Phosphatidylinositol 3,5-bisphosphate \([\text{PtdIns}(3,5)P_2]\) is a phospholipid that has a role in controlling membrane trafficking events in yeast and animal cells. The function of this lipid in plants is unknown, although its synthesis has been shown to be up-regulated upon osmotic stress in plant cells. PtdIns(3,5)P_2 is synthesized by the PIKfyve/Fab1 family of proteins, with two orthologs, FAB1A and FAB1B, being present in Arabidopsis \((\text{Arabidopsis thaliana})\). In this study, we attempt to address the role of this lipid by analyzing the phenotypes of plants mutated in FAB1A and FAB1B. It was not possible to generate plants homozygous for mutations in both genes, although single mutants were isolated. Both homozygous single mutant plant lines exhibited a leaf curl phenotype that was more marked in homozygous for mutations in both genes, although single mutants were isolated. Both homozygous single mutant plant lines exhibited a leaf curl phenotype that was more marked in FAB1B mutants. Genetic transmission analysis revealed that failure to generate double mutant lines was entirely due to inviability of pollen carrying mutant alleles of both FAB1A and FAB1B. This pollen displayed severe defects in vacuolar reorganization following the first mitotic division of development. The presence of abnormally large vacuoles in pollen at the tricellular stage resulted in the collapse of the majority of grains carrying both mutant alleles. This demonstrates a crucial role for PtdIns(3,5)P_2 in modulating the dynamics of vacuolar rearrangement essential for successful pollen development. Taken together, our results are consistent with PtdIns(3,5)P_2 production being central to cellular responses to changes in osmotic conditions.

Phosphoinositides make up a minor fraction of total membrane lipids in all eukaryotic organisms. Their production is spatially restricted to the cytoplasmic leaflet of specific organellar membranes and temporally regulated by phosphatidylinositol (PtdIns) kinases and phosphatases. Three of the five hydroxyl groups of PtdIns can be phosphorylated, either singly or combinatorially, to produce seven different phosphoinositides. These different phosphoinositides can recruit and/or activate proteins with specific phosphoinositide-binding domains and have been implicated in the regulation of many important cellular functions, including membrane trafficking, cell growth, and cytoskeleton remodeling \(\text{(Di Paolo and De Camilli, 2006)}\).

In animal cells, phosphorylation at the 3 position of PtdIns and its phosphorylated derivatives can be carried out by three different classes of PtdIns 3-kinases (classes I–III; Cantley, 2002). Plants and yeast only have class III PtdIns 3-kinases that are orthologs of the \(\text{Saccharomyces cerevisiae} \) protein Vps34p \(\text{(Mueller-Roeber and Pical, 2002)}\). Vps34p orthologs are thought to use PtdIns as their sole lipid substrate and produce PtdIns 3-phosphate \((\text{PtdIns3P})\). PtdIns3P is involved in endosomal/lysosomal protein sorting in eukaryotic cells in addition to cellular signaling events \(\text{(Backer, 2008)}\).

In plants, PtdIns3P is essential for normal growth and development. Arabidopsis \((\text{Arabidopsis thaliana})\) plants carrying a \(\text{VPS34} \) antisense construct have severe developmental defects \(\text{(Welters et al., 1994)}\). Furthermore, using pharmacological inhibitors of PtdIns3P production and analysis of transgenic plants defective in downstream signaling from PtdIns3P, it has been shown that this lipid has a role to play in many diverse physiological processes, such as root hair growth \(\text{(Lee et al., 2008a)}\). The phenotypes observed in studies of PtdIns3P function in plants are consistent with a role in endosomal and vacuolar trafficking in plants \(\text{(Kim et al., 2001; Lee et al., 2008a)}\), as in other eukaryotes. Recently, an attempt to generate \(\text{vps34} \) homozygous mutant plant lines was unsuccessful due to failure of the mutant \(\text{vps34} \) allele to transmit through the male germ line \(\text{(Lee et al., 2008b)}\).

Importantly, PtdIns3P is the precursor to another phosphoinositide, PtdIns 3,5-bisphosphate \((\text{PtdIns}(3,5)P_2)\), which also has vital roles in endosomal trafficking in eukaryotes \(\text{(Dove et al., 2009)}\). Thus, it is possible that some of the effects in plants attributed to PtdIns3P in previous studies may actually be due to an inability of cells to produce PtdIns(3,5)P_2. PtdIns(3,5)P_2 is produced by the PtdIns3P 5-kinases PIKfyve and Fab1p in animal and yeast cells, respectively. PIKfyve/Fab1p proteins have an N-terminal FYVE domain necessary for binding to PtdIns3P-containing membranes, a central Cpn60_TCP1 (for HSP chaperonin T complex 1) homology domain, and a C-terminal kinase domain. In Arabidopsis, there are a number of genes encoding...
putative Fab1p homologs, but only two of them, FAB1A (At4g33240) and FAB1B (At5g14270), encode proteins having FYVE domains at their N termini (Mueller-Roeber and Pical, 2002). It is likely that these proteins are PtdIns3P 5-kinases in Arabidopsis. Despite the importance of PtdIns(3,5)P2 in yeast and animals, very little is known about its function in plants. However, it has been shown that hyperosmotic stress can induce the rapid synthesis of PtdIns(3,5)P2 in cell suspension cultures from a number of plant species (Meijer and Munnik, 2003) and in pollen tubes from tobacco (Nicotiana tabacum; Zonia and Munnik, 2004). This production is consistent with a requirement for PtdIns(3,5)P2 in vacuolar membranes for further investigation as they contained insertions in these genes were identified. A number of candidates have been identified, including yeast Svp1p (Dove et al., 2004), its mammalian homolog WIP149 (Jeffries et al., 2004), CHMP3 (Whitely et al., 2003), and Ent1p (Friant et al., 2003).

