ABSTRACT

An Investigation on the Effects of Mutations in the ATG12 Autophagy Pathway of *Arabidopsis Thaliana*

Under nutrient-deficient conditions, plants are forced to recycle secondary cellular constituents to conserve energy for the more essential processes (Klionsky, *et al.* 2000). Plants accomplish this through a process called autophagy, which involves the capture of cytosolic constituents in vesicles called autophagosomes and transportation of these vesicles to lytic vacuoles for degradation. Macroautophagy, the main autophagy pathway, requires an ATG12-ATG5 protein conjugate for autophagosome formation (Thompson & Vierstra, 2005). Extensive research has been done on the ATG12 pathway in organisms such as yeast, but research is yet to be done on ATG12 disruption in plants. By studying protein expression and performing phenotypic analysis of the model plant *Arabidopsis thaliana* with mutations in ATG12 and other members of the ATG12 pathway, we can begin to understand the role of the pathway in autophagic recycling during plant growth and development. Since autophagy controls many important aspects of crop physiology (e.g. seed germination, pathogen defense, and growth under nutrient-limiting conditions), this work will likely provide new venues for improving crop production.

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COVER SHEET

TITLE: An Investigation on the Effects of Mutations in the ATG12 Autophagy Pathway of *Arabidopsis Thaliana*

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ABSTRACT

Under nutrient-deficient conditions, plants are forced to recycle secondary cellular constituents to conserve energy for the more essential processes (Klionsky, *et al.* 2000). Plants accomplish this through a process called autophagy, which involves the capture of cytosolic constituents in vesicles called autophagosomes and transportation of these vesicles to lytic vacuoles for degradation. Macroautophagy, the main autophagy pathway, requires an ATG12-ATG5 protein conjugate for autophagosome formation (Thompson & Vierstra, 2005). Extensive research has been done on the ATG12 pathway in organisms such as yeast, but research is yet to be done on ATG12 disruption in plants. By studying protein expression and performing phenotypic analysis of the model plant *Arabidopsis thaliana* with mutations in ATG12 and other members of the ATG12 pathway, we can begin to understand the role of the pathway in autophagic recycling during plant growth and development. Since autophagy controls many important aspects of crop physiology (e.g. seed germination, pathogen defense, and growth under nutrient-limiting conditions), this work will likely provide new venues for improving crop production.

INTRODUCTION

Cellular processes require substantial amounts of energy to function (Klionsky, *et al.* 2000), thus it is advantageous for cells to degrade unnecessary secondary cytosolic components when under environmental stresses in order to conserve energy. Autophagy is a process by which eukaryotic organisms degrade intracellular constituents for nutrient turnover when in a nutrient-deficient environment, cell development and differentiation (Muzushima, *et al.* 1998). It involves transporting cytoplasmic components including

organelles to vacuoles, which are acidic organelles that encase proteases and other hydrolytic enzymes that aid in degradation (Thompson & Vierstra, 2005).

Macroautophagy is the main pathway by which autophagy occurs. This pathway begins by converting tubules called preautophagic structures (PAS) into vesicles called autophagosomes, which carry cytosolic components to the vacuole for degradation (Figure 1). Through phenotypic observations, it has been determined that fusing PAS ends to create autophagosomes requires the ATG12-ATG5 protein conjugate, though the actual molecular mechanism for this process is still unknown (Thompson & Vierstra, 2005). However, it is known that ATG12-ATG5 conjugate formation in yeast requires a cascade of events similar to ubiquitination. Importantly, ATG12 is activated by ATG7 and conjugated to ATG5 by ATG10 (Klionsky, *et al.* 2000) (Figure 2).

This study seeks to determine phenotypic effects caused by disrupting different components of the ATG12 pathway in *Arabidopsis thaliana*? Because previous studies have shown the importance of ATG12 in vesicle formation in yeast (Klionsky, *et al.* 2000), Arabidopsis with a mutation in *atg12* would likely exhibit early senescence under nutrient-deficient conditions similar to *atg5* and *atg7* mutants (Thompson, *et al.* 2005), due to their inability to efficiently regenerate free nutrients via cytosolic degradation within their cells. By analyzing mutant phenotypes of different genes in the ATG12 pathway, we have begun to elucidate the roles of the pathway in plant autophagy.

METHODS

Double mutant generation and identification. There are two isoforms of ATG12 in *Arabidopsis*: ATG12a and ATG12b (Thompson, et al. 2005), therefore, it was necessary that

we observe single mutants to research the effect of disrupting ATG12. Single T-DNA insertion lines of ATG12a and ATG12b were identified by searching databases of *Arabidopsis* T-DNA insertion libraries. Segregating populations of *Arabidopsis* seed were screened by polymerase chain reaction (PCR) to identify homozygous atg12a and atg12b single mutant individuals. These individuals were self-fertilized to obtain homozygous populations.

