An Arabidopsis Homolog of Yeast *ATG6/VPS30* Is Essential for Pollen Germination[1][W]

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Yeast (*Saccharomyces cerevisiae*) Atg6/Vps30 is required for autophagy and the sorting of vacuolar hydrolases, such as carboxypeptidase Y. In higher eukaryotes, however, roles for *ATG6/VPS30* homologs in vesicle sorting have remained obscure. Here, we show that *AtATG6*, an Arabidopsis (*Arabidopsis thaliana*) homolog of yeast *ATG6/VPS30*, restored both autophagy and vacuolar sorting of carboxypeptidase Y in a yeast *atg6/vps30* mutant. In Arabidopsis cells, green fluorescent protein-AtAtg6 protein localized to punctate structures and colocalized with AtAtg8, a marker protein of the preautophagosomal structure. Disruption of *AtATG6* by T-DNA insertion resulted in male sterility that was confirmed by reciprocal crossing experiments. Microscopic analyses of *AtATG6* heterozygous plants (*AtATG6/atatg6*) crossed with the *quartet* mutant revealed that *AtATG6*-deficient pollen developed normally, but did not germinate. Because other *atatg* mutants are fertile, AtAtg6 likely mediates pollen germination in a manner independent of autophagy. We propose that Arabidopsis Atg6/Vps30 functions not only in autophagy, but also plays a pivotal role in pollen germination.

Autophagy is a bulk degradation mechanism by which cytoplasmic constituents are delivered to lytic compartments for degradation and recycling (Klionsky and Ohsumi, 1999; Ohsumi, 2001; Levine and Klionsky, 2004). The process involves sequestration of the cytoplasm into double-membrane vesicles called autophagosomes, which subsequently fuse with lysosomes or vacuoles. Genetic studies in yeast (*Saccharomyces cerevisiae*) have identified a set of *ATG* (autophagy) genes involved in this process (Klionsky and Ohsumi, 1999; Ohsumi, 2001). Various Atg proteins reside on the preautophagosomal structure (PAS) from which autophagosomes originate (Suzuki et al., 2001). The molecular mechanisms underlying the formation of autophagosomes, however, have not yet been fully elucidated.

Most of the yeast Atg proteins are specifically engaged in autophagy and/or the cytoplasm-to-vacuole targeting pathway, which is mechanistically similar to autophagy (Klionsky and Ohsumi, 1999; Levine and Klionsky, 2004). By contrast, *ATG6* is allelic to the previously characterized *VPS30* (vacuolar protein sorting), which is required for the vacuolar sorting of carboxypeptidase Y (CPY; Seaman et al., 1997; Kametaka et al., 1998). *ATG6/VPS30* is conserved in higher eukaryotes and its ortholog (*BECLIN 1*) can restore autophagy in *ATG6/VPS30*-deficient yeast (Liang et al., 1999; Meléndez et al., 2003; Liu et al., 2005). Suppression of *BECLIN 1* homologs blocked autophagy and disturbed various biological processes (Meléndez et al., 2003; Yu et al., 2004; Liu et al., 2005). On the other hand, the putative role of *BECLIN 1* in membrane trafficking is controversial (Zeng et al., 2006).

Many Atg proteins are conserved in plants, and reverse genetic studies have demonstrated that Arabidopsis (*Arabidopsis thaliana*) *atg* mutants are hypersensitive to nutrient starvation, as well as that *atg* plants exhibit accelerated senescence even under favorable growth conditions (for review, see Thompson and Vierstra, 2005; Bassham et al., 2006). Here, we are interested in *AtATG6*, an Arabidopsis homolog of yeast *ATG6/VPS30*, because it is a unique autophagy gene that also participates in trafficking events in yeast. We first investigated whether *AtATG6* was involved in autophagy and found that *AtATG6* restored autophagy in *atg6/vps30* yeast and that it localized in plant cells to a punctate structure containing Arabidopsis Atg8, a marker protein of the PAS (Suzuki et al., 2001). We also show that Arabidopsis Atg6/Vps30 partially complemented VPS function in *atg6/vps30* mutant yeast. Several other Arabidopsis *VPS* homologs are required in pollen tubes (Hicks et al., 2004; Lobstein et al., 2004) because the polarized growth of pollen tubes requires membrane trafficking (Hepler et al., 2001; Monteiro et al., 2005). We found that disruption of *AtATG6/VPS30* resulted in male sterility because the mutant pollen did not germinate. By contrast, all previously characterized *atatg* mutants are fertile. Our results support the idea that Atg6/Vps30 in plants has distinct functions in addition to autophagy: vesicle trafficking and pollen germination.