In this study, we aimed to further investigate the role of PtdIns(3,5)P2 in plant physiology and the function of PIKfyve/Fab1p orthologs in Arabidopsis by generating mutant plant lines homozygous for T-DNA insertions in both FAB1A and FAB1B. We failed to generate double homozygous fab1afab1b knockout plants but observed subtle phenotypes in both fab1a and fab1b single homozygous plants. The data show that pollen with a fab1afab1b genotype has an abnormal vacuolar phenotype and does not contribute to the next generation. Our data are consistent with the hypothesis that the male gametophytic defect observed in vps34 mutant pollen (Lee et al., 2008b) is due to an inability of this pollen to generate PtdIns(3,5)P2 and is not a direct result of the lack of PtdIns3P.

RESULTS

Phylogeny and Expression of PtdIns3P 5-Kinases in Arabidopsis

The availability of the complete Arabidopsis genome sequence has allowed the identification of enzymes involved in phosphoinositide metabolism based on comparison with the wealth of data from studies in animals and yeast (for review, see Mueller-Roeber and Pical, 2002). It has been proposed that there are four proteins in Arabidopsis that are putative homologs of PIKfyve and Fab1p, the enzymes responsible for the conversion of PtdIns3P to PtdIns(3,5)P2 (Fig. 1A), in animals and yeast, respectively (Mueller-Roeber and Pical, 2002). Animals and yeast contain a single copy of genes encoding PIKfyve/Fab1p. The genes encoding the four putative PIKfyve/Fab1p homologs in Arabidopsis are At4g33240 (FAB1A), At5g14270 (FAB1B), At1g71010 (FAB1C), and At1g34260 (FAB1D). However, only FAB1A and FAB1B encode proteins that contain predicted FYVE domains at their N termini and therefore have the same domain organization as PIKfyve/Fab1p (Fig. 1B). Phylogenetic analysis indicates that proteins similar to FAB1C and FAB1D, without a FYVE domain, are likely to exist in all higher plant species and that these proteins cluster in a plant-specific group distinct from plant FYVE domain-containing proteins (Fig. 1C). We propose that FAB1A and FAB1B encode bona fide PtdIns3P 5-kinases, whereas the role of FAB1C and FAB1D in the production of PtdIns(3,5)P2 is more debatable. FAB1C and FAB1D are predicted to encode polypeptides with PtdIns3P 5-kinase activity, but as they lack a FYVE domain, it is unlikely that they can be efficiently targeted to PtdIns3P substrate-containing membranes.

The transcript levels of FAB1A and FAB1B were investigated in a wide range of tissue types utilizing publicly available microarray data with the GENEVESTIGATOR software (Zimmermann et al., 2004). FAB1A and FAB1B transcripts were found in all plant tissues to varying degrees (Supplemental Fig. S1). FAB1A showed highest expression levels in pollen, seed, and senescent leaves, whereas FAB1B transcript levels were highest in the root hair zone, pollen, and stamens. The microarray data reveal that FAB1A expression levels are significantly lower than FAB1B in all tissue categories (Supplemental Fig. S1).

Isolation of FAB1A and FAB1B Knockout Lines

In order to obtain Arabidopsis lines with mutations in FAB1A and FAB1B, SALK T-DNA lines carrying insertions in these genes were identified. A number of lines with T-DNA insertions in these genes were identified. SALK_013923 and SALK_066673 were chosen for further investigation as they contained insertions in exons of FAB1A (At4g33240; exon 4 of 11) and FAB1B (At5g14270; exon 9 of 11), respectively (Fig. 2A). We predicted that these alleles (fab1a-1 and fab1b-1) would be unable to produce functional protein due to the T-DNA insertions being positioned 5′ of the lipid kinase domain. PCR-based genotyping confirmed T-DNA insertions in FAB1A and FAB1B. Homozygous fab1a-1 and fab1b-1 mutant plant lines were generated from these stocks and were checked for transcripts by reverse transcription (RT)-PCR. FAB1A and FAB1B transcripts could not be detected in fab1a-1/fab1a-1 or fab1b-1/fab1b-1 plants, respectively, but were present in wild-type plants (Supplemental Fig. S2). It should be noted that the primers used to amplify cDNA by RT-PCR were 3′ of the T-DNA insertion sites; thus, short transcripts coding for truncated proteins could potentially be produced in the homozygous T-DNA insertion lines. However, even if this were the case,
truncated proteins would lack the C-terminal lipid kinase domain and thus be unable to convert PtdIns3P to PtdIns(3,5)P₂. Therefore, we consider the fab1a⁻¹/fab1a⁻¹ and fab1b⁻¹/fab1b⁻¹ plants to be null mutants of FAB1A and FAB1B, respectively.

fab1a⁻¹ and fab1b⁻¹ Lines Display Subtle Leaf-Curling Phenotypes and Are Reproductively Viable

Seeds from fab1a⁻¹/fab1a⁻¹ and fab1b⁻¹/fab1b⁻¹ plants exhibited normal germination rates and frequencies. Germination frequencies for both lines were not significantly different from the wild type, being greater than 95% on soil and greater than 98% on 1× Murashige and Skoog medium. Both mutant lines appeared healthy and developed without displaying any gross morphological defects. However, by 4 weeks postgermination, it was noted that the leaves of both fab1a⁻¹/fab1a⁻¹ and fab1b⁻¹/fab1b⁻¹ mutant lines appeared different from those of wild-type plants. Leaves of mutant plants were noticeably curled at their margins (Fig. 2B). A quantitative analysis of leaf curling (Fig. 2C) revealed that there was a statistically significant difference in the degree of curling of fab1b⁻¹/fab1b⁻¹ compared with the wild type (P = 0.027). Although there was a similar trend in the data for
fab1a-1/fab1a-1, the phenotype was not as severe \( (P = 0.066) \). Both fab1a-1/fab1a-1 and fab1b-1/fab1b-1 plant lines were fully reproductively viable, displaying seed set values indistinguishable from that of the wild type.

