In Vitro Reconstitution of Plant Atg8 and Atg12 Conjugation Systems Essential for Autophagy*

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Genetic and biochemical analyses using yeast Saccharomyces cerevisiae showed that two ubiquitin-like conjugation systems, the Atg8 and Atg12 systems, exist and play essential roles in autophagy, the bulk degradation system conserved in yeast and mammals. These conjugation systems are also conserved in Arabidopsis thaliana; however, further detailed study of plant ATG (autophagy-related) conjugation systems in relation to those in yeast and mammals is needed. Here, we describe the in vitro reconstitution of Arabidopsis thaliana Atg8 and Atg12 (AtATG8 and AtATG12) conjugation systems using purified recombinant proteins. AtATG12b was conjugated to AtATG5 in a manner dependent on AtATG7, AtATG10, and ATP, whereas AtATG8a was conjugated to phosphatidylethanolamine (PE) in a manner dependent on AtATG7, AtATG3, and ATP. Other AtATG8 homologs (AtATG8b–8i) were similarly conjugated to PE. The AtATG8 conjugates were deconjugated by AtATG4a and AtATG4b. These results support the hypothesis that the ATG conjugation systems in Arabidopsis are very similar to those in yeast and mammals. Intriguingly, in vitro analyses showed that AtATG12-AtATG5 conjugates accelerated the formation of AtATG8-PE, whereas AtATG3 inhibited the formation of AtATG12-AtATG5 conjugates. The in vitro conjugation systems reported here will afford a tool with which to investigate the cross-talk mechanism between two conjugation systems.

Autophagy is responsible for bulk protein degradation in the vacuole/lysosome and plays a crucial role in fundamental biological processes (1–3). In the autophagic process, double membrane vesicles called autophagosomes sequester cargos destined for degradation. Then the outer membrane of the autophagosomes fuses with the lysosomal/vacuolar membrane to deliver the cargo into the lumen together with the inner membrane.

In yeast, detailed analysis of autophagy-defective mutants has helped clarify the molecular mechanisms of autophagy (4).

Most autophagy-defective mutants display an abnormality in autophagosome formation and cannot survive under starvation conditions. Characterization of the mutants has revealed two protein conjugation pathways required for autophagosome formation, namely the Atg8 and Atg12 conjugation systems (5, 6), which are similar to the ubiquitin system. Atg12, a ubiquitin-like protein, is covalently linked to Atg5 via an isopeptide bond between the C-terminal glycine of Atg12 and Lys-149 of Atg5 by sequential reactions catalyzed by Atg7 (an E12-like enzyme) and Atg10 (an E2-like enzyme). Atg12-Atg5 conjugates further associate with Atg16 to form Atg12-Atg5-Atg16 complexes (7). Another ubiquitin-like protein, Atg8, is first processed by the cysteine protease Atg4 (8). Atg4 removes a C-terminal arginine residue of Atg8 to expose a glycine residue. Processed Atg8 is covalently linked to phosphatidylethanolamine (PE) via an amide bond between the C-terminal glycine of Atg8 and the terminal amino group of PE by sequential reactions catalyzed by Atg7 and Atg3 (an E2-like enzyme) (6).

To date, more than 30 different ATG (autophagy-related) genes specifically affecting various steps of autophagy have been identified (9). Although most of these genes were initially identified in yeast, many of them are functionally conserved in higher eukaryotes, including mammals and plants. In plants, autophagy has been suggested to play a critical role in physiological and developmental responses, such as nutrient recycling during starvation, senescence, and apoptotic processes as well as the pathogen-induced hypersensitive response (10). Genome analysis of Arabidopsis identified at least 24 AtATG genes that are homologous to 31 yeast ATG genes (9). Several genes are present as multigene families and are predicted to express splicing variants. The functional domains and essential amino acids of yeast Atg proteins are well conserved in Arabidopsis homologs, suggesting that the molecular mechanisms of autophagosome formation are conserved between yeast and plants. Arabidopsis possesses all of the genes involved in the two conjugation systems essential for autophagosome formation and was shown to utilize the AtATG8 and AtATG12 conjugation systems for autophagy (11, 12). In Arabidopsis, there exist two forms of AtATG8, form I and form II. Form II migrates faster.
than form I in SDS-PAGE; therefore, it was thought to be the PE-conjugated form as in the case of yeast Atg8 (13). Arabidopsis lacking AtATG4s (Atatg4a4b), AtATG5, or AtATG7 displayed increased chlorosis, accelerated bolting, enhanced dark-induced senescence of detached leaves, and reduced seed yield under nutrient-limiting conditions (10). The AtATG7(C558S) mutant is hypersensitive to nutrient-limiting conditions and showed premature leaf senescence (11). In a double mutant of the AtATG4s, no autophagosomes were observed, and AtATG8s were not delivered to the vacuole under nitrogen-starved conditions (13). Further, sucrose starvation induces autophagy and transiently up-regulates the AtATG genes encoding the AtATG8 conjugation system in a sequential manner (14). However, the precise roles of the components of the conjugation systems in the formation of autophagosomes remain unknown.