Transfer assay. Seeds harvested from plants grown concurrently were sterilized and stratified for two days. Seeds were then grown on Gamborg's Medium (GM) for 7 days, after which they were transferred to media containing one of three compounds: 100 mM NaCl, 200 mM NaCl, 200 mM mannitol, 350 mM mannitol, 5 μM methyl violgen, or 10 μM methyl violgen for assessment. Controls were transferred to GM alone. An additional control was used that was not transferred to another plate. Three replicates per treatment were performed (N=45).

Germination assay. Seeds harvested from plants grown concurrently were sterilized and stratified for two days. These seeds were grown on either GM with 200 mM NaCl or GM only (as a control). Three replicates were used per treatment, each replicate with 15 seeds (N=45). Germination was assessed starting on day 3 after plating. Plants were examined under a microscope, and those showing roots that had emerged from the seed coat were considered to have germinated.

In the preliminary germination experiments, we used 100 mM, 150 mM, and 200 mM NaCl treatments, as well as 200 mM, 350 mM, and 400 mM mannitol treatments. Similar experiments were repeated with additional genotypes on 200 mM NaCl.

Liquid culture assays. Seeds harvested from plants grown concurrently were sterilized and stratified for two days. These seed were then grown in 1.5 mL liquid GM containing either 100 μM 2-bromopalmitate, 10 μM mevinolin, 20 μM MG132, or 40 μM MG132 (8-12 seeds per well, 3 replicates per treatment) for 7 days. DMSO treatments were used as controls for the 2-bromopalmitate and MG132 treatments. GM alone was used as the control treatment for mevinolin. In the preliminary trials, only Col-0, *atg5-1*, WS, and *atg7-1* lines were used (Col-0 and WS served as controls).

Root length assay. Seeds harvested from plants grown concurrently were sterilized and stratified for two days. These seeds were then plated on 10 μM and 15 μM canavanine (3 replicates per treatment, N=24). Seeds grown on GM media served as controls. Root lengths were measured at 11 and 17 days after plating using ImageJ software (NIH, http://www.nist.gov/lispix/imlab/prelim/dnld.html).

Cloning ATG genes for yeast two-hybrid (Y2H) analysis. For the yeast-two-hybrid assay, the full-length coding regions of the *ATG5*, *ATG10*, and *ATG7* cDNAs were amplified by PCR using the following primers:
ggggacaagtttgtacaaaaaagcaggcttcATGGATTCAGCTCGAGAGGTCA and
ggggaccactttgtacaagaaagctgggttCTAATTCAGCATCTCAAGAGGG for ATG10,

ggggacaagtttgtacaaaaaagcaggcttcATGGCGAAGGAAGCGGTCAAGT and ggggaccactttgtacaagaaagctgggttTCACCTTTGAGGAGCTTTCACA for ATG5, and ggggacaagtttgtacaaaaaagcaggcttcATGGCTGAGAAAGAAACTCCAG and ggggaccactttgtacaagaaagctgggttTTAAAGATCTACAGCTACATCG for ATG7. The primers were designed to introduce BP recombination sites at the 5' and 3' ends of the coding sequences (lower case) for ligating into cloned into the Gateway pDONR221 vector (Invitrogen, Carlsbad, CA). Using primers CTACATCCCTCTGGGACTGAGGACTG and CAGTCCTCAGTCCCAGAGGGATGTAG the ATG10 active site cysteine 178 (underlined) was changed to a serine by the Quick Change method (Stratagene, LaJolla, CA). The full-length coding regions of the ATG12a and ATG12b cDNAs were amplified by PCR using the following primers designed to introduce a 5' overhang (underlined) for subsequent cloning into the Gateway pENTR vector (Invitrogen):
CACCATGGCGACGGAGTCGTCGTCCCCGA and
TTAGCCCCATGCCATGGAACAAGC for ATG12a, and
CACCATGGCGACCGAATCTCCGAATTCT and TTAACCCCATGCCATGGAACAAGC for ATG12b. The positive controls amplified by PCR were *ASK1* and *u*nusual *f*loral *o*rgans (*UFO*).

The *ATG5*, *ATG7*, *ATG12a*, *ATG12b*, *ATG10*, and *ATG10C178S* coding regions were recombined into the Gateway pDEST22 and pDEST32 Y2H vectors (Invitrogen) which encode 5' *GAL4* activation and binding domains, respectively. The vectors were introduced into *Saccharomyces cerevisae* mating types 'a' and 'α', respectively, according to Gingerich, *et al.* (2005). The yeast transformants were grown on dropout media lacking tryptophan

(pDEST22) or leucine (pDEST32) to select for successful transformations. Colony PCR was performed on the resulting haploid colonies to confirm that they contained the vector.

