Supporting Information

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SI Materials and Methods

Plasmid Construction. The Citrine (C) coding sequence without the stop codon was PCR amplified and cloned into NheI-BamHI digested pET28(a) bacterial expression vector (Novagen) resulting in pET28-Citrine. The PCR amplified ShR (1) coding sequence with stop codon was cloned into SalI-NotI of pET28-Citrine vector, resulting in pET28-Citrine-ShR plasmid. To generate different AtAtg8 expression plasmids, individual AtAtg8 coding sequences without the stop codon were PCR amplified and introduced between BamHI and SalI sites of pET28-Citrine-ShR vector resulting in different C-AtAtg8-ShR plasmids. To generate C-hLC3a-ShR plasmid, PCR amplified hLC3a coding sequence was cloned into BamHI-SalI-cut pET28-Citrine-ShR vector. To generate C-AtAtg8a(GtoA)-ShR and C-AtAtg8i(GtoA)-ShR constructs, wild-type AtAtg8a and AtAtg8i cDNAs were PCR amplified with mutagenesis primers and amplified products were cloned to pET28-Citrine-ShR vector as described above. To clone AtAtg4a and AtAtg4b, PCR amplified coding sequences were cloned into BamHI-SalI cut pMal-C2 (New England Biolabs, NEB) resulting in maltose binding protein (MBP)-AtAtg4a and MBP-AtAtg4b fusion plasmids. To generate 35S::C-AtAtg8a-ShR plasmid, C-AtAtg8a-ShR was amplified from bacterial expression vector and cloned into pYL400 containing 35S promoter and NOS terminator cassette. Identities of inserts in all plasmids were confirmed by sequencing. Primer sequences used for cloning are available upon request.

AtAtg8 and AtAtg4 Protein Expression and Purification. The N terminus 6×HIS containing C-AtAtg8-ShR and MBP-fused AtAtg4 plasmids were transformed into *Escherichia coli* strain BL21 (DE3). Protein expression was induced with 1 mM IPTG at room temperature (RT) overnight. The soluble 6×HIS-C-AtAtg8-ShR and MBP-AtAtg4s were purified by column chromatography with cobalt resin (Thermo Scientific) and amylose resin (NEB), respectively, by following manufacturer recommendations. Affinity-purified recombinant proteins were dialyzed overnight in PBS (pH 7.2) buffer at 4 °C and the dialyzed proteins were concentrated using 10K cutoff-pore size centrifugal filter units (Millipore). Concentration of purified proteins was determined using BSA (Thermo Scientific) as a standard. Purified proteins were confirmed by SDS/PAGE followed by Coomassie brilliant blue staining (Fig. S1). The purified proteins were stored in aliquots in PBS buffer with 50% (vol/vol) glycerol at −80 °C.

NASCA and Immunoblot Assays. Approximately 250 ng of a purified AtAtg8 synthetic substrates and the same amount of AtAtg4 cysteine protease was incubated in 20 μL PBS at RT for 10 min and then 20 μL of 2× Tris/glycine native sample buffer (Invitrogen) was added. Typically, 8 μL of the reaction mixture (~50 ng) was separated in a 10% Mini-PROTEAN TGX precast gel (Bio-Rad) using Tris/glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) at 4 °C with 150 V for 1 h and 30 min. To the resulting gel, 2 μM of native coelenterazine (CLZ) (Biotium) in PBS was directly applied and incubated with agitation at RT for 10 min under darkness. After removing the excess CLZ substrate, the native gel was exposed to a Hyperfilm ECL (GE Healthcare). For assays with H_2O_2, the AtAtg4 purified proteins were preincubated with different amounts of H_2O_2 as indicated in 10 μL of PBS at RT for 5 min and then, ~300 ng of the C-AtAtg8f-ShR synthetic substrate was added and incubated for an additional 10 min. For AtAtg4 activity recovery assays 50 mM DTT (American Bioanalytical) was directly added to the preincubated reaction mixture containing AtAtg4s and different concentration of H_2O_2 for 5 min, the reaction was continued for another 5 min, and then the AtAtg8f synthetic substrate was incubated for 10 min. For immunoblot analyses, ~50 ng of the AtAtg8f synthetic substrates was boiled in 2× SDS sample buffer [125 mM Tris, pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 0.2 M DTT, 0.02% bromophenol blue] and separated on SDS/PAGE. After transferring the proteins onto PVDF membrane, immunoblot analyses were performed using monoclonal antibodies against RLUC (Millipore) or MBP (Sigma) and with SuperSignal west Pico Chemiluminescent Substrate (Thermo Scientific). For signal normalization of individual blots, cleavage products in 50 ng of the AtAtg8e substrate were used as an internal control. ImageJ (National Institutes of Health) and Prism 5.0 (GraphPad) software were used to determine signal intensity and kinetic constants from immunoblots, respectively.