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RESULTS

Arabidopsis Atg6 Restores Autophagy and Vacuolar Protein Sorting in *atg6/vps30* Mutant Yeast

To first investigate roles of Atg6/Vps30 in higher plants, we isolated a putative Arabidopsis homolog of *ATG6/VPS30* (*AtATG6*) encoded by a single gene (At3g61710) in the Arabidopsis genome. *AtATG6*-encoded protein shares 24% amino acid identity with yeast Atg6/Vps30 (Seaman et al., 1997; Kametaka et al., 1998) and 31% identity with human Beclin 1 (Liang et al., 1999). Animal homologs of *ATG6/VPS30* (*BECLIN 1*) restored autophagy, but not CPY sorting, in *atg6/vps30* mutant yeast (Liang et al., 1999; Meléndez et al., 2003). To test whether *AtATG6* is a functional homolog of *ATG6/VPS30*, we investigated whether AtAtg6 rescues defects of yeast *atg6/vps30* in autophagy and the VPS pathway. *AtATG6* cDNA containing the entire open reading frame (ORF) was fused with the yeast *ATG6/VPS30* promoter in a low-copy-number plasmid (pRS416) and expressed in *ATG6/VPS30*-deficient yeast (parental strain BY4741). Wild-type yeast cells accumulated numerous autophagic bodies within vacuoles after 5 h of nutrient starvation, whereas no such vesicles were found in the vacuoles of *atg6/vps30* yeast cells (Fig. 1A). Under these conditions, expression of *AtATG6* allowed *atg6/vps30* cells to accumulate autophagic bodies, albeit to a lesser extent than did wild-type cells (Fig. 1A).

We next measured the amount of intracellular and extracellular CPY protein to determine whether CPY was delivered into vacuoles or secreted. Wild-type or *atg6/vps30* cells transformed with *ATG6/VPS30* seemed to properly sort CPY into vacuoles, where CPY was processed to its mature form of 61 kD (Fig. 1B, mCPY). In *atg6/vps30* cells, on the other hand, mis-sorted CPY was secreted (Fig. 1B, pCPY) and concomitantly lower quantities of mature CPY were detected in the intracellular fraction (Fig. 1B, mCPY). In *AtATG6*-transformed *atg6/vps30* cells, secretion of CPY was suppressed, suggesting that AtAtg6 restores vacuolar sorting of CPY in *ATG6/VPS30*-disrupted yeast.

AtAtg6 Colocalizes with AtAtg8 at a Punctate Structure

In yeast cells, green fluorescent protein (GFP)-tagged Atg6/Vps30, like other Atg proteins, partially localizes to the punctate structure called the PAS (Obara et al., 2006). To visualize AtAtg6 protein in plant cells, a GFP-AtAtg6 fusion protein was expressed in Arabidopsis protoplasts under the control of the cauliflower mosaic virus 35S promoter. As shown in Figure 2A, the fluorescent signal from GFP-AtAtg6 was distributed in the cytoplasm and to a single or a few bright dots. Similar dot signals were observed when AtAtg6 was fused to the N terminus of GFP (AtAtg6-GFP; data not shown). Such punctation was not found in cells transformed with the empty vector (35S-GFP alone; Fig. 2A; see also Supplemental Fig. S1B).

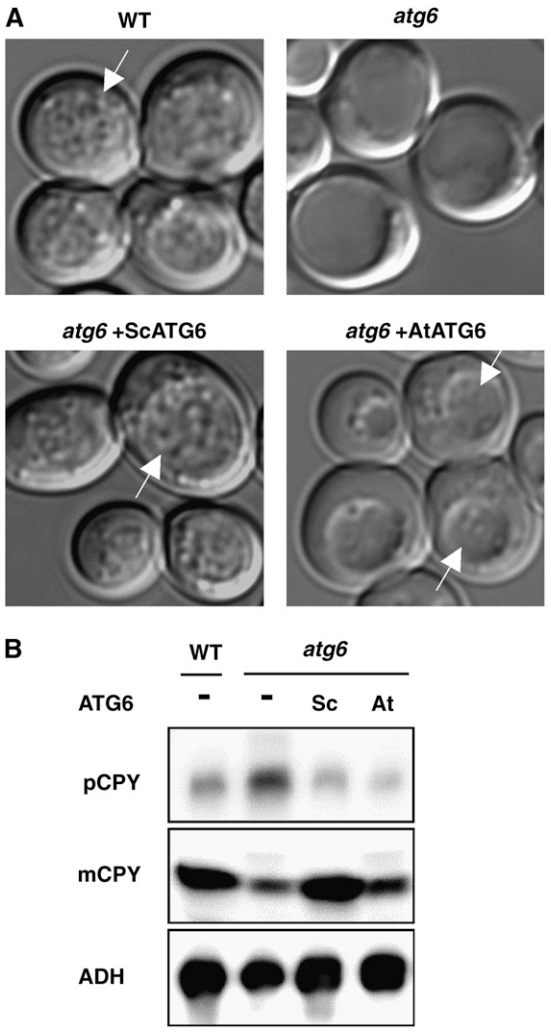

Figure 1. Functional complementation by Arabidopsis *ATG6/VPS30* of autophagy and vacuolar protein sorting in a yeast *atg6/vps30* mutant. The Arabidopsis *ATG6/VPS30* (*AtATG6*) gene was cloned in the low-copy-number plasmid pRS416 and expressed in *atg6/vps30* yeast cells. Yeast *ATG6/VPS30* (*ScATG6*) and the empty vector (pRS416) were used as controls. A, Cells grown to mid-log stage were collected, washed, and subsequently incubated for another 5 h in nutrient-free medium in the presence of 1 mM phenylmethylsulfonyl fluoride. Autophagic bodies accumulated within vacuoles (arrows). B, Intracellular or extracellular extracts were prepared from wild-type or *atg6/vps30* cells that were transformed with the empty vector (−), yeast (Sc), or Arabidopsis (At) *ATG6/VPS30*. Secreted CPY (pCPY) in the extracellular extracts and vacuolar-targeted CPY (mCPY) or alcohol dehydrogenase (ADH) in the intracellular extracts were detected by immunoblotting.