The yeast α cells were then mated with the yeast a cells on YPAD media, and the diploid progeny from successful matings were selected for on dropout media lacking both Leu and Trp. These diploid cells were tested for protein-protein interactions using both β-galactosidase and 3-AT (His-) assays, as described in Gingerich, *et al.* (2005). Growth of diploids on 3-AT dropout media or the production of β-galactosidase indicates positive physical interaction between the proteins encoded by pDEST22 and pDEST32 vectors.

RESULTS

No significant phenotypic differences were seen in ATG12 pathway mutants in transfer assays. Preliminary transfer assay experiments were done using 100 mM and 200 mM NaCl, 200 mM and 350 mM mannitol, and 5 μM and 10 μM methyl violgen. The four lines used were Col-0, *atg5-1*, *GFP:ATG8a*, and *atg7-1*. No visible phenotypes significantly different from Col-0 (wildtype) were observed under these different conditions (data not shown).

Reduced germination was found in atg5-1, atg7-1, and atg12a-1. Preliminary germination assay experiments using different concentrations of NaCl and mannitol were done on Col-0, *atg5-1*, and *GFP:8a* (see Methods). No observable phenotypes were seen in plants grown on any of the tested concentrations of mannitol, but a potential phenotype for *atg5-1* was seen on 200 mM NaCl, where the percentage germination for *atg5-1* was significantly higher that for Col-0 (data not shown).

We then repeated the assay with more repetitions using Col-0, *atg5-1*, *atg7-1*, *atg10-1*, *atg12a-1*, *atg12b-3* (an *ATG12b* overexpressor), and *atg12b-5* on 200 mM NaCl. Contrary to results in the preliminary experiments, significantly reduced germination was observed in *atg5-1*, *atg7-1*, and *atg12a-1* mutants at day 13 compared to wildtype Col-0. Interestingly, germination in the *atg10-1* mutant was significantly higher than in the other lines until day 7 post-plating (Figure 3 and Table 1). None of these genotypes displayed a germination rate significantly different from wildtype on GM media without 200 mM NaCl (data not shown).

Reduced resistance to 2-bromopalmitate was observed in atg12a and atg12b mutants.

Preliminary liquid culture assay experiments were done using Col-0, *atg5-1*, WS, and *atg7-1* in 2-bromopalmitate, mevinolin, and two different concentrations of MG132 (see Methods). No phenotype was apparent for any of the genotypes in either concentration of MG132. A potential phenotype was observed in *atg7-1* grown in the presence of 10 μM mevinolin (faster senescence in *atg7-1* compared to WS, data not shown). Therefore, we repeated the experiment using Col-0 and the *atg5-1*, *atg7-1*, *atg10-1*, *atg12a-1*, *atg12b-3*, and *atg12b-5*. However, no phenotype was seen in this second trial in any of the lines (data not shown).

A phenotype was also observed for *atg5-1* and *atg7-1* in the 2-bromopalmitate preliminary experiment. Again, we repeated the experiment using the same lines as in the second mevinolin trial described above. This time, we discovered that the *atg12a-1*, *atg12b-3*, and *atg12b-5* mutants were all smaller and more yellow than Col-0. On GM, all seedlings developed normally (Figure 4 and Table 1).

Root lengths in atg12a-1 and atg12b-3 are least affected by exposure to canavanine. The mutant lines were also grown on medium with canavanine and resulting root lengths were measured at 11 and 17 days after plating. On 10 μM canavanine, the *atg12b-3* root lengths were significantly shorter and the *atg7-1* root lengths significantly longer than Col-0 root lengths. Additionally, on 15 μM canavanine, *atg12b-5* and *atg7-1* root lengths were significantly longer and *atg5-1* root lengths significantly shorter than Col-0. However, normalized data with respect to GM control root lengths show that *atg12a-1* and *atg12b-3* exhibit the least change in root length in both concentrations compared to controls, while *atg5-1*, *atg7-1*, and *atg12b-5* exhibited fold changes comparable to those of Col-0 (Figure 5 and Table 1).

ATG7 physically interacts with ATG12a and ATG12b. *ATG5*, *ATG7*, *ATG10*, *ATG10C178S*, *ATG12a*, and *ATG12b* genes were inserted in both pDEST22 and pDEST32 vectors and transformed into yeast for mating. A 3-AT assay and β-galactosidase assay were done on the resulting diploid yeast to test for protein-protein interactions. We observed blue staining in ATG7/ATG12a and ATG7/ATG12b crosses in both directions in the β-galactosidase assay, and we observed the growth of robust yeast colonies in those aforementioned crosses in the 3-AT assay (Figure 6). These two results indicate the presence of extremely strong interactions between ATG7 and ATG12a, as well as between ATG7 and ATG12b. This strong interaction occurred much earlier than the interaction of the positive controls, which appeared after at least 24 hours (results not shown).

DISCUSSION

Autophagy is crucial to a plant's survival when deprived of essential nutrients. In this study, we have examined the effects of mutations in different components of the ATG12 pathway in *Arabidopsis*. It is known that several autophagy mutants are compromised under various environmental stresses, such as under nitrogen- and carbon-deficient environments (Doelling, *et al.* 2002; Thompson, *et al.* 2005). The conditions tested in this paper created additional environmental stresses on the plants to determine whether autophagy mutants, specifically those in the ATG12 pathway, were further compromised.

