in pollen grains. Suppression of *PTEN1* by RNA interference silencing

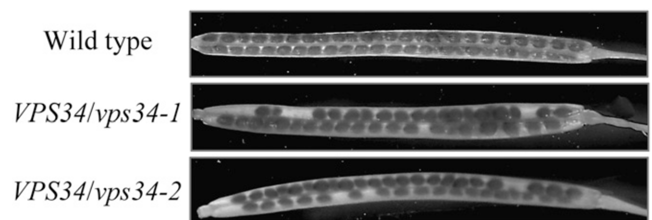

Figure 8. Seed set within mature siliques from wild-type, *VPS34/vps34-1*, and *VPS34/vps34-2* plants. Mature siliques of *VPS34/vps34* mutants showed slightly sparser seed set than those of the wild type.

Table III. *Analysis of seed set in mature siliques*

The number of developed seeds and unfertilized ovules were counted in the 10th to 12th siliques of the main inflorescence from 10 plants of the indicated genotype.

Genotype	Developed Seeds	Unfertilized Ovules	Percentage of Developed Seeds	Seed No. per Silique
Wild type	1,508	30	97.8 ± 0.8	50.3 ± 1.1
VPS34/vps34-1	1,326	104	92.2 ± 1.5	44.2 ± 1.3
VPS34/vps34-2	1,068	149	86.8 ± 2.0	35.6 ± 1.5

causes cell death at the tricellular stage in Arabidopsis pollen (Gupta et al., 2002). BECLIN1, an Arabidopsis homolog of yeast Beclin1/Atg6/Vps30, which associates with VPS34 and plays a role in autophagy and vacuolar protein sorting, is expressed predominantly in mature pollen, and disruption of this gene by T-DNA insertion results in male sterility due to severely reduced pollen germination (Fujiki et al., 2007; Qin et al., 2007). *VACUOLESS1* (*VPS16*) and *POKY POLLEN TUBE* (*VPS52*), Arabidopsis VPS homologs, also play roles in pollen tube elongation (Hicks et al., 2004; Lobstein et al., 2004). Together, these findings strongly support a central role for the PI3K-associated pathway in pollen development and/or tube elongation. It is interesting that the PI3K mutant showed aberrant phenotypes at earlier stages of pollen development than mutants of any other genes associated with PI3K. This difference may reflect the central role of PI3K and/or the fact that there is only one PI3K gene, in contrast to multiple members of other genes that work redundantly.

In summary, we showed that PI3K is important for male gametophyte development and successful reproduction of plants. Consequently, genetic and biochemical analyses of PI3K have been difficult. Knockdown of transcript levels with RNA interference silencing techniques may be helpful in this regard, but in our experience, this is also difficult because only the lines of plants with slightly reduced levels of PI3K transcript survive, and they do not show distinct phenotypes. Use of tissue-specific or inducible promoters may be useful for further characterization of the roles of this important enzyme in plant development and physiology.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were grown in a greenhouse with 16-h-light and 8-h-dark cycles at 22°C ± 2°C. Plants were watered with Hyponex solution (1 g L^{-1}).

Identification of *VPS34/vps34-1* and *VPS34/vps34-2*

Genotyping of *VPS34/vps34-1* (SALK_007281) and *VPS34/vps34-2* (GABI_418H01) plants and progeny was performed with a PCR-based approach. Genomic DNA was isolated and used as a template for PCR amplification of DNA fragments corresponding to the wild type and the insertion alleles. The following primers were used: LB (5′-GCGTGGACCGCTTGCTGCAACT-3′), LP (5′-TTCGGAATCTCTTCACGGCAC-3′), and RP (5′-CTCCGGAAATCGAGCAACCTT-3′) for SALK_007281, and LB (5′-GGGCTACACTGAATTGGTAGCTC-3′), LP (5′-ACTCCACTCCACACAACGAAG-3′), and RP (5′-AAGCAGAAGCTAAGGCTCTGG-3′) for GABI_418H01.