We next attempted to determine the organelle to which AtAtg6 localized. Punctate localization of GFP-AtAtg6 was not influenced by brefeldin A (BFA; Fig. 2B). Venus-tagged SYP31, SYP41, and SYP81 served as marker proteins for the Golgi, trans-Golgi network (TGN), and endoplasmic reticulum (ER), respectively (Uemura et al., 2004), and each of these marker proteins showed intracellular distribution patterns that were clearly distinguished from that of AtAtg6 (Fig. 2,

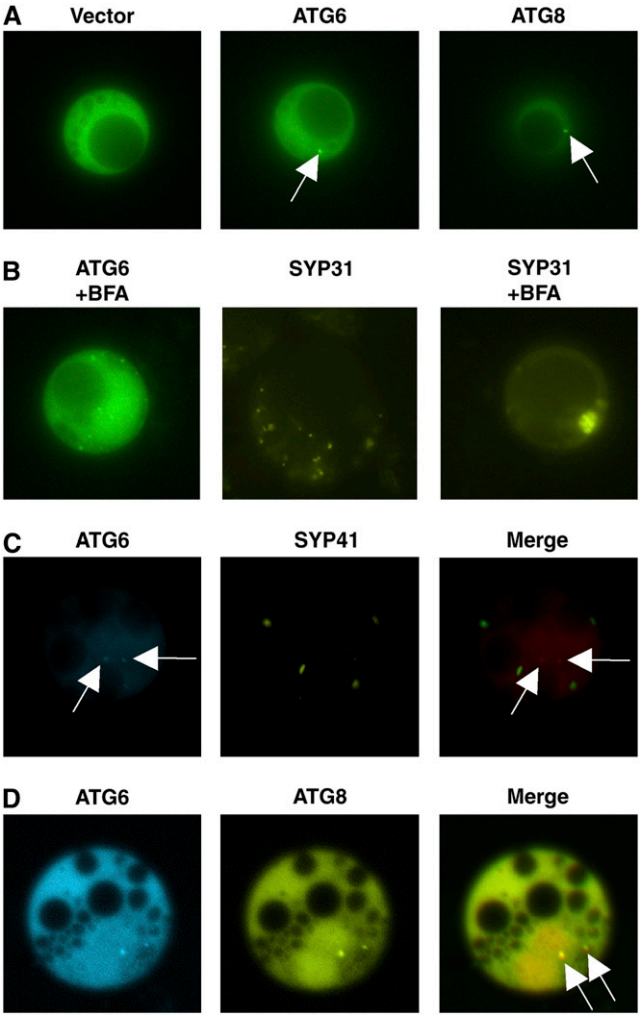

Figure 2. Intracellular localization of AtAtg6 in Arabidopsis cells. AtAtg proteins tagged with GFP derivatives were expressed in protoplasts prepared from Arabidopsis suspension cells. Protoplasts were incubated for 24 h in Suc-free medium. A, Localization of GFP-AtAtg6 (middle) and GFP-AtAtg8 (right), a marker protein of the PAS (arrows). The vector control (pEZS-CL) is shown at left. B, Effects of BFA on the localization of GFP-AtAtg6. Cells were incubated for 2 h in the presence of 200 μM BFA. Venus-tagged SYP31 was used as a Golgi marker. C, CFP-AtAtg6 and Venus-tagged SYP41, a marker protein of TGN, were expressed together in Arabidopsis cells. Arrows indicate punctate signals of CFP-AtAtg6. D, Punctate colocalization (arrows) of CFP-AtAtg6 and YFP-AtAtg8.

B and C; data not shown). Bright dots of cyan fluorescent protein (CFP)-AtAtg6 also did not overlap with any of these organelle markers (Fig. 2C; data not shown). In contrast, the PAS marker protein, GFP-AtAtg8, exhibited a punctate localization profile similar to that of GFP-AtAtg6 (Fig. 2A; see also Supplemental Fig. S1). We then compared the localization of CFP-AtAtg6 with that of yellow fluorescent protein (YFP)-AtAtg8. Each fusion protein again exhibited clear enrichment in the punctate structure within the cytoplasm, and the coincidence of CFP and YFP signals on the bright dots (Fig. 2D, arrows; Supplemental Fig. S2) suggested the colocalization of AtAtg6 with AtAtg8 in plant cells, similar to that shown in yeast cells (Obara et al., 2006).