Generation of Transgenic Plants Expressing the C-AtAtg8a-ShR Synthetic Substrate. T-DNA construct with the C-AtAtg8a-ShR cassette was transformed into Agrobacterium GV3101 strain by electroporation. The Agrobacterium with C-AtAtg8a-ShR was used to transform Col-0 or *atg4a4b* mutant plants using the floral dip method (2). Gentamycin (50 μg/mL)-resistant T1 seedlings were selected and selfed.

Bioluminescence Resonance Energy Transfer Measurement and Confocal Microscopy. Individual transgenic seedlings of Col-0::C-AtAtg8a-ShR and *atg4a4b*::C-AtAtg8a-ShR were subjected to nitrogen starvation for 5 d and then the seedlings were washed with water and each seedling was placed in a single well of 96-well microtiter plate (Costar) to set up bioluminescence resonance energy transfer (BRET) measurement using Tristar LB941 microplate reader (Berthold technology). Briefly, 25 μM of native CLZ was injected and diluted to 2.5 μM in each well by the automation mode. BRET measurement was started after 1-min incubation under darkness. F485 and F530 filters were used to measure blue luminescence and yellow fluorescence for 1 s operated by BRET measurement program, respectively. Raw data generated by MikroWin software (Berthold technology) were analyzed.

For microscopy, 7-d-old seedlings were grown in the selection media and then transferred to either MS −N (nitrogen starvation) or control liquid media for 5 d. To stabilize autophagic bodies in the vacuole, 1 μM concanamycin A or dimethyl sulfoxide (DMSO) as a solvent control was treated for 12 h before microscopic analyses. Leaves were imbibed with water and confocal images were obtained using Zeiss LSM710 confocal microscope equipped with a LDC-apochromat 40×/1.1W Korr M27 water-immersion objective (NA 1.1). 5 μM FM4-64 was applied to samples followed by a 10-min incubation and samples were visualized using 5% 488-nm laser excitation and 640- to 750-nm spectral detection. Citrine was observed using 20% 514-nm laser excitation and 519- to 568-nm spectral detection. For colocalization of C-AtAtg8a with monodansylcadaverine (MDC), 50 μM of MDC was directly added to the nutrient media and incubated under darkness for 2 h. MDC-stained autophagic bodies were visualized using diode 405-nm UV laser excitation and 467- to 541-nm spectral detection. Images were captured by sequential channel detection individually and processed for display by ImageJ (NIH).

1. Woo J, von Arnim AG (2008) Mutational optimization of the coelenterazine-dependent luciferase from Renilla. *Plant Methods* 4:23.

2. Clough SJ, Bent AF (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* 16(6):735–743.

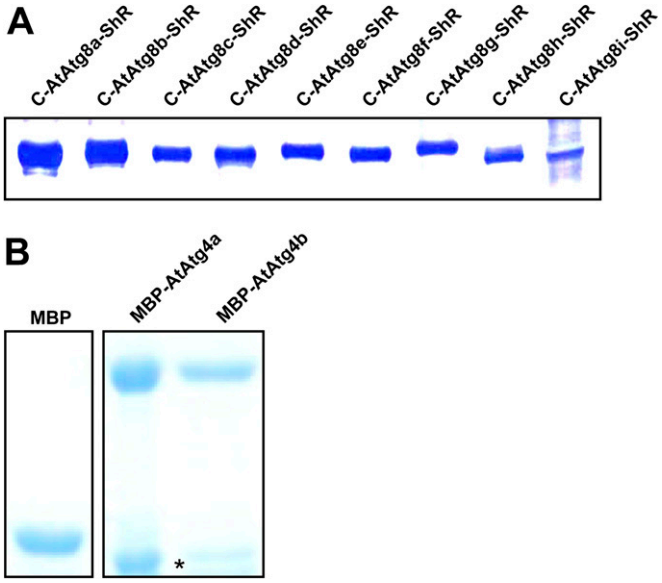

Fig. S1. Purification of the C-AtAtg8-ShR synthetic substrates and MBP-AtAtg4 cysteine proteases. Nine 6×HIS-C-AtAtg8-ShR synthetic substrates (*A*) and two MBP-AtAtg4 cysteine proteases (*B*) were purified in bacterial expression system as described in *SI Materials and Methods*. Purified recombinant proteins were separated on SDS/PAGE and gels were stained with Coomassie brilliant blue. Asterisk in *B* indicates degradation product.