VPS34 Promoter::GUS Assay

The promoter region of *AtVPS34* (−1,391 to +125 relative to ATG) was amplified from the genomic DNA of wild-type plants with the specific primers VPS34_Pr_For (5′-GGATCCGTATGAAATTTAGACGATCAAAATTAGGGC-3′) and VPS34_Pr_Rev (5′-AAGCTTCCGATCGTTATATGAAGATATAATCGGTGC-3′) and then ligated to the *GUS* gene containing pBI121 vector from which the 35S promoter was eliminated using the restriction enzymes *Hin*dIII and *Bam*HI. This construct was transformed into Arabidopsis by the floral-dip method (Clough and Bent, 1998). The transformed seeds were selected on half-strength Murashige and Skoog agar plates containing 30 $\mu g\ \mu L^{-1}$ kanamycin, and the selected plants were grown in soil and then incubated for 2 d in GUS solution with 5-bromo-4-chloro-3-indolyl-β-*O*-glucopyranoside at 37°C. Chlorophyll was extracted in 70% ethanol solution at 65°C for 50 min.

Pollen Analysis

For pollen staining, grains were released from anthers and incubated in different histochemical stains as described previously (Regan and Moffatt, 1990) with some modifications. Pollen grains were soaked in 0.02% neutral red for 30 min for visualization of vacuoles. Viability was assayed by double staining pollen grains with 1 $\mu g\ mL^{-1}$ propidium iodide and 0.5 $\mu g\ mL^{-1}$ FDA.

To visualize nuclei, pollen grains were processed essentially as described by Park et al. (1998). Sufficient mature pollen was obtained by placing an open flower in a microfuge tube containing 200 μL of DAPI staining solution (0.1 M sodium phosphate, pH 7, 1 mM EDTA, 0.1% Triton X-100, and 0.4 $\mu g\ mL^{-1}$ DAPI). Pollen grains were released from the anther by a brief vortexing, transferred to a microscope slide, and observed with an epifluorescence microscope (Axioskop 2; Carl Zeiss).

In Vitro Pollen Germination and Growth Measurement

In vitro pollen germination and pollen tube growth were analyzed as described previously (Li et al., 1999) with some modifications. Flowers collected from Arabidopsis plants 1 to 2 weeks after bolting were used for the examinations. Pollen was germinated on agar medium containing 18% Suc, 0.01% boric acid, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 1 mM $Ca(NO_3)_2$, and 0.5% agar, pH 7. Open flowers were dehydrated at room temperature for 1 h. Pollen grains were transferred to agar medium by dipping the flowers on agar. Pollen was germinated at 28°C for 4 h, examined with a light microscope, and photographed using a bright-field microscope (Axioskop 2) with a CCD camera (Axio Cam; Carl Zeiss). Pollen tube length was measured from photographs using the Interactive Measurement software package AxioVision 3.0.6 (Carl Zeiss).

Light Microscopy and Transmission Electron Microscopy

For microscopic observation, anthers from wild-type and heterozygous plants were fixed in a solution containing 2.5% glutaraldehyde and 4% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.4) at 4°C for 4 h. Anthers were rinsed in the phosphate buffer and fixed further in 1% (w/v) osmium

tetroxide for 4 h at 4°C. The samples were then dehydrated in an alcohol series (final dehydration was done in absolute alcohol) and embedded in LR White resin (London Resin Company). One-micrometer and 40- to 50-nm sections for light microscopy and transmission electron microscopy, respectively, were prepared with an ultramicrotome (Bromma 2088; LKB). The thin sections (40–50 nm) were collected on a nickel grid (1-GN, 150 mesh) and stained with uranyl acetate and lead citrate. Observations were made with a transmission electron microscope (JEM-100CX-1).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number U10669.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of the *VPS34* gene in Arabidopsis.

Supplemental Figure S2. Abnormal vacuoles in mature pollen from *VPS34*/*vps34-1* and *VPS34*/*vps34-2* Arabidopsis plants.

Supplemental Table S1. Number of nuclei in mature pollen.

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