Deletion of *AtATG6* Causes a Male Gametophytic Mutation

To evaluate the functions of AtAtg6 in planta, we employed a reverse genetic approach. Our initial attempts to screen Arabidopsis mutants (two independent lines, SALK_051168 and SALK_109281) harboring T-DNA insertions within the *AtATG6* gene identified no homozygous mutant lines. *AtATG6* heterozygous plants exhibited reduced *AtATG6* mRNA levels relative to wild-type plants (Supplemental Fig. S3), but appeared normal in appearance. Each *AtATG6* heterozygous plant showed approximately a 1:1 segregation ratio for transmission of the T-DNA in the first selfing progeny (Table I). A Mendelian transmission ratio of 3:1 was rejected by a χ^2 test ($P = 7.4 \times 10^{-25}$; $P = 3.6 \times 10^{-17}$). Embryos produced by selfing of *AtATG6/atatg6* plants grew normally and no aborted seeds were found in the siliques. Therefore, failures of transmission of T-DNA to the progeny seemed to be due to defects in the gametes, not embryonic lethality. Next, we performed crossing experiments to determine the gametophytes that were affected by *atg6*. When *AtATG6* heterozygous plants (background Columbia; the line SALK_051168 was used in the following experiments) were used as pollinators of wild-type plants (Wassilewskija [Ws] was used to make it possible to confirm crossing by PCR), no T-DNA-inserted progeny were obtained (Table I). This suggested the failure of transmission of *atg6* through the male gametophyte, which was statistically supported by a χ^2 test ($P = 9.2 \times 10^{-9}$). In contrast, pollination of *AtATG6* heterozygous stigmas with wild-type pollen yielded progeny with a 1:1 segregation ratio of transmission of T-DNA (Table I). The observed ratio was nearly identical to the expected T-DNA transmission ratio of 1:1 through the female gametophyte ($\chi^2 = 0.44$; $P = 0.51$). Deletion of *AtATG6*, therefore, appeared specifically to influence male gametophytes but not to compromise the function of female reproductive structures.

Table I. *Genetic transmission analysis of atg6 mutations*

Numbers of progeny containing T-DNAs (*atg6-1*, SALK_051168; *atg6-2*, SALK_109281) were counted using PCR and actual transmission efficiency (TE; the number of T-DNA-containing progeny/total number of seedlings × 100) was calculated. The χ^2 value is shown for the predicted ratio of 3:1 (self-crosses) or 1:1 (reciprocal crosses).

Genotype	Numbers		TE (%)	χ^2 (*P*)
	T-DNA$^+$	T-DNA$^-$		
ATG6/atg6-1 (selfed)	131	142	48.0	106 (7.4×10^{-25})
ATG6/atg6-2 (selfed)	33	36	47.8	71 (3.6×10^{-17})
♂ATG6/atg6-1 × ♀Ws	0	33	0.0	33 (9.2×10^{-9})
♂Ws × ♀ATG6/atg6-1	16	20	44.4	0.44 (0.51)

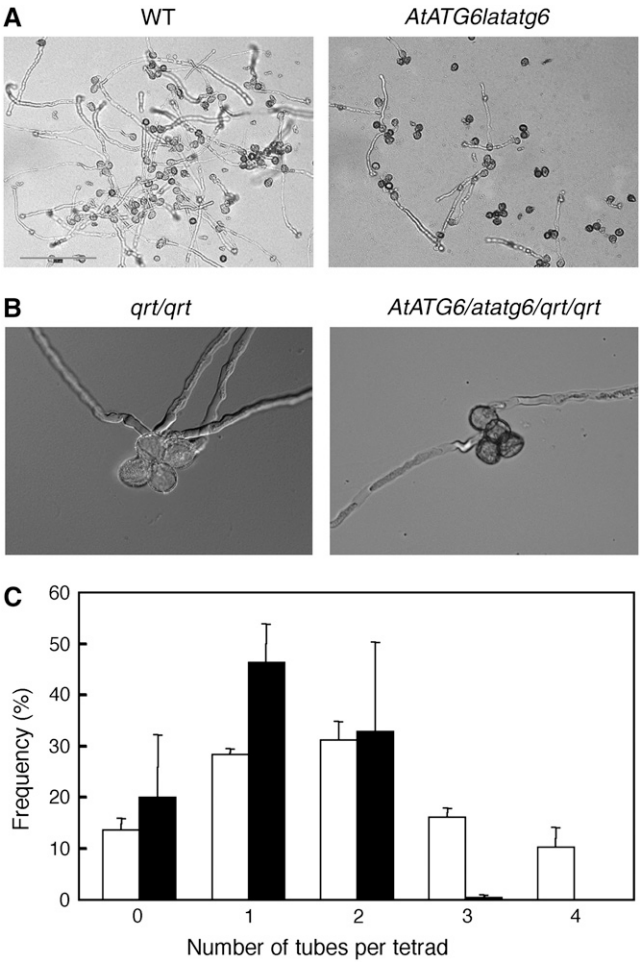

Figure 3. Pollen germination efficiency of *AtATG6/atatg6* heterozygotes. A, In vitro pollen germination of the *AtATG6/atatg6* heterozygous mutant (right) and the wild-type control (left). B, In vitro pollen germination of *AtATG6/atatg6/qrt/qrt* (right) and *AtATG6/AtATG6/qrt/qrt* (left). C, Frequency (%) of wild-type (*AtATG6/AtATG6/qrt/qrt*; white bars) and heterozygous mutant (*AtATG6/atatg6/qrt/qrt*; black bars, $n > 500$) tetrads with zero to four pollen tubes. The values shown represent the means of three different experiments (±SD).