In vivo studies using mammalian and yeast cells have so far supported the presence of cross-talk between the Atg8 and Atg12 conjugation systems (15). In mammals, LC3 (microtubule-associated protein 1 light chain 3), a mammalian ortholog of yeast Atg8, is converted to LC3-II, a PE-conjugated form. The formation of LC3-II was impaired in ATG5-deficient mouse embryonic stem cells (16). The formation of yeast Atg8-PE was also abolished in yeast cells deficient in any components of the Atg12 system (17). However, an excess amount of Atg12-Atg5 conjugates was reported to inhibit the formation of yeast Atg8, is converted to LC3-II, a PE-conjugated form. The formation of LC3-II was impaired in ATG5-deficient mouse embryonic stem cells (16). The formation of yeast Atg8-PE was also abolished in yeast cells deficient in any components of the Atg12 system (17). However, an excess amount of Atg12-Atg5 conjugates was reported to inhibit the formation of LC3-II, whereas Atg12 alone facilitated the formation of LC3-II in HEK293 cells (18). Despite substantial efforts, the molecular details and biological significance of the cross-talk between the two conjugation systems remain elusive.

In order to clarify the cross-talk mechanism between the AtATG8 and AtATG12 conjugation systems, we reconstituted the AtATG8 and AtATG12 conjugation systems in vitro. Furthermore, using the in vitro systems, we showed that AtATG12-AtATG5 conjugates accelerated the formation of AtATG8-PE, and AtATG3 inhibited the AtATG12 conjugation system. The reconstitution systems we present herein allow us not only to study the relationship between the two conjugation systems but also to identify and characterize any other factors that can regulate these conjugation systems.

EXPERIMENTAL PROCEDURES

Plasmid Construction—To construct Escherichia coli expression plasmids encoding AtATG3-FLAG, AtATG5-HA (hemagglutinin), AtATG10-myc, AtATG12b, and the C-terminal glycinexposed form of AtATG8a–8i (AtATG8-form I), the appropriate genes were amplified by PCR and cloned into pGEX-6P-1 (GE Healthcare). The reverse primers containing FLAG, HA, and Myc epitope coding sequences were used to amplify the AtATG3, AtATG5, and AtATG10 genes, respectively. The AtATG7 gene was amplified by PCR and cloned into pGEX-His, a modified version of pGEX-6P-1 (19).

To construct E. coli co-expression plasmids encoding AtATG3 and AtATG7, the AtATG3 and AtATG7 genes were amplified by PCR and cloned into pRSFDuet-1 (Novagen) and pACYC184 with the T7 promoter, respectively. The reverse primers containing FLAG and His\textsubscript{a} coding sequences were used to amplify the AtATG3 and AtATG7 gene, respectively. The genes of the full-length AtATG8a and AtATG8a-form I were amplified by PCR and cloned into pET-11a.

Mutations leading to the specific amino acid substitutions were introduced by PCR-mediated site-directed mutagenesis. All of the constructs were sequenced to confirm their identities and transformed into E. coli BL21 (DE3).

Protein Expression and Purification—Expression of recombinant AtATG proteins was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside. After induction, cells were disrupted by sonication in PBS(−) containing 5 mM dithiothreitol, 0.1 mM EDTA, and 0.1 mM phenylmethylsulfonyl fluoride.