**fab1a-1/fab1a-1/fab1b-1/fab1b-1 Double Mutants Cannot Be Generated**

Due to the ubiquitous expression profile of \( FAB1A \) and \( FAB1B \) and the lack of severe developmental defects in mutant plant lines, it remains feasible that \( FAB1A \) and \( FAB1B \) proteins are functionally redundant. In order to generate a double mutant (\( fab1a-1/fab1a-1/fab1b-1/fab1b-1 \)), both single mutant lines were crossed with one another. The resulting F1 plants (\( fab1a-1/+;fab1b-1/+ \)) were allowed to self-pollinate, and their progeny were screened by PCR for the presence of double homozygous mutants. Of 86 plants that were screened (Supplemental Table S1), not a single double mutant was detected, despite there being a theoretical one in 16 probability of recovering such a plant (assuming no transmission defects). However, \( fab1a-1/+;fab1b-1/fab1b-1 \) and \( fab1a-1/fab1a-1;fab1b-1/+ \) plants were identified, and these lines developed to maturity, again with no severe vegetative morphological defects. This suggests that a single copy of either \( FAB1A \) or \( FAB1B \) is sufficient to support growth and development. In order to further increase the chances of obtaining \( fab1a-1/fab1a-1/fab1b-1/fab1b-1 \) double mutants, \( fab1a-1/+;fab1b-1/fab1b-1 \) and \( fab1a-1/fab1a-1/fab1b-1/+ \) plants were allowed to set self seed. In these cases, potentially one-fourth of the progeny should be homozygous for both mutant alleles, again assuming no transmission defects. Fifty plants from each parent were screened by PCR to determine their genotype, and again no double mutants were recovered.

Importantly, the seed set of both \( fab1a-1/+;fab1b-1/fab1b-1 \) and \( fab1a-1/fab1a-1/fab1b-1/+ \) plants was normal (Fig. 3). As no gaps were observed in the developing siliques, this indicates that our inability to detect double mutants was not due to failures in the ability of plants to (1) produce female gametes or (2) undergo seed development following fertilization. Furthermore, seeds harvested from these siliques germinated

![Figure 2](Figure 2. Identification of a leaf curl phenotype in fab1a-1/fab1a-1 and fab1b-1/fab1b-1 plants. A, Exon and intron structure of Arabidopsis \( FAB1A \) and \( FAB1B \) genes. Boxes represent exons and intervening lines represent introns. The locations and orientations of SALK T-DNA insertions are indicated for both \( fab1a-1 \) and \( fab1b-1 \) alleles. LB and RB indicate the left border and right border of the T-DNA, respectively. Inward-facing arrows indicate the positions and directions of primers used in RT-PCR experiments to verify that the T-DNA insertions disrupted gene expression. The region of the genes encoding the catalytic PtdIns(3)P 5-kinase domain are indicated (PIP5K). B, Plant lines homozygous for \( fab1a-1 \) and \( fab1b-1 \) mutant alleles have a distinct leaf curl phenotype in comparison with wild-type (WT) plants; plants shown are 4 weeks old. C, The degree of curling was quantified by comparing the ratio of the uncurled leaf area with the visible curled surface area for each line. Leaf areas were determined by digital image analysis using ImageJ software. Curling was most extreme in \( fab1b-1/fab1b-1 \) plants, being significantly different from the wild type \( (P = 0.027) \). \( fab1a-1/fab1a-1 \) plants displayed a less extreme, although consistent, phenotype in comparison with the wild type \( (P = 0.066) \). Plotted are the ratio means with 95% confidence intervals indicated by vertical bars. Leaves were analyzed from three randomly selected plants from each line (numbers of leaves measured: wild type, \( n = 30 \); \( fab1a-1/fab1a-1 \), \( n = 32 \); \( fab1b-1/fab1b-1 \), \( n = 32 \).)

![Figure 3](Figure 3. Seed set in mature siliques of wild-type (WT), \( fab1a-1/+;fab1b-1/fab1b-1 \) and \( fab1a-1/fab1a-1/fab1b-1/+ \) plant lines. \( fab1a-1/+;fab1b-1/fab1b-1 \) and \( fab1a-1/fab1a-1/fab1b-1/+ \) plants have seed set indistinguishable from the wild type.)
with frequencies comparable to the wild type (99% on 1x Murashige and Skoog medium; n = 100). Taken together, these data are strongly indicative of a transmission defect affecting only fab1a-1/fab1b-1 pollen.