Pollen Lacking *AtATG6* Does Not Germinate

To determine the biological basis of the male gametophytic mutant phenotype of *atatg6*, we performed microscopic analysis of *atatg6* pollen. Pollen grains from *AtATG6* heterozygous flowers were indistinguishable from wild-type pollen in shape and size (data not shown). However, the efficiency of the in vitro pollen germination of *AtATG6* heterozygous flowers (39.8 ± 1.4% of pollen grains germinated; $n = 977$) was lower than that of wild-type controls (76.7 ± 2.5%; $n = 1{,}715$; for each genotype, SES for three independent experiments are shown; Fig. 3A). Under these conditions, pollen from other *atatg* mutants (*atatg4a4b-1* and *atatg2* were tested) germinated with efficiency similar to that of wild-type controls (data not shown).

We crossed *quartet1* (*qrt1*) mutant plants to *AtATG6/atatg6* plants to further address the role of AtAtg6 in pollen germination. The *qrt* mutation yielded four microspores derived from a single pollen mother cell that stay attached to one another for the duration of pollen development, which makes it easy to perform tetrad analyses (Preuss et al., 1994; McCormick, 2004). We expected that if AtAtg6 were essential for pollen germination, at most two pollen grains in each *AtATG6/atatg6/qrt/qrt* tetrad would germinate. This prediction was correct; only one or two pollen grains germinated from each *AtATG6/atatg6/qrt/qrt* tetrad, whereas up to four pollen grains germinated from each wild-type (*AtATG6/AtATG6/qrt/qrt*) tetrad (Fig. 3, B and C). Apparently, there were no severe defects in the elongation of germinated pollen tubes of *AtATG6/atatg6/qrt/qrt* tetrads (Fig. 3B; data not shown), possibly because only the pollen-carrying wild-type *AtATG6* could germinate. These results strongly suggested that *atatg6* mutant pollen fails to germinate.

Despite the defects in pollen germination, transmission electron microscopy and environmental scanning electron microscopy (ESEM) showed that all four pollen grains in an *AtATG6/atatg6/qrt/qrt* tetrad were normal in appearance and indistinguishable from one another (Fig. 4). Each mutant pollen grain contains three normal nuclei: one vegetative and two sperm nuclei (Fig. 4F). We also stained pollen with aniline blue to investigate whether disorganization of cell wall composition (e.g. callose) was associated with failures of pollen germination (Johnson and McCormick, 2001). Each pollen grain of an *AtATG6/atatg6/qrt/qrt* tetrad, even if not germinated, was able to perform local outgrowth of the pollen wall or polarized deposition of callose in a manner similar to that of *AtATG6/AtATG6/qrt/qrt* pollen (Fig. 4, G and H). It was therefore likely that the polarity of the pollen germination site was determined normally in *atatg6* pollen. Taken together, these results suggested that AtAtg6 is not essential for male meiosis or pollen development prior to germination.

Expression of *ATG6* in Arabidopsis

We previously showed that the *AtATG4* and *AtATG8* genes were ubiquitously expressed in whole plants (Yoshimoto et al., 2004). Similarly, reverse transcription (RT)-PCR revealed that *AtATG6* mRNA was expressed in all organs tested (Fig. 5A). Expression of *AtATG6* mRNA was not up-regulated during floral development (Fig. 5B). Likewise, *POK/AtVPS52*, another Arabidopsis VPS homolog implicated in pollen tube elongation (Lobstein et al., 2004), was expressed in different tissues and continuously expressed throughout pollen development (Lobstein et al., 2004; Fig. 5B). These results suggest the occurrence of autophagy and vesicle trafficking in various tissues.

DISCUSSION

We have identified *AtATG6* as a functional homolog of yeast *ATG6/VPS30* and characterized its function in