Defense against drought conditions is important to plant survival. NaCl creates drought-like conditions; hence we grew *Arabidopsis* in 200 mM NaCl to assess ATG12 pathway mutants under drought stress. Significant reductions in *atg5-1*, *atg7-1*, and *atg12a-1* germination suggest that mutations in these genes compromise germination rates in the plants (Figure 3). Interestingly, different germination assays yielded different results in *atg5-1*. In our preliminary studies, *atg5-1* was shown to have significantly higher germination rates than Col-0, yet the opposite was observed later in our second trial (this paper; Suttangakul, unpublished data). It is possible that cold-treating sterilized seeds on experiment plates before beginning the assay versus cold-treating the seeds in water before plating could affect germination. However, it is important to note that the preliminary germination assays done used seeds harvested from plants grown at different times, which could introduce confounding variables in our initial experiments. In the second assay, all seeds were grown concurrently and treated similarly in an attempt to eliminate unknown environmental variable. Further study should be done using all ATG12 pathway mutants to determine if the conflicting data obtained is due to the former or latter explanation.

Exposure to 2-bromopalmitate inhibits fatty acid oxidation, thereby eliminating one source of energy for the plant. Observations of ATG12 pathway mutants grown in the presence of 2-bromopalmitate show that *atg12a-1*, *atg12b-3*, and *atg12b-5* mutants have smaller and more yellow leaves than Col-0 grown under the same conditions (Figure 4), suggesting that turnover by autophagy may help plants get sufficient energy when unable to obtain other sources of energy.

Canavanine is an anti-arginine metabolite. When exposed to canavanine, plants incorporate the compound in place of arginine. This substitution can impair protein folding and degradation. This treatment is likely to obstruct degradation by the proteasome, thus introducing more stress for the plant by blocking two major protein degradation pathways. Our results show that though different concentrations of canavanine can have variable effects on root lengths in ATG12 pathway mutants, *atg12a-1* and *atg12b-3* mutants consistently showed the least change in root length compared to controls (Figure 5). Because *atg12a-1* is a null gene, it is possible that lack of ATG12 upregulates *ATG12b*. This compensation may cause the minimal change in root length observed in canavanine treatments. The significantly lesser degree of root change in the overexpressor *atg12b-3* mutant likewise supports the claim that ATG12b is important for plant response to canavanine. Overexpression of ATG12b may enable the plant to survive better under these conditions.

Positive results in our Y2H β-galactosidase and histidine assays suggest a strong interaction between ATG7 and ATG12a, as well as between ATG7 and ATG12b (Figure 6). These results are expected based on the currently accepted pathway, where ATG7 functions as an E1 enzyme for activating ATG12. Until now, it has only been speculated that ATG12 is the peptide tag used in the ATG12-ATG5 conjugation in plants. These results verify the

role of ATG12 in the autophagy pathway in plants. However, these assays do not reveal specifically how the two proteins interact. We are currently creating pDEST constructs containing an ATG7 mutant with a C557A mutation at a possible site of interaction to determine if the ATG7 and ATG12 have a covalent or noncovalent interaction.

Because there are two known isoforms of ATG12 that are likely functionally redundant, testing single null mutants of the gene does not offer insight into how important the total protein is to autophagy and plant survival. To address this issue, we have recently isolated an *atg12a-1/atg12b-5* double mutant that we will analyze. This double mutant combination will be phenotypically characterized under nitrogen and carbon starvation, as well as in canavanine and NaCl treatments.

The presence of autophagy pathways in other plant species such as tobacco, rice, and corn (Takatsuka, *et al*. 2004; Suttangakul, unpublished) suggests that the results of this proposed study in *A. thaliana* could be extended to other plant species as well. Through a better understanding of the function of ATG12 pathway, we can improve our understanding of how plants cope with nutrient-deficient conditions and other environmental stresses through autophagy and perhaps one day manipulate this process to increase plant viability for agricultural purposes.