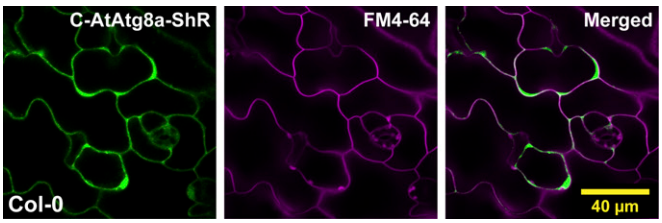

Fig. S2. Subcellular localization of the C-AtAtg8a-ShR synthetic substrate in transgenic Col-0 *Arabidopsis* plants. Leaves from 2-wk-old plants grown in normal condition were imaged under confocal microscope. Citrine fluorescence was observed in the cytoplasm (*Left*). FM4-64 dye was used to visualize the plasma membrane (*Center*). Merged image is shown (*Right*). (Scale bar = 40 μm.)

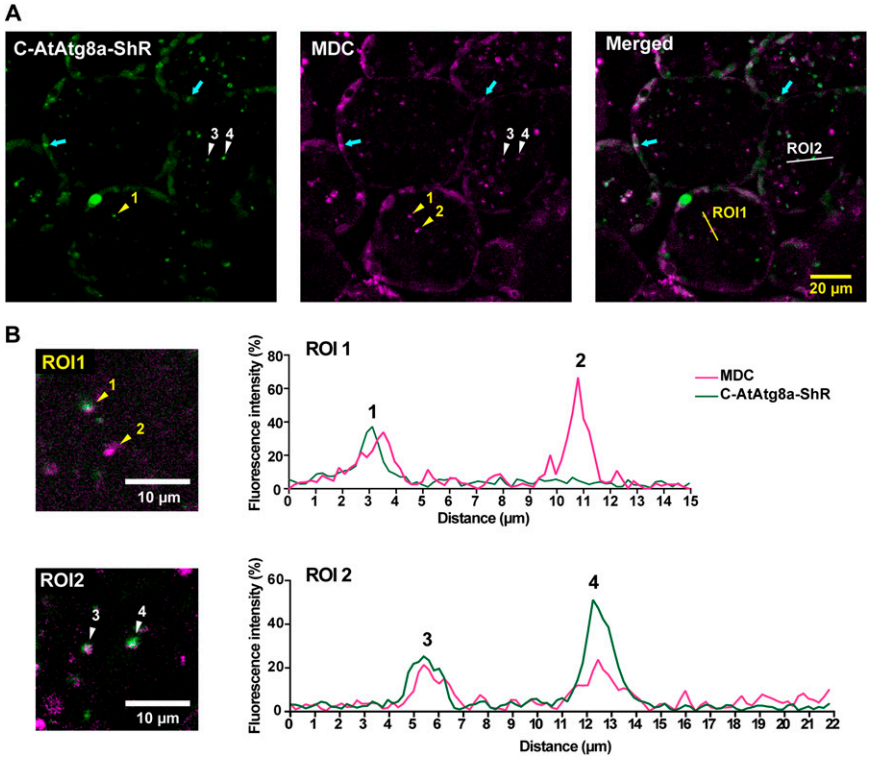

Fig. S3. Colocalization of mature synthetic substrate (C-AtAtg8a) and MDC to autophagic bodies. (*A*) One-wk-old transgenic Col-0::C-AtAtg8a-ShR plants were subjected to nitrogen starvation for 3 d followed by concanamycin A and monodansylcadaverine (MDC) treatment. Citrine fluorescence (false colored to green; *Left*) and MDC (false colored to magenta; *Center*), and merged image (*Right*) are shown. Citrine-labeled vesicles frequently colocalized with MDC-labeled autophagic bodies both in the cytoplasm (cyan arrows) and in the vacuole (yellow and white arrowheads). Note that vesicle labeled 1 (yellow arrowhead) and vesicles 3 and 4 (white arrowheads) contain both C-AtAtg8a and MDC signal in the vacuole compared with vesicle 2 (yellow arrowhead) that only contains MDC signal. Yellow and white lines in the merged *Right* panel indicate two regions of interest (ROI1 and ROI2) described below in *B*. (Scale bar = 20 μm.) (*B*) Two ROIs indicated in the merged image of *A* were enlarged for better visualization. (Scale bar = 10 μm.) Signal intensities of Citrine fluorescence and MDC of corresponding ROIs were plotted as green (Citrine from C-AtAtg8a-ShR) and magenta (MDC). Intensity maxima of Citrine and MDC clearly coalign in peaks 1, 3, and 4 but not in peak 2. Note that intensity difference of two colors on the same pixel result in pronounced green- or magenta-colored vesicles, depending on the relative intensities of Citrine and MDC (see vesicle 4 and peak 4 in ROI2). *X* axis is the distance within ROIs and *y* axis indicates signal intensities.