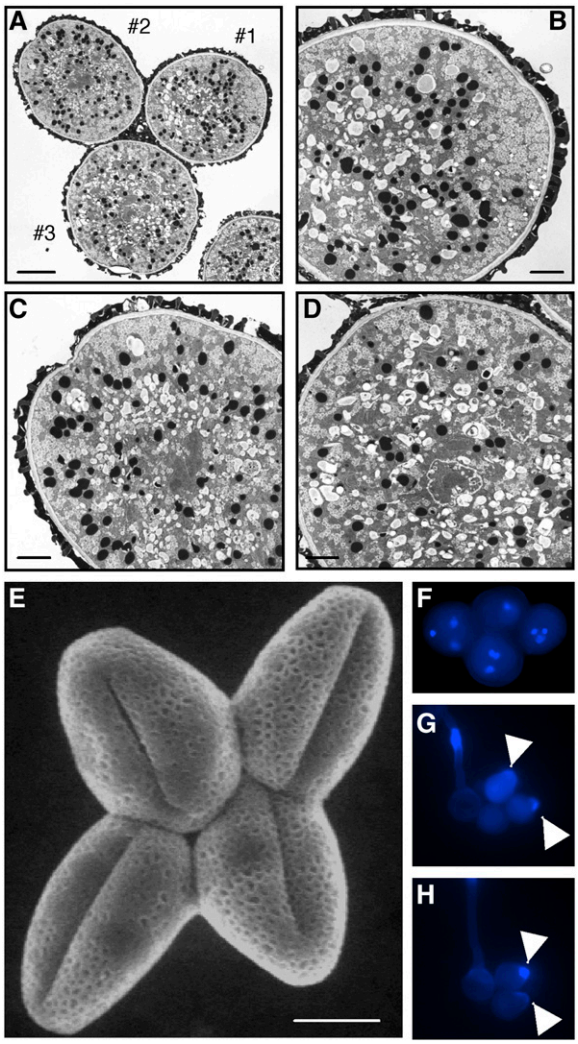

Figure 4. Morphology of *AtATG6/atatg6/qrt/qrt* pollen grains. A, Transmission electron micrograph of an *AtATG6/atatg6/qrt/qrt* tetrad. The pollen grains (1, 2, and 3) are enlarged in B, C, and D, respectively. The fourth pollen grain is out of focus. Bars = 5 μm in A; 2 μm in B to D. E, ESEM image of the *AtATG6/atatg6/qrt/qrt* tetrad. Bar = 10 μm. F, DAPI staining of the *AtATG6/atatg6/qrt/qrt* tetrad. G and H, Aniline blue staining of tetrads from *AtATG6/AtATG6/qrt/qrt* (G) and *AtATG6/atatg6/qrt/qrt* (H) plants. Note that the fourth pollen grain in H is out of focus. In both cases, more than three pollen grains in each tetrad either germinated or exhibited polarized accumulation of callose (arrowheads).

plants. First, a yeast complementation assay demonstrated that expression of *AtATG6* partially restored autophagy in *atg6/vps30* yeast. Liu et al. (2005) recently showed that Arabidopsis *BECLIN 1* (*AtATG6*) rescued autophagy in *atg6/vps30* yeast cells, in agreement with our results. However, it has thus far been unclear as to whether plant or mammal *BECLIN 1* homologs are effective in trafficking events, such as CPY sorting. In this article, we show that expression of *AtATG6* rescued *atg6/vps30* yeast from the mis-sorting of CPY protein. Although it has not been determined in plant cells how AtAtg6 regulates vesicular trafficking, we found that *AtATG6*-deleted plants displayed a male gametophytic mutant phenotype, which was similarly exemplified by another *atvps* mutant (see below). In contrast, none of the previously characterized *atatg* mutants has exhibited male gametophytic defects. Taken together, our results raise the possibility that Arabidopsis Atg6/Vps30 may be involved in other biological pathways (e.g. vesicle trafficking) in addition to autophagy.

In Arabidopsis cells, GFP-AtAtg6 proteins were predominantly distributed throughout the cytoplasm in accordance with biochemical studies identifying cytosolic Atg6/Vps30 in yeast cells (Seaman et al., 1997; Kihara et al., 2001a). In addition, recent microscopic observations revealed that Atg6/Vps30 is partially targeted to the punctate structure upon which the PAS marker protein Atg8 resides (Obara et al., 2006). As expected, CFP-AtAtg6 colocalized with YFP-AtAtg8 at the dot-like structures, which were reminiscent of the PAS. By contrast, in whole plants, GFP-AtAtg8 localizes to many dot structures in each cell, some of which may represent autophagosomes (Yoshimoto et al., 2004). The appearance of autophagosomes might depend on cell type: An isolated cell, such as a yeast cell, contains only a few autophagosomes and Atg8 predominantly localizes at a single dot (PAS) rather than to autophagosomes (Suzuki et al., 2001), mimicking the punctate localization of AtAtg proteins in Arabidopsis suspension cells.

In addition to the PAS, yeast Atg6/Vps30 also resides at the endosome and on the vacuolar membrane (Obara et al., 2006), and mammalian Beclin 1 localizes to the TGN (Kihara et al., 2001b). Although the localization of AtAtg6 to endomembranes was not detected under our experimental conditions, we cannot exclude the possibility that plant Atg6/Vps30/Beclin 1 may function in part through the endomembrane system to control vesicle trafficking.

Precise molecular mechanisms by which Atg6/Vps30/Beclin 1 proteins regulate autophagosome formation

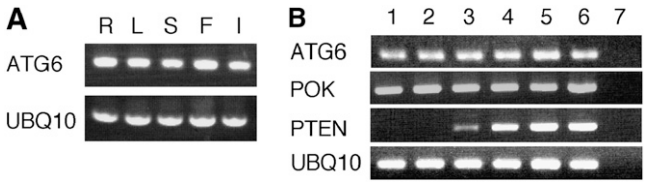

Figure 5. Expression of *AtATG6* in Arabidopsis. RT-PCR analyses of *AtATG6* mRNA levels in various organs (A) and during floral development (B). A, Each organ was collected from plants grown hydroponically for 4 to 5 weeks. R, Roots; L, leaves; S, stems; F, fruits; I, inflorescences. B, Flowers at different stages of development (Gupta et al., 2002) and leaves were collected from plants grown in soil for 5 to 6 weeks. 1, Leaves; 2, small green buds; 3, large green buds; 4, white buds; 5, opening flowers; 6, opened flowers; 7, leaf mRNA (prior to RT). *AtATG6* and *POK/VPS52* cDNAs were amplified with 25 cycles of PCR. *AtPTEN1*, a pollen-specific cDNA (Gupta et al., 2002), was amplified with 27 cycles of PCR. UBQ10 cDNA was amplified with 20 cycles.