All of the glutathione S-transferase-fused proteins were first purified using a glutathione-Sepharose 4B column (GE Healthcare), followed by excision of glutathione S-transferase from the proteins with PreScission protease (GE Healthcare). All of the proteins except for AtATG7 were again applied to a glutathione-Sepharose 4B column in order to remove the excised glutathione S-transferase. AtATG7 was applied to an Ni\textsuperscript{2+}-ni-trilotriacetic acid column (Qiagen) equilibrated with 20 mM Tris-HCl at pH 8.0, 500 mM NaCl, and 10 mM imidazole and eluted with 20 mM Tris-HCl at pH 8.0, 100 mM NaCl, and 200 mM imidazole. Further purification was performed using the following procedures. AtATG3 was applied to a Resource Q column (GE Healthcare) equilibrated with 20 mM Tris-HCl at pH 8.0, and the protein was eluted with a 0–400 mM NaCl gradient in the same buffer. AtATG5, AtATG7, and AtATG10 were purified on a Superdex 200 gel filtration column (GE Healthcare) and eluted with 20 mM Tris-HCl at pH 8.0, 150 mM NaCl, and 2 mM dithiothreitol. AtATG8a–8i-form I were purified on a Superdex 75 gel filtration column (GE Healthcare) and eluted with 20 mM Tris-HCl at pH 8.0 and 150 mM NaCl. Purification of AtATG12b was performed as described previously (20). AtATG12-AtATG5 conjugates formed by in vitro reconstitution were applied to a HiTrap DEAE FF column (GE Healthcare) equilibrated with 20 mM Tris-HCl at pH 8.0, and eluted with a 0–400 mM NaCl gradient in the same buffer.

Liposome Preparation—To prepare liposomes with various phospholipid compositions, phospholipids were mixed in a glass tube at the appropriate ratios in chloroform. Dioleoylphosphatidylethanolamine (DOPE), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), dioleoyl phosphatidylglycerol, phosphatidylinositol from bovine liver, and dioleoylphosphatidic acid (DOPA) were purchased from Avanti Polar Lipids. The chloroform solvent was removed using a rotary evaporator. Samples were further dried in a desiccator under vacuum for 12 h. The resulting lipid film was suspended in a buffer (25 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2.7 mM KCl) at a final concentration of 1 mM phospholipid by mixing vigorously. Samples were then subjected to sonication for 5 min to obtain small unilamellar liposomes.

Antibodies and Immunoblotting—Polyclonal antibodies against AtATG8a and AtATG12b have been previously described (13, 20). Horseradish peroxidase-conjugated monoclonal antibodies against HA and FLAG M2 were purchased from Sigma. Monoclonal antibodies against Myc (9E10) and Penta–His were purchased from Covance, Research Products, Inc., and Qiagen, respectively. Horseradish peroxidase-conjugated anti-mouse IgG and horseradish peroxidase-conjugated anti-rabbit IgG were purchased from Pierce. Monoclonal antibodies against AtATG8 and AtATG12 were purchased from Covance.
In Vitro Reconstitution of Plant ATG Conjugation Systems

RESULTS

Reconstitution of the AtATG12 Conjugation System in Vitro—In our previous report, we showed that in the plant extracts, AtATG12 was detected as two bands corresponding to the proper molecular weight and a higher molecular weight not detected in homozygous Atatg5-1 or Atatg10-1 mutant plants (20). This suggests that the slower migrating band corresponds to AtATG12-AtATG5 conjugates, and, therefore, Arabidopsis possesses the AtATG12 conjugation system. Thus, we examined whether AtATG5, AtATG7, AtATG10, and AtATG12 represent the minimum components of the AtATG12 conjugation system.

Purified recombinant proteins for AtATG7, AtATG10, AtATG12, and AtATG12b were mixed with ATP and incubated for 1 h at 30 °C. As shown in Fig. 1A, lane 6, a new 50 kDa band was detected only when all of the components were present in the reaction mixture. The theoretical molecular mass of the AtATG12-AtATG5 conjugate is ~50 kDa. Additionally, the 50 kDa band was detected by both anti-AtATG12b and anti-HA antibodies (Fig. 1A, middle and bottom). Therefore, we concluded that the 50 kDa band corresponded to the AtATG12-AtATG5 conjugates, indicating that AtATG5, AtATG7, AtATG10, AtATG12, and ATP are sufficient for the formation of an AtATG12-AtATG5 conjugate.