**fab1a-1/fab1b-1 Pollen Is Not Viable**

Plants carrying three mutant alleles (fab1a-1/+; fab1b-1/ fab1b-1 and fab1a-1/fab1a-1/fab1b-1/) will produce pollen of which 50% will lack both wild-type FAB1A and FAB1B genes (i.e. will be of the genotype fab1a-1/ fab1b-1). Pollen derived from these plants was checked for viability by staining with fluorescein diacetate (FDA). A large proportion of pollen isolated from fab1a-1/+; fab1b-1/fab1b-1 (34%) and fab1a-1/fab1a-1; fab1b-1/+ (43%) plants failed to stain with FDA and therefore was likely to be dead (Fig. 4; Table I). Many of these grains were seen to be collapsed and were frequently devoid of cytoplasmic content (Fig. 4B). Staining of pollen with the nucleic acid stain 4’-6-diamidino-phenylindole (DAPI) supported the FDA data, as many grains lacked distinct sperm and vegetative cell nuclei. However, of those grains that remained intact, none was found to have abnormalities in the number of nuclei (two sperm nuclei and one vegetative cell nucleus), suggesting that gametogenesis progresses normally for both wild-type and mutant grains.

To further investigate whether fab1a-1/fab1b-1 pollen is capable of effecting fertilization, restricted pollinations were carried out between fab1a-1/+; fab1b-1/ fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ pollen parents and a transgenic male-sterile line (A9::barnase) as the mother. Fifty progeny resulting from each of these pollinations were screened for the presence of fab1a-1 or fab1b-1 mutant alleles, and none of the resulting plants were found to have been sired by fab1a-1/ fab1b-1 pollen. Thus, we conclude that the failure to generate fab1a-1/fab1a-1/fab1b-1/fab1b-1 double mutant plants is entirely attributable to fab1a-1/fab1b-1 pollen being inviable and not a case of it being at a competitive disadvantage in pollinations of mixed pollen genotype.

**fab1a-1/fab1b-1 Pollen Displays an Abnormal Vacuolar Phenotype Late in Pollen Development**

To further investigate fab1a-1/fab1b-1 pollen inviability, fresh mature pollen, derived from fab1a-1/+; fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants, was stained with Neutral Red, which rapidly accumulates in vacuoles and vesicles. Shortly after pollen hydration in the stain, approximately 50% of these pollen grains had either an abnormal vacuolar phenotype or were unstained/dead (Table II; Fig. 4, F–I, K, and L), whereas the vast majority of pollen grains from wild-type plants (98%) displayed numerous small vacuoles of relatively uniform size and morphology (Table II; Fig. 4, E and J). The majority of grains that were deemed abnormal were collapsed (Fig. 4, K and L).

**Figure 4.** Phenotypic analysis of mature pollen from wild-type, fab1a-1/++; fab1b-1/fab1b-1, and fab1a-1/fab1a-1/fab1b-1/+ plants. A and B, Fluorescence microscopy of FDA-stained pollen derived from wild-type (A) and fab1a-1/++; fab1b-1/fab1b-1 (B) plants. FDA stains live cells green when imaged under UV light. A large proportion of pollen from fab1a-1/++; fab1b-1/fab1b-1 plants was collapsed and failed to stain with FDA (white arrowheads in B), indicating that they were dead. This was also observed for pollen from fab1a-1/fab1a-1/fab1b-1/+ (data not shown). C and D, DAPI staining of pollen nuclei revealed that uncollapsed pollen from fab1a-1/++; fab1b-1/fab1b-1/+ plants (D) were trinucleate, as they were for wild-type pollen (C). This was also observed for pollen from fab1a-1/fab1a-1/fab1b-1/+ plants. White arrows indicate the diffuse vegetative nucleus in close proximity to the more intensely fluorescing pair of sperm nuclei. E to L, Neutral Red staining of pollen vacuoles observed by light differential interference contrast microscopy. Wild-type pollen is characterized as containing many small, regular vacuoles (E and J). Pollen samples from fab1a-1/++; fab1b-1/+ (K) and fab1a-1/++; fab1b-1/fab1b-1 (L) plants contained a large number of collapsed grains (black arrowheads). A small proportion of the collapsed grains from fab1a-1/++; fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants had a range of highly abnormal vacuolar phenotypes, typified by the presence of relatively few, large, and frequently irregular vacuoles (F–I).
L), although some remained expanded and displayed a range of phenotypes typified by the presence of relatively few vacuoles that were irregular in size and morphology, with some vacuoles being exceedingly large (Fig. 4, F–I).

In order to assess at which stage of pollen development the fab1a-1/fab1b-1 mutations exerted their effects, chemically fixed floral tissues from fab1a-1/+; fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants were sectioned and observations were made with a light microscope. No evidence of aberrant pollen/vacuolar morphology could be detected at early developmental stages, with tetrads and the highly vacuolated uninucleate microspores looking uniform and identical to pollen in anthers from wild-type plants (data not shown). Uninucleate microspores then divide to form bicellular pollen consisting of a large vegetative cell, which fills the pollen wall cavity, and the generative cell, which resides within the vegetative cell cytoplasm. The transition from the uninucleate to the bicellular stage of development is followed immediately by fragmentation of the large vacuole that predominated in uninucleate microspores (McCormick, 1993; Yamamoto et al., 2003). We observed no significant differences between wild-type pollen and pollen from fab1a-1/+; fab1b-1/fab1b-1 and fab1a-1/fab1a-1; fab1b-1/+ plants at this stage of development. As development proceeded toward the second mitotic division, which produces two sperm cells from the generative cell, there was some indication that vacuoles were enlarged and irregularly sized in a proportion of grains from the two mutant lines compared with the wild type (Fig. 5, A, C, and E). This abnormal vacuolar phenotype became more marked just prior to and after the second mitotic division in both mutant lines (Fig. 5, G and H), whereas vacuoles of wild-type grains appeared to fragment into many smaller vacuoles and disperse at the transition from the bicellular to the tricellular stage of development (Yamamoto et al., 2003). As tricellular pollen matured in wild-type anthers, the pollen cytoplasm took on a smoother appearance, with vacuoles being difficult to detect with certainty among many other membrane-bound structures. In contrast, nearly 50% of grains from mutant lines underwent rapid collapse or exhibited extreme vacuolar morphologies as tricellular pollen matured (Fig. 5, D and F). Taken together, these observations strongly suggest that fab1a-1/fab1b-1 pollen grains develop normally until just after the first mitotic division. However, following this, during the phase of extensive vacuole fragmentation that occurs prior to the second mitotic division, these grains fail to regulate this process properly.