have not yet been described in any organisms. In yeast, Atg6/Vps30 associates with Vps34, a phosphatidylinositol 3-kinase (PI3-kinase; Kihara et al., 2001a). Recent studies have suggested that targeting of Atg6/Vps30 and Vps34 to the PAS is critical for subsequent autophagosome formation (Obara et al., 2006). Vps34 may be recruited to the PAS by Atg14, a connector protein that binds Vps34 and Atg6/Vps30, and then phosphatidlylinositol-3 phosphate (PI3-P) produced by PI3-kinase at the PAS could subsequently recruit other proteins involved in autophagosome formation (Obara et al., 2006). Atg6/Vps30 seems indispensable for Atg14 function because Atg14 is unstable in *atg6/vps30* yeast cells (Kihara et al., 2001a). Although no *ATG14* homologs have been found in plants or animals, it is tempting to speculate that AtAtg6 may interact with an unidentified PI3-kinase regulatory protein that would direct the specificity of PI3-kinase activity toward autophagosome formation, possibly by recruiting the PI3-kinase complex to the putative PAS in Arabidopsis cells.

A number of Arabidopsis *atg* mutants have been isolated thus far, each of which has exhibited retarded growth under nutrient starvation and accelerated senescence even under nutrient-rich conditions (Thompson and Vierstra, 2005; Bassham et al., 2006). Similarly, virus-induced gene silencing of tobacco (*Nicotiana tabacum*) autophagy genes, including a *BECLIN 1/ATG6* homolog (*NbATG6*), enhanced yellowing of leaves under environmental stress, such as pathogen attack or dark-induced senescence (Liu et al., 2005). In this particular gene-silencing system, no difference was apparent between the phenotypes of *NbATG6*- and other *NbATG*-silenced plants. In contrast, we assumed that the *atg6*/vps30 mutant would reveal more severe phenotypes than other *atg* mutants because yeast Atg6/Vps30 mediates various biological pathways, including autophagy. Accordingly, of the *atatg* plants examined thus far, only *atatg6* mutants exhibited pollen tube germination abnormalities. This phenotype was not ascribed to autophagy because none of the *atatg* mutants (except *atatg6*) has exhibited male gametophytic defects (Thompson and Vierstra, 2005; Bassham et al., 2006; this study). As described above, this finding supports our hypothesis that AtAtg6/Vps30 may have an autophagy-independent role responsible for pollen germination.

The compelling question, then, is how AtAtg6 mediates pollen germination. Extensive studies have identified several key components for pollen tube growth; one of these is vesicular trafficking (Hepler et al., 2001; Monteiro et al., 2005). The tip of the pollen tube contains numerous vesicles that could provide precursor materials for the rapidly expanding cell wall of the growing cells. Arabidopsis *VPS* homologs, including *VCL1* (*VPS16*) and *POK* (*VPS52*), seem to play roles in germinating pollen tubes (Hicks et al., 2004; Lobstein et al., 2004). Similarly, it is possible that *AtATG6/VPS30* may be involved in the trafficking machinery that is crucial for pollen tube elongation.

Another possible explanation is that AtAtg6-mediated PI3-kinase signaling might be involved in pollen germination because PI3-P is known to act as a second messenger and modulate a variety of cellular activities in plants (Meijer and Munnik, 2003). Previous studies described roles of another phosphoinositide, phosphatidylinositol 4,5-bisphosphate, during cytoskeletal organization and membrane trafficking, which may regulate cell elongation of root hairs and of pollen tubes (Kost et al., 1999; Vincent et al., 2005). To our knowledge, however, the involvement of PI3-P in pollen tube germination has not been reported. *AtATG6*-disrupted pollen, which completely lacks *AtATG6* but is still viable, would serve as a useful system to further investigate the molecular mechanisms underlying PI3-kinase-regulated polarized cell growth.

Finally, despite its inhibitory effects on pollen germination, the *atatg6* mutation affected neither the functions of female gametophytes nor the viability of pollen, suggesting that AtAtg6 is not essential for growth of germ cells. Nevertheless, this does not necessarily exclude the possibility that disruption of *AtATG6* drastically affects various developmental processes in plants. For instance, if *atatg6* homozygous plants were available, their early embryonic development would be severely damaged, as shown in *AtVPS34*-suppressed transgenic Arabidopsis (Welters et al., 1994). In animals, suppression of *BECLIN 1* affected dauer development (Meléndez et al., 2003) and cell death (Yu et al., 2004). Suppression of *AtATG6* by antisense or RNAi techniques would unveil another unanticipated physiological significance of *ATG6/VPS30/BECLIN1* genes, in addition to *AtATG6*-mediated pollen germination.

MATERIALS AND METHODS

Complementation of Yeast Mutants

An ORF of Arabidopsis (*Arabidopsis thaliana*) *ATG6* (At3g61710; The Arabidopsis Information Resource [TAIR]; http://www.arabidopsis.org) was fused with the 5′ flanking sequence (−816 to −1 relative to the initiation codon) of yeast (*Saccharomyces cerevisiae*) *ATG6/VPS30* in the pRS416 vector. A genomic fragment of yeast *ATG6/VPS30* (−952 to +2,325; Kametaka et al., 1998) was inserted into pRS416 as a control. Wild-type yeast (strain BY4741) and an *atg6/vps30* deletion mutant (Y2132; Giaever et al., 2002) were then transformed with these plasmids.