Next, to confirm the catalytic residues of AtATG7 and AtATG10 required for AtATG12 conjugation and the conjugation site of AtATG5, we prepared recombinant mutant proteins that were subsequently applied to an in vitro reconstitution reaction. AtATG12-AtATG5 conjugates were formed in a time-dependent manner (Fig. 1B); however, as shown in Fig. 1C, the AtATG12-AtATG5 conjugates were not formed when Atatg7c558s, Atatg10c178s, or Atatg5k128r was used instead of wild-type proteins. These results suggest that AtATG7 and AtATG10 function as enzymes, presumably E1 and E2, respectively, and conjugate AtATG12 to the side chain of Lys-128 of AtATG5.

Reconstitution of the AtATG8 Conjugation System in Vitro—In vitro reconstitution of the Atg8 conjugation system was established using purified yeast and mammalian recombinant proteins (21, 22). Since yeast Atg8-PE was formed efficiently in the presence of Atg3, Atg7, ATP, and 70% DOPE, 30% POPC liposomes (21), we attempted to reconstitute the AtATG8a conjugation system in vitro under the same conditions. Recombinant proteins of AtATG3, AtATG7, and AtATG8a-form I were mixed with 70% DOPE, 30% POPC liposomes in the presence of ATP and incubated for 1 h at 30 °C. We failed to detect any change in AtATG8a under these conditions. Since the addition of negatively charged phospholipids to liposomes was known to enhance the level of yeast Atg8-PE formation in vitro (21), we next attempted to reconstitute the AtATG8 system using liposomes containing negatively charged phospholipids.
AtATG3, AtATG7, AtATG8a-form I, and ATP were mixed with liposomes, the lipid components of which are shown in Fig. 2A, and incubated for 1 h at 30 °C. In the reconstitution system using yeast proteins, liposomes containing yeast Atg8-PE were precipitable by centrifugation (21); therefore, the reconstitution system was reconstituted with mutant proteins in vitro. The reaction solution was centrifuged at 20,630 × g for 10 min, and then the supernatants and precipitates were subjected to urea-SDS-PAGE sample buffer. Subsequently, the reaction solutions were subjected to SDS-PAGE and stained by CBB. In the middle and lower panels, immunoblotting was performed on samples using antibodies against AtATG12b and HA, respectively. As shown in Fig. 2B, when ATP was present, it was assigned to AtATG8a-form I (13,514.6). Since the mass peak at 14,242 was observed only when ATP was present, it was assigned to AtATG8a-form II. The difference in molecular mass between AtATG8a-PE and AtATG8a-form I should be 726 Da. This is in good agreement with the observed difference in molecular mass between AtATG8a-form I and form I, and ATP but not liposomes (Fig. 2B, top, lanes 3 and 5). The apparent molecular mass of the protein was consistent with the molecular mass estimated for the AtATG3-AtATG8a intermediate. Furthermore, the band reacted with both anti-FLAG and anti-AtATG8a antibodies (Fig. 2B). Taken together, these results suggest that the 50-kDa band is the AtATG3-AtATG8a intermediate and that AtATG3, AtATG7, AtATG8a-form I, ATP, and liposomes are necessary and sufficient for the formation of AtATG8a-form II.

To examine whether AtATG8a-form II is a PE-modified form of AtATG8a, MALDI-TOF-MS analysis was performed on the reaction mixtures. MALDI-TOF-MS analysis gave two signals at m/z 13,516 and 14,242 (Fig. 2C). The former value was assigned to AtATG8a-form I (13,514.6). Since the mass peak at 14,242 was observed only when ATP was present, it was assigned to AtATG8a-form II. The difference in molecular mass between AtATG8a-PE and AtATG8a-form I should be 726 Da. This is in good agreement with the observed difference in molecular mass between AtATG8a-form I and form II (726 Da). The result clearly shows that AtATG8a is, in fact, modified by PE in vitro.

