

Detection of Autophagy in Plants by Fluorescence Microscopy

Yunting Pu and Diane C. Bassham

Abstract

Autophagy is a key process for degradation and recycling of proteins or organelles in eukaryotes. Autophagy in plants has been shown to function in stress responses, pathogen immunity, and senescence, while a basal level of autophagy plays a housekeeping role in cells. Upon activation of autophagy, vesicles termed autophagosomes are formed to deliver proteins or organelles to the vacuole for degradation. The number of autophagosomes can thus be used to indicate the level of autophagy. Here, we describe two common methods used for detection of autophagosomes, staining of autophagosomes with the fluorescent dye monodansylcadaverine, and expression of a fusion between GFP and the autophagosomal membrane protein ATG8.

Key words Autophagy, *Arabidopsis thaliana*, Autophagosome, Monodansylcadaverine, GFP-ATG8, Vacuole

1 Introduction

Autophagy is an important process for delivering macromolecules or organelles to be degraded and recycled in animal and plant cells. Three types of autophagic pathway have been identified with distinct mechanisms: macroautophagy, microautophagy, and chaperone-mediated autophagy [1–3]. In plants, although both macroautophagy and microautophagy have been identified, the function and mechanism of macroautophagy is better studied. In this protocol, autophagy hereafter refers to macroautophagy. Upon activation of autophagy, a cup-shaped double-membrane vesicle called a phagophore forms to engulf cargo that will be degraded. Expansion and closure of the phagophore leads to the formation of an intact vesicle called an autophagosome. Autophagosomes then deliver the cargo into the lysosome in animal cells or the vacuole in plant cells for degradation. In plant cells, the outer membrane of the autophagosome fuses with the tonoplast, or vacuole membrane, while the inner membrane along with the cargo is delivered into the vacuole as an

autophagic body and degraded by lytic enzymes. The products of degradation are released into the cytoplasm for reuse [1].

Initiation and formation of autophagosomes involves a series of autophagy-related (*ATG*) genes. The ATG8-PE conjugation system plays a key role in autophagosome formation [4]. In *Arabidopsis thaliana*, nine isoforms within an *ATG8* gene family have been identified, *AtATG8a–AtATG8i* [5]. Upon induction of autophagy, the C-terminus of ATG8 is cleaved by the ATG4 protease and eventually conjugated to the membrane lipid phosphatidylethanolamine (PE). ATG8 is therefore attached to the autophagosome membrane, enabling it to participate in the formation of autophagosomes [4, 6]. The conjugation is reversible via cleavage by ATG4 for ATG8 recycling [7]. ATG8 has also been characterized in other photosynthetic organisms, including *Chlamydomonas*, rice, and maize, where it acts via a similar mechanism [8–10].

Autophagy is maintained at a basal level in cells as a housekeeping process. It is induced in both biotic and abiotic stress conditions, including nutrient starvation, salt and drought stress, oxidative stress, endoplasmic reticulum (ER) stress, pathogen infection, and senescence [1, 11]. Several assays have been established to monitor autophagy in plant cells, such as detection of ATG8 lipidation by immunoblot with ATG8 antibodies, visualization of autophagosomes using a GFP-ATG8 fusion protein, and staining of autophagosomes with acidotropic dyes such as LysoTracker Red and monodansylcadaverine (MDC), followed by fluorescence microscopy [12]. In this protocol, we describe two methods to monitor autophagy *in vivo* by fluorescence microscopy. ATG8 decorates both the outer and inner membranes of autophagosomes through ATG8-PE adduct formation via lipidation, and remains on the inner membrane upon its delivery into the vacuole as an autophagic body. Therefore, fluorescent protein-fused ATG8 can be used as a marker both of autophagosomes and of autophagic bodies inside the vacuole [4, 13]. Due to the rapid degradation of autophagic bodies, their visualization is sometimes facilitated by incubation with degradation inhibitors such as Concanamycin A, a V-ATPase inhibitor that blocks hydrolase activity by elevating vacuolar pH [4, 14]. MDC is an acidotropic dye that stains acidic cellular compartments, including autophagosomes [13]. Although other acidic vesicles might also be stained by MDC and thus be confused with autophagosomes, the simplicity and time-saving advantages make it a good method for rapid autophagy detection when combined with other approaches [15].

2 Materials

2.1 Plant Growth Materials

1. Seed sterilizing solution: 33% (v/v) Bleach and 0.1% (v/v) Triton X-100 (Sigma, St. Louis, MO, USA). Mix 1 mL Triton X-100 with 9 mL water to prepare a 10% stock solution.

Add 30 μL 10% Triton X-100 solution and 10 mL household bleach to a tube containing 20 mL water. Store at room temperature.