For autophagy complementation experiments, cells were grown at 30°C to the mid-log stage (OD_{600} of 1.0–1.5) in synthetic medium (0.67% [w/v] yeast nitrogen base without amino acids and ammonium) supplemented with 2% (w/v) Glc, 0.5% (w/v) ammonium sulfate, 0.5% (w/v) casamino acid, and 0.002% (w/v) Trp. Cells were then collected, washed, and incubated for another 5 h in the synthetic medium in the presence of 1 mM phenylmethylsulfonyl fluoride. Accumulation of autophagic bodies was observed using a differential interference contrast microscope (IX81; Olympus).

For VPS complementation, cells under 5 h of nitrogen starvation were collected by centrifugation and used to prepare intracellular extracts, whereas the supernatant was collected as the extracellular fraction. Preparation of cell extracts and immunoblot analyses were performed as described previously (Hamasaki et al., 2005).

Plant Materials

Wild-type Arabidopsis (strain Columbia) plants were grown at 22°C with 16-h-light/8-h-dark photoperiods. Two distinct T-DNA insertion lines of

AtATG6 (SALK_051168 and SALK_109281) were generated by the SALK Institute, and *AtATG6* heterozygous plants were identified by PCR. The primer 5′-TGGTTCACGTAGTGGGCCATCG-3′ was designed for the T-DNA sequence and others were used for *AtATG6* (see below). *AtATG6* heterozygous plants (SALK_051168) were crossed with *qrt* mutant plants (*qrt1-2*; Arabidopsis Biological Resource Center [ABRC] CS8846).

Arabidopsis suspension-cultured cells were maintained as described (Ueda et al., 2001).

Subcellular Localization of AtAtg6 Protein Fused with GFP Derivatives

The ORF of *AtATG6* or *AtATG8a* (Yoshimoto et al., 2004) was fused to the C terminus of enhanced GFP in the pEZS-CL plasmid (Carnegie Institution of Washington). The ORFs of *AtATG6* and *AtATG8i* were C-terminally fused to enhanced CFP or enhanced YFP (p2CGW7 and p2YGW7, Karimi et al., 2005) using Gateway technology (Invitrogen). Venus-tagged SYP31, SYP41, and SYP81 were used as the Golgi, TGN, and ER marker proteins, respectively (Uemura et al., 2004). Transient expression of fluorescence proteins in Arabidopsis suspension cells was performed as described in Ueda et al. (2001). BFA treatment was performed as described previously (Uemura et al., 2004). Cells were observed using an epifluorescence microscope (Olympus IX81).

Microscopic Observation of Pollen

Pollen grains were germinated in the dark at 22°C on an agar plate containing 0.6% agar, 13.5% Suc, 30 μg mL^{-1} myoinositol, 0.4 mM boric acid, 10 mM $CaCl_2$, and 1 mM KCl.

Sections of mature pollen were observed using a JEM-2000EX transmission electron microscope (JEOL) as described by Yoshimoto et al. (2004).

Pollen was observed in the chamber of an ESEM (PHILIPS XL30; FEI Electron Optics) at 2°C under 650 to 660 Pa of pressure with an acceleration voltage of 15 kV. Images were processed using Adobe Photoshop 6.0 (Adobe Systems).

Staining of pollen with 4′,6-diamidino-2-phenylindole (DAPI) or aniline blue was performed as described previously (Regan and Moffatt, 1990; Shimizu and Okada, 2000).

RT-PCR

Arabidopsis plants were grown hydroponically for 4 to 5 weeks (Hanaoka et al., 2002) and each organ was harvested. Flowers at different developmental stages were collected as described by Gupta et al. (2002). RNA was extracted from each sample using TRIzol reagent (GIBCO-BRL) and treated with DNase I. cDNA was synthesized using a ProSTAR first-strand RT-PCR kit (Stratagene). The primers used were *AtATG6*, 5′-GCTGAAGTAAACCATCAACTG-3′ and 5′-GTTACACATCGTATGGAGGAG-3′; *UBIQUITIN10* (*UBQ10*, At4g05320), 5′-GCAGATCTTTGTTAAGACTC-3′ and 5′-CCAAAGTGATAGTTTTCCCAG-3′; *POK* (At1g71270), 5′-GGTGTTAAGTGCACATTTTCGTG-3′ and 5′-TCACCAAAGAAATCATCACAAAA-3′; and *AtPTEN1* (At5g39400), 5′-ATGGGTCTCAAGCTCTCACG-3′ and 5′-ACCGTAAACAAGGTACGCCGA-3′.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AY039613 (*AtATG6*) and BT024554 (*AtATG8i*).

Supplemental Data

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

Supplemental Figure S1. Intracellular localization of AtAtg8 in Arabidopsis cells.

Supplemental Figure S2. Colocalization of AtAtg6 and AtAtg8 proteins in Arabidopsis cells.

Supplemental Figure S3. Reduced *AtATG6* mRNA levels in *AtATG6* heterozygous plants.

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