Next, in order to identify the catalytic residues of AtATG3 and AtATG7 required for the AtATG8 conjugation reaction, recombinant mutant proteins were prepared and used for in vitro reconstitution reaction. AtATG8a-PE was formed in a time-dependent manner (Fig. 2D) but was not formed when AtATG7C558S or AtATG3C258S was used instead of the respective wild-type proteins (Fig. 2E). These results suggest that AtATG7 and AtATG3 function as enzymes, presumably E1 and E2, respectively, and conjugate the C-terminal glycine of AtATG8a-form I to PE.

Arabidopsis has nine AtATG8 homologs, all of which display significant homology to yeast Atg8 and have a glycine residue corresponding to the Gly-116 of yeast Atg8, the conjugation site for PE (11, 12). We next examined whether all of the AtATG8 homologs can be modified using the in vitro reconstitution system. We confirmed that all of the AtATG8 homologs were converted to AtATG8-form II (AtATG8-PE) when AtATG8s were incubated with AtATG7, AtATG3, liposomes, and ATP, although the formation of AtATG8-form II was low for AtATG8c, AtATG8g, and AtATG8i (Fig. 3).
FIGURE 2. A, in vitro reconstitution of the AtATG8 conjugation system with varying phospholipid content. Liposomes were prepared from DOPE, POPC, phosphatidylinositol (PI), dioleoyl phosphatidylglycerol, and DOPA at the indicated compositions. The formation of AtATG8a-form II was performed using the generated liposomes in the presence of ATP and incubated for 1 h at 30 °C. The reaction solutions were centrifuged at 20,630 × g for 10 min and subjected to urea-SDS-PAGE, followed by staining with CBB. S, supernatant; P, pellet. B, reconstitution of the AtATG8 conjugation system in vitro. Where indicated (+), 10 μM AtATG7-His, 10 μM AtATG3-FLAG, 50 μM AtATG8a-form I, and 70% DOPE, 10% POPC, 20% DOPA liposomes were mixed in the presence (ATP +) or absence (ATP −) of ATP and incubated for 1 h at 30 °C. The reaction solutions were centrifuged at 20,630 × g for 10 min, and then the precipitates were subjected to urea-SDS-PAGE, followed by staining with CBB (precipitates). Immunoblotting was performed on the reaction solutions using antibodies against FLAG and AtATG8a (WB: anti-FLAG and WB: anti-AtATG8a). C, MALDI-TOF mass spectra of the AtATG8 conjugation reaction samples. The samples mixed in the presence or absence of ATP (B, lanes 4 and 5) were centrifuged, and the resultant precipitates were used for MALDI-TOF-MS analysis with 2,5-dihydroxybenzoic acid as matrix. The calculated m/z values of the protonated molecule [M + H]+ for AtATG8a-form I and AtATG8a-PE are 13,515.6 and 14,241.6, based on their average molecular weights, respectively. D, 10 μM AtATG7-His, 10 μM AtATG3-FLAG, 50 μM AtATG8a-form I, and 70% DOPE, 10% POPC, 20% DOPA liposomes were mixed in the presence of ATP and incubated for the indicated time at 30 °C. The reaction solutions were centrifuged at 20,630 × g for 10 min, and then the precipitates were subjected to urea-SDS-PAGE, followed by staining with CBB. The asterisk indicates the degradation product of AtATG8a-form II. E, reconstitution of the AtATG8 conjugation system with mutant proteins in vitro. Wild type (WT) or the C558S mutant (CS) of AtATG7-His, wild type (WT) or the C258S mutant (CS) of AtATG3-FLAG, the C-terminal glycine-exposed form (Gly) or full length (Full) of AtATG8a, and 70% DOPE, 10% POPC, 20% DOPA liposomes were mixed in the presence of ATP and incubated for 1 h at 30 °C. The reaction solutions were centrifuged at 20,630 × g for 10 min, and then the precipitates were subjected to urea-SDS-PAGE, followed by staining with CBB. PC, phosphatidylcholine; PA, phosphatidic acid; PI, phosphatidylinositol.
were cleaved by AtATG4s in vivo (13). AtATG4s have significant sequence homology with yeast Atg4, which deconjugates yeast Atg8-PE conjugates both in vivo and in vitro (8). Therefore, we first examined whether AtATG8a-PE could be cleaved by AtATG4s. AtATG8a-PE was formed using the in vitro reconstitution system and subsequently incubated with AtATG4s. The reaction solutions were centrifuged, and then the precipitates were subjected to urea-SDS-PAGE and stained with CBB. The amount of AtATG8a-PE obtained when treated with AtATG4a and AtATG4b was significantly reduced, which is in contrast to the absence of any change in AtATG8a-PE when treated with water and is similar to the degree of reduction observed in AtATG8a-PE when treated with phospholipase D, which releases phosphatidic acid from the conjugate (Fig. 4A). The result shows that AtATG8a-PE is deconjugated by both AtATG4a and AtATG4b. We examined other AtATG8s in the same manner and again detected decreases in AtATG8-form II, except AtATG8g and AtATG8i, by incubation with AtATG4s (Fig. 4B). AtATG8g and AtATG8i readily aggregated prior to conjugation; therefore, it is possible that their respective form II compounds are too strongly aggregated to be deconjugated by AtATG4s.