Further analysis of pollen development by transmission electron microscopy for fab1a-1/+; fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants confirmed the observations obtained by light microscopy. The ultrastructure of “early” bicellular pollen was highly similar between wild-type (Fig. 6, A and B) and mutant (Fig. 6, E, F, I, and J) lines. However, highly aberrant vacuolar phenotypes were observed in grains from mutant plant lines at the tricellular stage of development (Fig. 6, G, H, K, and L). These vacuoles were frequently very large and irregular in shape with uneven boundaries (Fig. 6, G and H), starkly contrasting with the small, more uniform vacuoles with smooth peripheries present in wild-type grains (Fig. 6, C and D). Many of these large vacuoles contained granular deposits that appeared to be degraded cytoplasm. In many cases, these vacuoles filled the majority of the pollen cavity, suggesting large-scale loss of cytoplasm and degradation of cellular components. Again, many grains were completely collapsed and contained little or no cytoplasm (Fig. 6K).

Table I. Analysis of pollen viability by FDA staining

The effect of fab1a-1 and fab1b-1 mutant alleles on pollen viability was assessed by staining pollen from freshly dehisced anthers with FDA as described in “Materials and Methods.” Viable grains stain FDA positive (Fig. 4, A and B). Actual numbers of grains scored are indicated in parentheses.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Pollen FDA Positive</th>
<th>Pollen FDA Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>99 (198)</td>
<td>1 (2)</td>
</tr>
<tr>
<td>fab1a-1/+; fab1b-1/fab1b-1</td>
<td>66 (145)</td>
<td>34 (75)</td>
</tr>
<tr>
<td>fab1a-1/fab1a-1/fab1b-1/+</td>
<td>57 (166)</td>
<td>43 (125)</td>
</tr>
</tbody>
</table>

Table II. Assessment of pollen vacuolar phenotype by Neutral Red staining

The effect of fab1a-1 and fab1b-1 mutant alleles on the morphology of vacuoles in pollen collected from freshly dehisced anthers was assessed by staining with Neutral Red as described in “Materials and Methods.” Pollen scored as “normal” contained numerous small vacuoles (Fig. 4E), whereas grains scored as “abnormal” typically contained fewer irregularly sized large vacuoles (Fig. 4, F–I) or were collapsed. “Unstained” pollen grains lacked cytoplasm and were frequently collapsed. Actual numbers of grains scored are indicated in parentheses.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Normal Pollen</th>
<th>Abnormal Pollen</th>
<th>Unstained Pollen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>98 (191)</td>
<td>1.5 (3)</td>
<td>0.5 (1)</td>
</tr>
<tr>
<td>fab1a-1/+; fab1b-1/fab1b-1</td>
<td>55.4 (210)</td>
<td>30.1 (114)</td>
<td>14.5 (55)</td>
</tr>
<tr>
<td>fab1a-1/fab1a-1/fab1b-1/+</td>
<td>48.1 (140)</td>
<td>19.6 (57)</td>
<td>32.3 (94)</td>
</tr>
</tbody>
</table>
DISCUSSION

PtdIns(3,5)P_2 is a ubiquitous but minor membrane phospholipid in plants, animals, and yeast (Dove et al., 1997; Whiteford et al., 1997; Meijer et al., 1999). It has a conserved role in regulating endomembrane trafficking events in animals and yeast (for review, see Dove et al., 2009). PtdIns(3,5)P_2 production is tightly regulated (Sbrissa et al., 2007; Botelho et al., 2008; Ikonomov et al., 2009) and inducible by a number of different stimuli. For example, hyperosmotic stress increases levels of PtdIns(3,5)P_2 by over 20-fold in yeast (Dove et al., 1997) and significantly increases levels in animal cells and plants (Meijer et al., 1999; Zonia and Munnik, 2004; Sbrissa and Shisheva, 2005). Hormonal stimuli, such as insulin in mammalian cells, can also lead to an increased production of PtdIns(3,5)P_2 (Ikonomov et al., 2007).

In animals and yeast, PtdIns(3,5)P_2 is produced from PtdIns3P by the action of PtdIns3P 5-kinases called PIKfyve and Fab1p, respectively (Shisheva, 2008). PIKfyve and Fab1p have similar domain architecture, consisting of an N-terminal FYVE domain that recruits the protein to specific membranes by binding PtdIns3P (Sbrissa et al., 2002), a central Cpn60_TCP1 domain that probably plays a regulatory role, and a kinase domain that is responsible for the conversion of PtdIns3P to PtdIns(3,5)P_2. In Arabidopsis, the two genes encoding proteins with this domain architecture are FAB1A (At4g33240) and FAB1B (At3g14270). In addition, there are two genes, FAB1C (At1g71010) and FAB1D (At1g34260), that contain a Cpn60_TCP1 domain and a C-terminal lipid kinase domain but lack a FYVE domain. As the FYVE domain is not essential for yeast Fab1p to phenotypically rescue a fab1D strain (Botelho et al., 2008), the possibility that FAB1C and FAB1D are PtdIns3P 5-kinases cannot be ruled out.