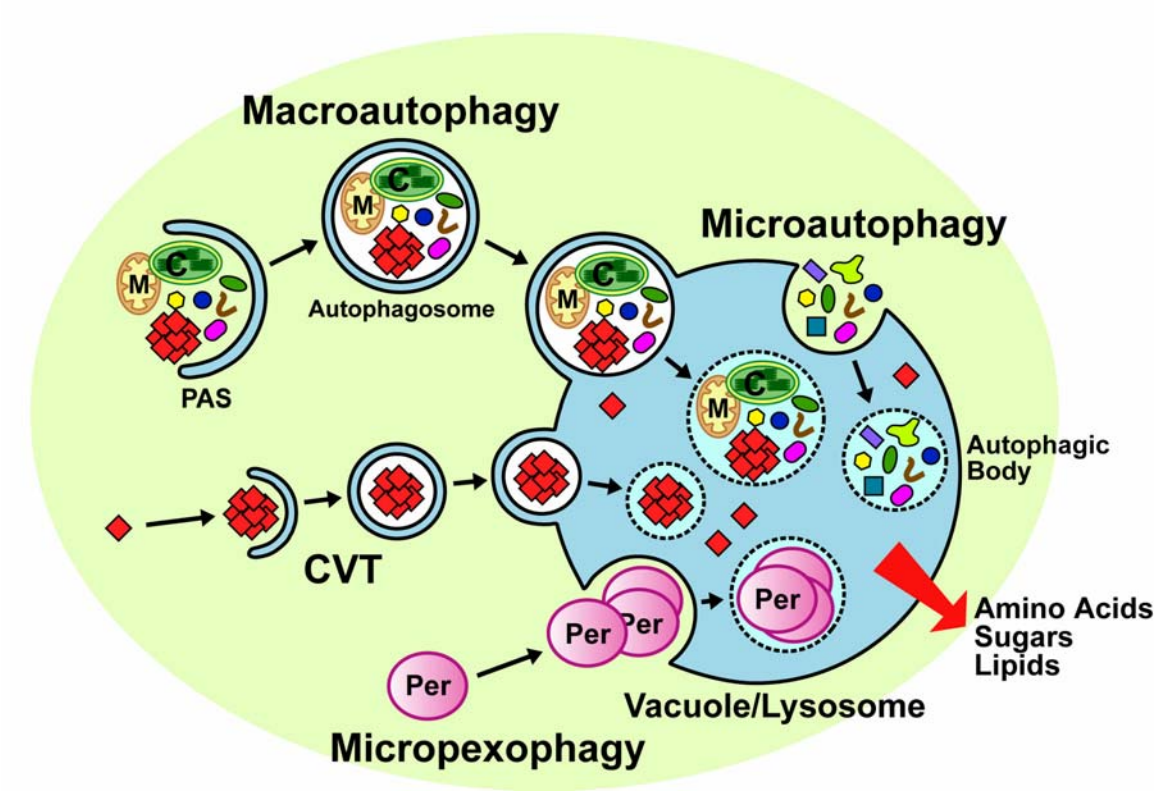

Figure 1. Morphological steps during microautophagy, macroautophagy, CVT, and micropexophagy. Micropexophagy and microautophagy proceed by invagination of the tonoplast to engulf portions of the cytosol and peroxisomes to create autophagic bodies within the vacuole. Conversely, both macroautophagy and CVT sequester cystolic components in the double membrane-bound vesicle, which then fuses with the tonoplast to release its contents into the vacuolar lumen. For macroautophagy, this vesicle is called the autophagosome. Where appropriate, the cargo is degraded by resident vacuolar hydrolases; the products are either stored in the vacoule or transported back to the cytosol for reuse. C, chloroplast. M, mitochondrion. Per, peroxisome. (Thompson and Vierstra, 2005).