The Effect of AtATG12-AtATG5 Conjugates on the AtATG8 Conjugation System—As previously reported, the absence of yeast Atg12-Atg5 conjugates results in a severe decrease in yeast Atg8-PE in vivo (17). In addition, yeast Atg12-Atg5 conjugates were recently shown to enhance the formation of yeast Atg8-PE in vitro (3). To confirm the effect of AtATG12-AtATG5 conjugates on the formation of AtATG8-PE, we attempted to introduce AtATG12-AtATG5 conjugates into the AtATG8 conjugation system. AtATG12-AtATG5 conjugates were prepared using the in vitro reconstitution system and subsequently purified by anion exchange chromatography. Formation of AtATG8a-PE was significantly increased in a dose-dependent manner in the presence of AtATG12-AtATG5 conjugates (Fig. 5A). On the other hand, the introduction of AtATG12 or AtATG5 alone or in combination did not increase the level of AtATG8-PE (Fig. 5B), indicating that the covalent linkage between AtATG12 and AtATG5 is essential for their acceleration of AtATG8-PE formation.

The Effect of AtATG3 on the AtATG12 Conjugation System—The amount of mammalian Atg12-Atg5 conjugates formed was reported to increase significantly in COS7 cells when Atg3 was overexpressed together with Atg7 and Atg12 (23). In order to verify the effect of AtATG3 on AtATG12-AtATG5 conjugation efficiency, we introduced AtATG3 into the AtATG12 conjuga-

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**In Vitro Reconstitution of Plant ATG Conjugation Systems**

AtATG12-AtATG5 conjugates were formed by the enzymatic reactions of AtATG7 (E1-like) and AtATG10 (E2-like) *in vitro*, although the reaction was not as efficient as that *in vivo*. Some additional factor(s) might further enhance this conjugation reaction *in vitro*. *In vitro* reconstitution of the mammalian Atg12 conjugation system was reported recently (24). In that case, the Atg12-Atg5 conjugation efficiency was extremely low, and ribonucleic acids were required to enhance the conjugation reaction, although the mechanism underlying this effect was elusive. We also introduced ribonucleic acids into the AtATG12 conjugation system, but no improvement in the efficiency of AtATG12-AtATG5 formation was detected (data not shown). Although the basic mechanism of the Atg12 system is conserved between mammals and *Arabidopsis*, differences in the regulation of the system may exist.

AtATG8-PE was also formed by the enzymatic reactions of AtATG7 and AtATG3. *Arabidopsis* has nine AtATG8 homologs, all of which were converted to form II, the PE-conjugated form, *in vitro* but with different efficiency. Furthermore, form II of seven homologs among nine was deconjugated by AtATG4a and -4b *in vitro*, again with different efficiency. These results suggest that nine AtATG8 homologs might have distinct functions in *Arabidopsis*. In fact, nine AtATG8 homologs showed distinct spatial and temporal expression patterns in roots of young *Arabidopsis* (25). Detailed functional analyses of each AtATG8 homolog *in vivo* are required to reveal each function of these homologs.