The only obvious abnormal feature of FAB1A and FAB1B homozygous T-DNA insertion lines was a leaf-curving phenotype. Given the apparent evolutionarily conserved role of PtdIns(3,5)P_2 in osmoregulation, the inability of fab1a-1/fab1a-1 and fab1b-1/fab1b-1 lines to produce sufficient levels of this lipid to correctly regulate turgor pressure may explain the leaf curling. The severity of leaf curl was most pronounced in fab1b-1/fab1b-1 plants, consistent with FAB1B being more highly expressed than FAB1A in leaves (Supplemental Fig. S1). Despite phenotypic differences between both the homozygous single mutant lines and wild-type plants, the mutants developed to maturity and exhibited normal seed set. The broadly overlapping expression profiles of FAB1A and FAB1B, together with similar phenotypes being exhibited by fab1a-1/fab1a-1 and fab1b-1/fab1b-1 mutants, suggest that FAB1A and FAB1B are, to a large extent, functionally redundant. We were unable to assess developmental

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Figure 5. Phenotypic analysis by light microscopy of pollen development in wild-type, fab1a-1/fab1a-1;fab1b-1/+; and fab1a-1/+;fab1b-1/ fab1b-1 plant lines. Pollen grains in anthers at bicellular and tricellular stages of development are shown, respectively, for wild-type (A and B), fab1a-1/fab1a-1;fab1b-1/+ (C and D), and fab1a-1/+;fab1b-1/ fab1b-1 (E and F) plant lines. Wild-type pollen displayed multiple small vacuoles at both bicellular and tricellular stages of development (A and B). Some bicellular pollen grains in mutant lines contained abnormally large vacuoles (black arrowheads in C and E). A large proportion of tricellular grains in mutant lines were collapsed (black stars in D and F), although some were only partially collapsed and contained very large vacuoles (black arrowheads in F). The abnormal vacuolar phenotype was noted to be more marked in bicellular grains approaching the second mitotic division in both fab1a-1/fab1a-1;fab1b-1/+ (G) and fab1a-1/+;fab1b-1/fab1b-1 (H) plant lines (black arrowheads indicate abnormally large vacuoles). GC, Generative cell; SC, sperm cell; Tp, tapetum; VN, vegetative nucleus. Bars = 20 μm.
phenotypes in fab1a-1/fab1a-1/fab1b-1/fab1b-1 double mutant plants, as attempts to generate such mutants were unsuccessful. Genetic analyses revealed unambiguously that fab1a-1/fab1b-1 pollen was incapable of transmitting these alleles to the next generation. If the role of FAB1A and FAB1B in growth and development is to be fully elucidated, transgenic approaches aimed at abolishing their respective transcripts will have to be employed.

Importantly, it has been previously reported that Arabidopsis pollen lacking the PtdIns 3-kinase VPS34 have severe developmental defects (Lee et al., 2008b). VPS34 produces PtdIns3P from PtdIns; as PtdIns3P is a substrate for Fab1/PIKfyve enzymes, and therefore required for PtdIns(3,5)_2 synthesis, the defects seen in VPS34-deficient pollen could be primarily due to a lack of PtdIns(3,5)_2 production (rather than an absence of PtdIns3P). In order to test this hypothesis, pollen development in plants carrying fab1a-1 and fab1b-1 mutant alleles was investigated. In fab1a-1/+; fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants, 50% of the pollen will have a fab1a-1/fab1b-1 genotype. A large proportion of pollen from these plants did not stain with FDA and therefore was considered to be nonviable/dead pollen (34% and 43%, respectively). We predict that the dead pollen is mainly fab1a-1/fab1b-1 in genotype, as pollen from fab1a-1/fab1a-1 (fab1a-1/FAB1B pollen) and fab1b-1/fab1b-1 (FAB1A/ fab1b-1 pollen) plants appear normal (>98% FDA positive). Although some fab1a-1/fab1b-1 pollen survives to maturity, Neutral Red staining revealed a further population of grains with aberrant vacuolar morphologies. Together, these grains and those that were observed to be dead account for the 50% of fab1a-1/fab1b-1 pollen in the population. Another notable feature of pollen from fab1a-1/+;fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants was that it tended to aggregate and was difficult to remove from anthers. No such problems were noted for pollen derived from either fab1a-1/fab1a-1 or fab1b-1/fab1b-1 plants.

The phenotypes observed in pollen from fab1a-1/+; fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants are very similar to those described for VPS34 mutant plants (Lee et al., 2008b). However, there are some important differences. Some transmission of the vps34-1 allele was observed by Lee et al. (2008b), but despite extensive efforts, transmission of fab1a-1/fab1b-1 pollen was never observed in our study. In addition to the abnormal vacuolar phenotype, vps34 mutant plants produce an increased proportion of binucleate pollen (Lee et al., 2008b). It was proposed that abnormal vacuoles could physically prevent normal cell division in developing mutant pollen or alternatively that production of PtdIns3P may have a more specific role in directly regulating the cell cycle. As fab1a-1/fab1b-1 pollen has abnormal vacuoles but no cell division defect, we favor the latter of these explanations. We propose that the vacuolar defects observed in pollen from vps34-1 mutant plants and in fab1a-1/fab1b-1 pollen can be entirely attributable to the inabil-