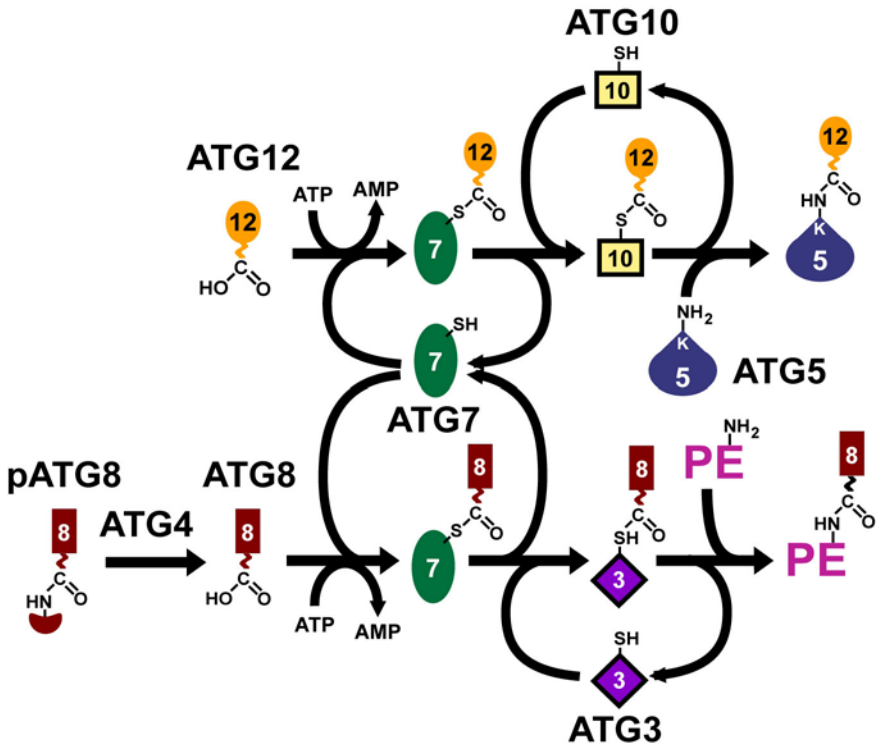

Figure 2. Description of the ATG12 conjugation pathway in yeast. ATP-dependent reaction cascades involving a single E1 and multiple E2 enzymes covalently attach ATG12 and ATG8 to ATG5 and phosphatidylethanolamine (PE), respectively. Prior to activation, the ATG8 precursor is processed by the ATG4 protease to remove the extra C-terminal residues, thus exposing a penultimate glycine. Both ATG12 and ATG8 are activated by the E1 (ATG7) and become bound to ATG7 by a thiol-ester bond. The activated tags are then donated to their respective E2s, ATG10 and ATG3 by transesterification, and finally attached to their targets by an amide bond. A specific lysine (K) residue is necessary for formation of the isopeptide bond between ATG5 and ATG12. The amide group of ethanolamine is used for attachment of PE to ATG8 (Doelling *et al*, 2002).

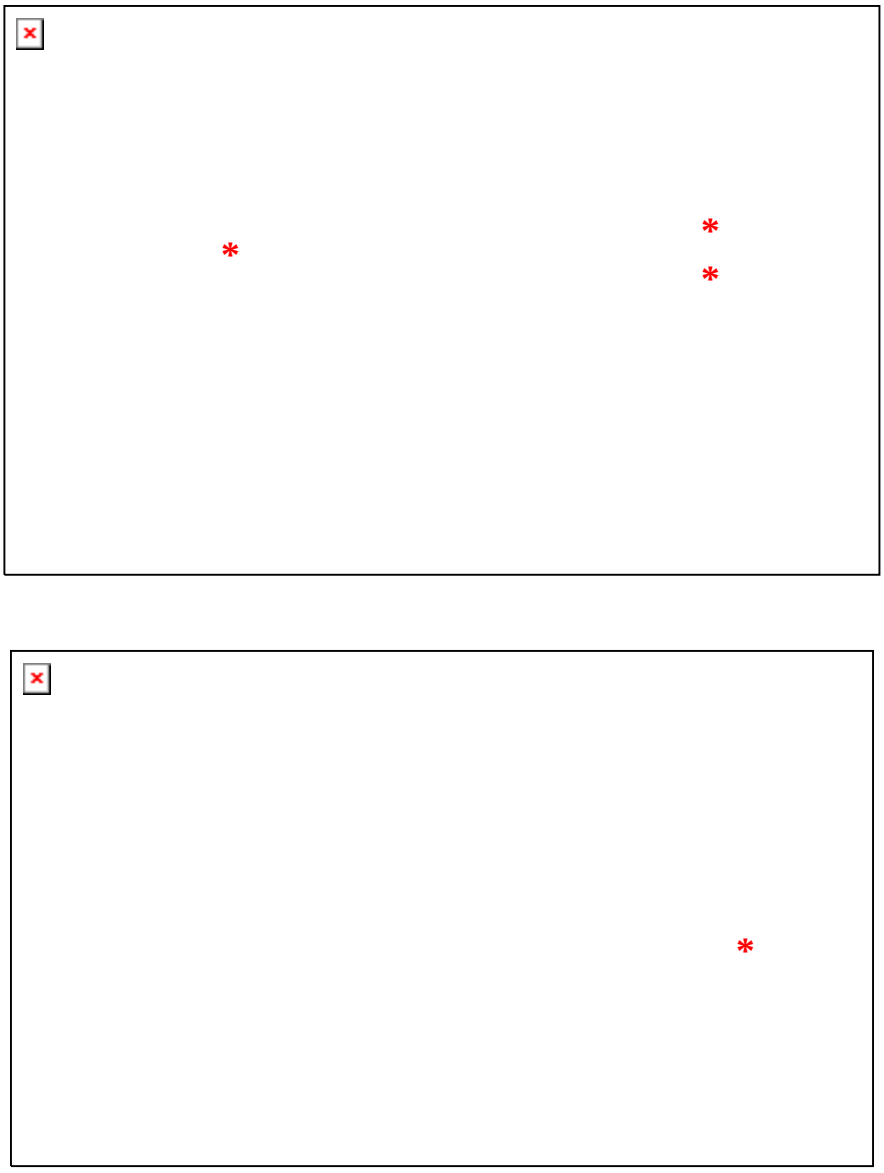

Figure 3. Germination analysis of ATG insertional T-DNA mutants on 200 mM NaCl. Seeds from wildtype Colombia (Col-0), *atg5-1*, *atg7-1*, *atg10-1*, *atg12a-1*, *atg12b-3*, and *atg12b-5* mutant alleles were grown on Gamborg's medium (GM) containing 200 mM NaCl. Seeds grown on GM-only served as controls. Each data point represents the average percentage germination of 3 replicates (N=45) relative to the average percentage germination GM controls. Red asterisks indicate genotypes with germination significantly different from Col-0, which include (a) *atg5-1*, *atg7-1*, *atg10-1*, and (b) *atg12a-1* mutants.