The efficiency of AtATG8-PE formation in the *in vitro* reconstitution reaction was much lower than that in plant cells. We used synthetic liposomes, which differ from plant membranes in various points, for *in vitro* reconstitution. In one case, plant membrane phospholipids consist of 35.5% POPC, 38.9% DOPE, 9% phosphatidylglycerol, 5.1% phosphatidylinositol, 5.1% phosphatidylserine, and 6.4% DOPA (26); therefore, we also prepared synthetic liposomes consisting of these lipids for use in *in vitro* reconstitution. However, the use of these synthetic lipids again failed to raise efficiency (data not shown). Since the endomembranes of plant cells are diverse, there might be a more appropriate ratio of phospholipids for AtATG8-PE formation. *In vitro* reconstitution of the yeast Atg8 system has been reported (21). Yeast Atg8-PE is formed efficiently with 70% PE, 30% phosphatidylcholine liposomes *in vitro*. However, the efficiency of the Atg8-PE formation is low, with 20% PE, 80% phosphatidylcholine liposomes, the PE content of which is similar to that of yeast organelle membranes, such as mitochondria and vacuoles. *In vitro* reconstitution of the LC3 conjugation system has also been reported, in which the efficiency of LC3-II formation was low (22). These studies, together with our results, raise the possibility that Atg8 conjugation systems require additional factor(s), such as an E3 enzyme, for efficient conjugation.

In yeast and mammals, the Atg12 conjugation system is known to be crucial for the efficient formation of Atg8-PE (16, 17). Recently, *in vitro* reconstitution experiments revealed that yeast Atg12-Atg5 conjugates, but not Atg12 or Atg5, directly enhance the conjugation of yeast Atg8 to PE, suggesting that Atg12-Atg5 conjugates act as the E3 enzyme in the Atg8 system.3 Using the *in vitro* reconstitution systems, we showed that...

**DISCUSSION**

In the present study, we reconstituted the AtATG12 and AtATG8 conjugation systems using recombinant proteins *in vitro*. We demonstrated that the cysteine residues of AtATG3, AtATG7, and AtATG10 as well as the lysine residue of AtATG5, all of which were conserved among *Arabidopsis*, yeast, and mammals, were actually crucial for the conjugation reactions, showing that the AtATG8 and AtATG12 conjugation systems in *Arabidopsis* are functionally homologous to the conjugation systems in yeast and mammals.
AtATG12-AtATG5 conjugates also significantly promote the conjugation of AtATG8 to PE (Fig. 5A). Furthermore, we also showed that AtATG12 and AtATG5 alone or in combination did not promote the conjugation reaction (Fig. 5B). Therefore, AtATG12-AtATG5 conjugates are also considered to act as the E3 enzyme in the AtATG8 system. These observations strongly suggest that the E3-like role of Atg12-Atg5 conjugates in the Atg8 system is evolutionarily conserved. It has been reported that excess amounts of mammalian Atg12-Atg5 conjugates inhibit the formation of LC3-II in HEK293 cells when mammalian Atg12, Atg5, Atg7, and LC3 are simultaneously overexpressed (18). Thus, the molar ratio between Atg proteins may be important for efficient Atg8-PE formation. To clarify this, mammalian systems should also be studied using in vitro reconstitution systems in a similar manner to yeast and Arabidopsis systems.

AtATG3 inhibited the formation of AtATG12-AtATG5 conjugates (Fig. 6A). The inhibition of AtATG12-AtATG5 conjugation by AtATG3 was recovered by excess amounts of AtATG10 (Fig. 6B). These results suggest that the two E2-like enzymes compete against each other in the conjugation reactions. In ubiquitin-like conjugation systems, the direct interaction between E1 and E2 is crucial for the transfer of a modifier from E1 to E2, which is an essential event prior to the conjugation reaction. The Atg8 and Atg12 conjugation systems employ the same E1-like enzyme, Atg7. In vitro pull-down assays showed that AtATG7 directly binds to AtATG3 and AtATG10 (data not shown). It is possible that the AtGT7 binding sites for AtATG3 and AtATG10 overlap with each other, resulting in competition between these E2 enzymes for binding to AtATG7. In fact, the inhibition of AtATG12-AtATG5 conjugation by competition between these E2 enzymes for binding to AtATG7 showed that AtATG7 directly binds to AtATG3 and AtATG10 (data not shown). The stoichiometry among Atg proteins might be crucial for such enhancement. Alternatively, there might be some differences in the regulation of the two conjugation systems between mammals and Arabidopsis. The in vitro reconstitution systems we present here will be a powerful tool in helping to address these problems.

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