Figure 6. Transmission electron microscopy of pollen from wild-type, fab1a-1/+; fab1b-1/fab1b-1, and fab1a-1/fab1a-1/fab1b-1/+ plants. Pollen was observed at bicellular (wild-type, A and B; fab1a-1/fab1a-1; fab1b-1/+; E and F; fab1a-1/+; fab1b-1/fab1b-1, I and J) and tricellular (wild-type, C and D; fab1a-1/fab1a-1; fab1b-1/+; G and H; fab1a-1/+; fab1b-1/fab1b-1, K and L) stages of development. Dashed boxes indicate enlarged regions in adjacent micrographs to the right. Many small vacuoles are present in bicellular pollen (white arrowheads in B, F, and J), although some abnormally large vacuoles were observed in a proportion of grains from mutant lines (I and J). Wild-type tricellular grains were characterized by the presence of many small regular vacuoles (white arrowhead in D), whereas approximately 50% of tricellular grains from both mutant lines were either collapsed or contained large irregular vacuoles (black stars in G, H, K, and L) in addition to enlarged regular vacuoles (white arrowhead in H). Ex, Exine; GC, generative cell; It, intine; LB, lipid body. Bars = 5 μm.
ity of mutant pollen to produce normal levels of PtdIns(3,5)P_2.

An important question to consider is how does the lack of FAB1A/FAB1B affect pollen viability? One possibility is that PtdIns(3,5)P_2 production may be required for pollen tube growth, although, as a large proportion of developing pollen in anthers of fab1a-1/+; fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants collapses long before dehiscence, it is apparent that the primary defect in pollen occurs while the pollen is still in the anthers. However, as it is known that membrane dynamics are important for polarized pollen tube elongation and that phosphoinositides play a key role in membrane trafficking and cytoskeletal reorganization during polarized cell growth (Monteiro et al., 2005; Samaj et al., 2006; Zonia and Munnik, 2008), PtdIns(3,5)P_2 may have such a role in surviving fab1a-1/fab1b-1 pollen. Experimental evidence to address the possibility of FAB1A and FAB1B being required for pollen tube growth was not obtained, as our data suggest that only a small proportion of pollen surviving beyond dehiscence was fab1a-1/fab1b-1, with the majority of surviving pollen being fab1a-1/FAB1B or FAB1A/fab1b-1 (dependent on parent).

The presence of large vacuolar structures at the late bicellular stage is the first sign that pollen development is perturbed in mutant plants. An increase in PtdIns(3,5)P_2 levels increases the surface area-to-volume ratio of intracellular organelles by mediating fission of large vacuoles (or equivalent organelles) into smaller vacuoles, protecting cells against rapid dehydration and lysis (Weisman, 2003; Sbrissa and Shisheva, 2005). In fab1a-1/fab1b-1 pollen, unable to produce normal levels of PtdIns(3,5)P_2, an inability of vacuoles to undergo fission can explain the accumulation of large vacuoles at the late bicellular stage of development. This would ultimately lead to pollen collapse during the dehydration phase that occurs just prior to anthesis. The cue for vacuole fission could be strictly developmentally regulated or induced by changes in solute concentration late in pollen development.

We are aware that the interpretation of these results relies on the assumption that Arabidopsis FAB1A and FAB1B are major sources of PtdIns(3,5)P_2 production, particularly in pollen. Due to the domain structure, we feel that we can safely assume that FAB1A and FAB1B will be PtdIns3P 5-kinases. However, it will be extremely difficult to biochemically quantify PtdIns(3,5)P_2 levels with accuracy in fab1a-1/fab1b-1 pollen by metabolic labeling methods (Zonia and Munnik, 2004), due to the high mortality of fab1a-1/fab1b-1 pollen among other genotypes from fab1a-1/+; fab1b-1/fab1b-1 and fab1a-1/fab1a-1/fab1b-1/+ plants.

Regarding the importance of FAB1A and FAB1B in plant growth and development, microarray data reveal that their expression levels are very high in pollen in comparison with most other tissues. This contrasts with FAB1C expression, which is negligible in pollen. Although FAB1D levels are relatively high in pollen, this seems likely to be due to extremely high expression in sperm cells (Supplemental Fig. S1). Interestingly, lack of FAB1A and FAB1B in the female gametophyte did not appear to have any measurable effect on fertility. Thus, it is not gametogenesis per se that requires FAB1A and FAB1B; rather, their function in reproduction is related to specific developmental processes unique to pollen. We speculate that this may be related to preparation for the dehydration of pollen prior to dehiscence, whereas no such stresses are developmentally imposed within the ovule.

In summary, we present, to our knowledge, the first genetic and phenotypic analysis of Fab1/PIKfyve family proteins in plants. We conclude that the activity of FAB1 proteins is essential for the development of viable pollen. Our results are consistent with a role for FAB1 proteins in responding to osmotic changes by controlling levels of PtdIns(3,5)P_2. This is most apparent in pollen, but the leaf curl phenotype in homozygous single mutant plants further hints at a defect in osmoregulation. Future studies will include investigations of cell and plant phenotypes grown under normal and stress conditions in conjunction with biochemical analysis of PtdIns(3,5)P_2 levels in mutant plants. In the future, it will be interesting to address whether plant orthologs of known yeast and animal effectors of PtdIns(3,5)P_2 are essential for pollen viability or whether plant-specific effectors are responsible.