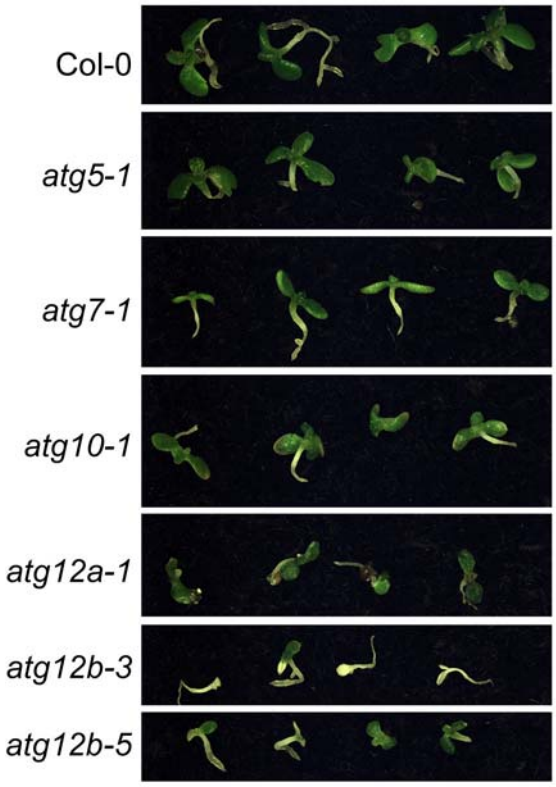

Figure 4. Growth assay of *atg* mutants in 100 μM 2-bromopalmitate. Seeds from Col-0, *atg5-1*, *atg7-1*, *atg10-1*, *atg12a-1*, *atg12b-3*, and *atg12b-5* mutant alleles were grown in GM liquid medium containing 100 μM 2-bromopalmitate. Three replicates were used, and each genotype had 8-12 seeds grown together per replicate. Photographs taken 7 days post-plating.