MATERIALS AND METHODS

Plant Growth Conditions

All Arabidopsis (Arabidopsis italiana) lines used in this study were grown in a controlled-environment room with a 16-h-day/8-h-night cycle. Temperature was maintained at 22°C ± 1°C, and relative humidity was 60%. Seeds were sown in petri dishes containing a fine-grade peat-based compost, and seedlings were transferred to pots containing the same compost 5 d after germination.

Phylogenetic Analysis of the FAB1/PIKfyve Family of Proteins

Protein sequences were aligned using ClustalX (Larkin et al., 2007), and trees were drawn using the NJPLOT program distributed with ClustalX software. The following UniProtKB protein sequences were used for the analysis: Schizosaccharomyces pombe, FAB1_SCHPO; Aspergillus fumigatus, Q4WN65_ASPEF; Kluyveromyces lactis, Q6CSS2_KLULA; Saccharomyces cerevisiae, FAB1_YEAST; Dictyostelium discoideum, BOG126_DICDI; Chlamydomonas reinhardtii, A8JR5S_CHLRE; Oryza sativa, Q6IZN4_ORYOS; Q8H3L4_ORYOS; and Q08DB6_ORYOS; Vitis vinifera, AB69E1_VITVI; A7PDP8_VITVI; and ASC675_VITVI; Arabidopsis, QWURS5_ARATH (FAB1A), Q9LUMO_ARATH (FAB1B), Q985J8_ARATH (FAB1C), and Q9XID0_ARATH (FAB1D); Populus trichocarpa, B9HJ42_POTR; and B9W7I0_POTR; Physcomitrella patens, A95S9F_PHYPA and A9RY16_PHYPA; Medicago truncatula, A4QQTQ_MEDTR; Drosophila melanogaster, FYV1_DROME; Danio rerio, B2KTE1_DANRE; Mus musculus, FYV1_MOUSE; and Homo sapiens, FYV1_HUMAN.

Isolation of FAB1A and FAB1B Mutant Plant Lines

SALK T-DNA insertion lines (Alonso et al., 2003) were obtained from the Nottingham Arabidopsis Stock Centre. Genotyping of plants was carried out.
by PCR using genomic DNA as a template. fab1a-1 alleles (SALK, 013923) were identified using the following primers: T-DNA-specific left border primer (5'-ATTGGTCGACCTTACGACC-3') and gene-specific left (LP: 5'-CTGCTGCTGTCTCGAG-3') and right (RP: 5'-CTGGTCGAGCTTGCAGTACC-3') primers. fab1b-1 alleles (SALK, 066673) were identified using the same left border primer in conjunction with gene-specific LP (5'-TGGACCATGCTGTCAGTACC-3') and RP (5'-GAGGACGGTACGAGACC-3') primers. Likewise fab1b-1/fab1b-1 plants were also tested by RT-PCR using LP (5'-TGGTCGAGCTTGCAGTACC-3') and RP (5'-GAGGACGGTACGAGACC-3') primers. Total RNA for RT-PCR was isolated using the RNeasy kit (Qiagen) with the manufacturer's instructions being followed.

Leaf Curl Analysis
The degree of leaf curl was quantified for wild-type, fab1a-1/fab1a-1 and fab1b-1/fab1b-1 plants by estimating the visible leaf area in photographs taken directly above plants and comparing this area with actual leaf area. Leaves were removed sequentially from the plants to permit a full analysis. Each leaf was then flattened under clear adhesive plastic and rephotographed. Image analysis was carried out using ImageJ software (Abramoff et al., 2004) to obtain accurate measurements.

Pollen Analysis
For all staining methods, pollen was initially collected from six open flowers placed in 400 μL of an 8% (w/v) Suc solution. The flowers were briefly vortexed to release pollen and then removed with tweezers. Pollen grains were immersed in 8% (w/v) Suc containing 0.5 mM the appropriate stain for microscopical analysis. Observations were carried out with a Nikon ECLIPSE 90i microscope (Nikon Instruments). For transmission electron microscopy operating at 80 kV. Nikon ECLIPSE 90i microscope (Nikon Instruments). For transmission electron microscopy operating at 80 kV. Toluidine Blue for light microscopy. Sections (100 nm thick) were cut for material was washed in distilled water. Samples were stained in 1% aqueous material was washed in distilled water. Samples were stained in 1% aqueous aqueous water. Sections were then rinsed in PIPES buffer. Postfixation was carried out in 1% (w/v) osmium tetroxide for 4 h at 4°C, following which the material was washed in distilled water. Samples were stained in 1% aqueous uranyl acetate for 1 h in the dark and then dehydrated in an ethanol series (30%, 50%, 70%, 80%, 90%, 95%, and 100%) and embedded in LR White resin (London Resin Company). Sections (0.5 μm thick) were cut and stained with Toluidine Blue for light microscopy. Sections (100 nm thick) were cut for transmission electron microscopy. Light microscopy was conducted with a Nikon ECLIPSE 90i microscope (Nikon Instruments). Transmission electron microscopy, observations were made with a JEOL 1200EXII transmission electron microscope operating at 80 kV.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_119478 and NM_112285.

Supplemental Data
The following materials are available in the online version of this article.
the trans-Golgi network to the lumen of the central vacuole in plant cells. Plant Cell 13: 287–301
Welters P, Takegawa K, Emr SD, Chrispeels MJ (1994) AtVPS34, a phosphatidylinositol 3-kinase of Arabidopsis thaliana, is an essential protein with homology to a calcium-dependent lipid binding domain. Proc Natl Acad Sci USA 91: 11399–11402