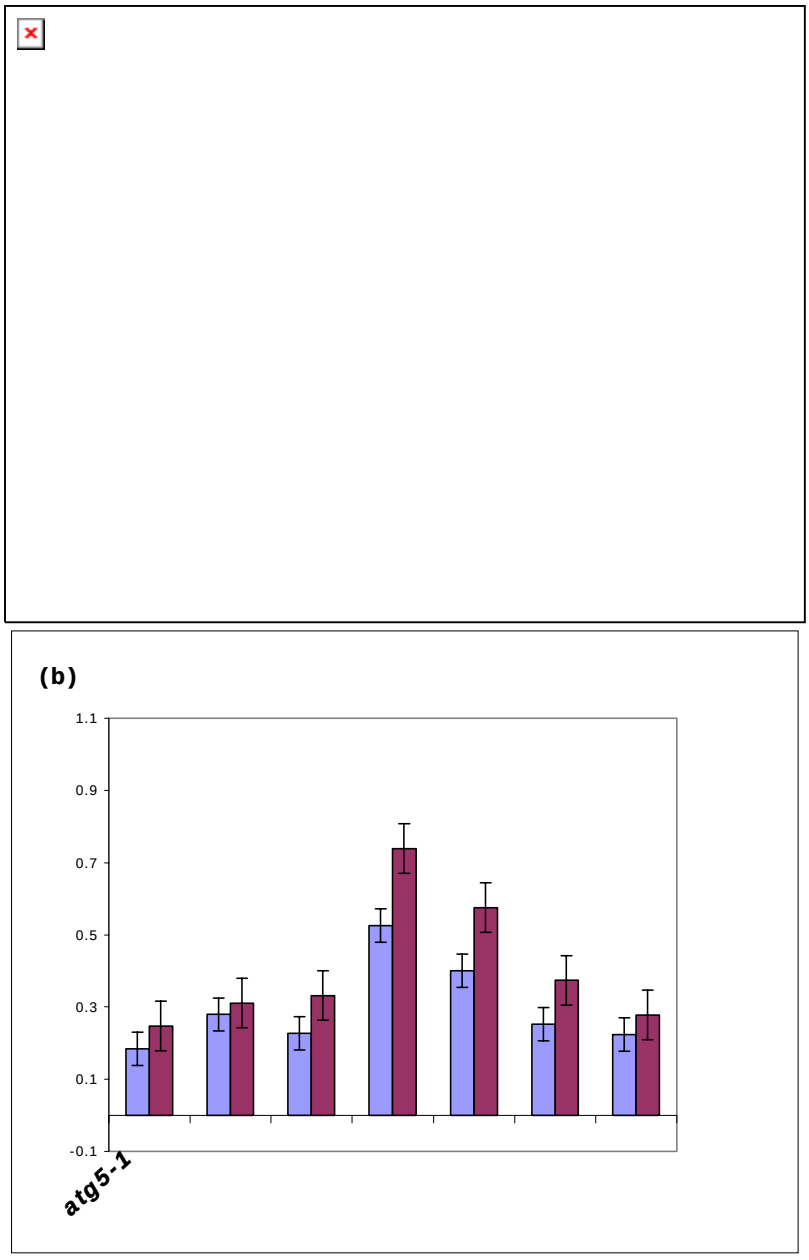

Figure 5. Root lengths of ATG12 pathway mutants in canavanine relative to GM control. Seeds from wildtype Colombia (Col-0), *atg5-1*, *atg7-1*, *atg10-1*, *atg12a-1*, *atg12b-3*, and *atg12b-5* were grown on GM (control), (a) GM + 10 μM canavanine, and (b) GM + 15 μM canavanine. Measurements were taken on day 11 and day 17. Each data point represents the average of at least 2 replicates (N = at least 2 per replicate) relative to the average root length in GM.

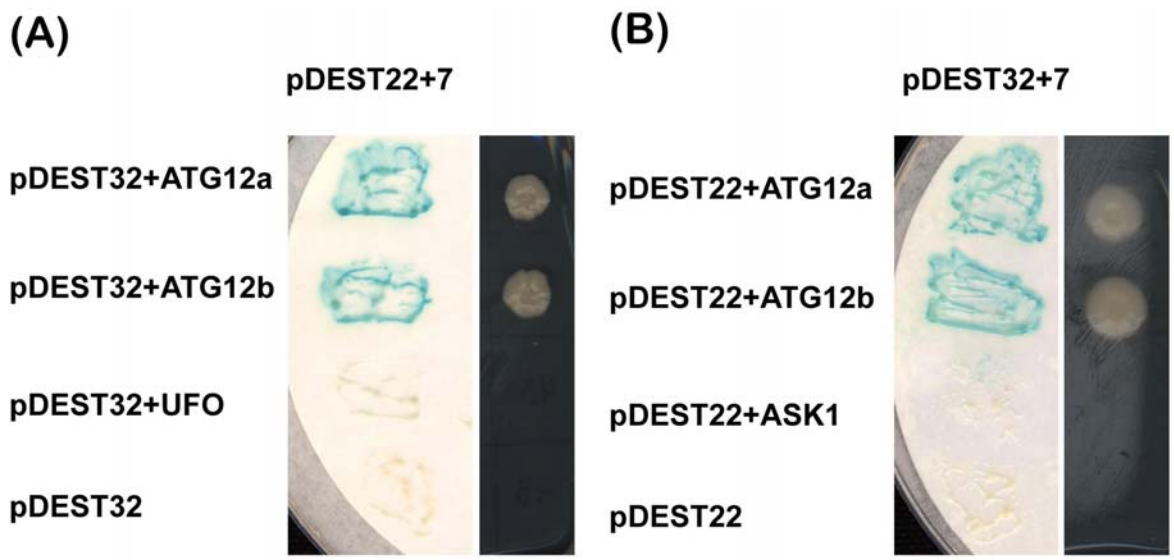

Figure 6. Protein-protein interactions analyzed by Y2H assay. (a) *Saccharomyces cerevisiae* mating-type a cells containing pDEST22+ATG7 were crossed with mating type α cells containing (i) pDEST32+ATG12a, (ii) pDEST32+ATG12b, (iii) pDEST32+UFO and (iv) pDEST32 control. (b) *S. cerevisiae* mating-type α cells containing pDEST32+ATG7 were crossed with a cells containing (i) pDEST22+ATG12a, (ii) pDEST22+ATG12b, (iii) pDEST22+ASK1, and (iv) pDEST22 control. β-galactosidase activity (blue staining) and growth on histidine (as indicated by the presence of yeast colonies) were measured as an indication of protein-protein interaction (binding at the GAL4 promoter and activation of the GAL4 reporter gene).

ASSAY	STRESS	*REAGENT*	***atg5-1***	***atg7-1***	***atg10-1***	***atg12a-1***	***atg12b-3***	***atg12b-5***
Germination	Drought	200 mM NaCl	3	3	3	3		
Root	Arginine anti-metabolite	10 μM and 15 μM Canavanine				3	3	
Liquid Culture	Mitochondrial fatty acid oxidation inhibitor	100 μM 2-bromopalmitate				3	3	3
	Proteasome inhibitor	40 μM MG132						
	Cholesterol synthesis inhibitor	10 μM mevinolin						

Table 1. Phenotypic analysis of ATG12 pathway mutants. All assays were performed in either solid growth media or liquid culture with stress reagents added. Check marks indicate the existence of a phenotype in a given mutant line.

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