2. Solid half-strength MS medium with sucrose: 0.22% (w/v) Murashige–Skoog vitamin mixture (Caisson Laboratories, North Logan, UT, USA), 2.4 mM 2-morpholinolothanesulfonic acid (MES) (Sigma), 0.6% (w/v) Phytoblend agar (Caisson Laboratories), 1% sucrose (Sigma). Adjust pH to 5.7 with KOH. Autoclave the medium at 121 °C for 20 min. Allow the medium to cool to 45–50 °C. Pour the medium into petri dishes in a laminar flow hood to approximately half the depth of the plate. Allow the medium to cool to room temperature for about an hour to solidify. Store plates at 4 °C (*see Note 1*).
3. Solid half-strength MS medium without nitrogen: 5% (v/v) Murashige–Skoog basal salt micronutrient solution (10 \times) (Sigma), 1.5 mM CaCl_2 , 0.75 mM MgSO_4 , 0.625 mM KH_2PO_4 , 2.5 mM KCl, 2 mM MES, 1% (w/v) sucrose. Adjust pH to 5.7 with KOH. Autoclave and pour the medium as for solid half-strength MS medium with sucrose.
4. 0.1% Agarose: 0.1% (w/v) Agarose (Fisher Scientific, Dallas, TX, USA) in water. Autoclave at 121 °C for 20 min. Store at room temperature.
5. Petri dishes (100 mm \times 20 mm) (Fisher Scientific).

2.2 Seedling Treatment and Staining

1. Phosphate-buffered saline (PBS, 10 \times): 8% (w/v) NaCl, 0.2% (w/v) KCl, 1.4% (w/v) Na_2HPO_4 , 0.24% (w/v) KH_2PO_4 , pH 7.4. Store at room temperature.
2. 20 \times MDC stock solution: 1 mM dansylcadaverine (Sigma). Aliquot 500 μL or 1 mL into microcentrifuge tubes. Store at -20 °C in the dark (*see Note 2*).
3. 6-well plates (BioExpress, Kaysville, UT, USA).
4. Mannitol (Sigma).
5. Dithiothreitol (DTT, 100 \times): 0.2 M DTT (Fisher Scientific) in water. Store at -20 °C.
6. Tunicamycin (200 \times): 1 g/mL tunicamycin (Sigma) in dimethyl sulfoxide (DMSO). Store at 4 °C.
7. Hydrogen peroxide (H_2O_2) 30% (w/w) (Sigma).
8. Methyl viologen (1000 \times): 10 mM methyl viologen dichloride hydrate (Sigma) in water. Filter the solution through a 0.22 μm sterile syringe filter (VWR, Radnor, PA, USA). Store at 4 °C.
9. Concanamycin A (1000 \times): 1 mM Concanamycin A (Sigma) in DMSO. Store at -20 °C.
10. Aluminum foil.

2.3 Fluorescence Microscopy

1. Glass slides (Fisher Scientific).
2. Glass cover slips (22 × 22 mm) (Fisher Scientific).
3. Light microscope: Zeiss Axio Imager.A2 upright microscope (Zeiss, Jena, Germany) with Zeiss Axiocam BW/color digital cameras (*see Note 3*).
4. Confocal microscope: Leica SP5 × MP confocal/multiphoton microscope system (Leica, Wetzlar, Germany) (*see Note 4*).
5. ZEN 2012 (blue edition) (Zeiss) (*see Note 5*).
6. Leica Application Suite (Leica) (*see Note 6*).

3 Methods

3.1 Plant Materials and Growth Conditions

1. Sterilize Arabidopsis seeds with seed sterilizing solution for 20 min with agitation or rocking, followed by five washes with sterile water for 5 min each. Sterilized seeds are stored at 4 °C in the dark for at least 2 days before plating to allow stratification.
2. Plate seeds in lines on solid half-strength MS medium with sucrose. Suspend seeds in a tube containing 0.1 % agarose. Use a pipette to spot 10–13 seeds per line and at most 8 lines per plate to allow sufficient growth space. Keep the plates vertically under long day conditions (16 h light) at 22 °C for 7 days.

3.2 Autophagy Activation in Seedlings by Abiotic Stresses (*See Note 7*)

1. To induce autophagy by starvation, transfer 7-day-old seedlings onto solid half-strength MS medium without sucrose for carbon starvation, or solid half-strength MS medium without nitrogen for nitrogen starvation. Meanwhile, transfer seedlings onto half-strength MS medium with sucrose as a control. Wrap the plates for sucrose starvation with aluminum foil to maintain darkness. Grow the transferred seedlings on sucrose starvation plates in the dark, and seedlings on control or nitrogen starvation plates in the light for an additional 2–4 days.
2. To induce autophagy by salt or osmotic stress, immerse 5–10 7-day-old seedlings in 2 mL liquid half-strength MS medium with sucrose plus 0.16 M NaCl or 0.35 M mannitol in a 6-well plate. To induce autophagy by ER stress, immerse 7-day-old seedlings in 2 mL liquid half-strength MS medium with sucrose plus 2 mM DTT or 5 µg/mL tunicamycin in a 6-well plate. When inducing ER stress with tunicamycin, add an equal volume of DMSO into liquid medium for a control treatment. Wrap the 6-well plate with aluminum foil and gently shake for 6–8 h (*see Note 8*).
3. To induce autophagy with oxidative stress, immerse 5–10 7-day-old seedlings in 2 mL liquid half-strength MS medium with sucrose plus 5 mM H₂O₂ or 10 µM methyl viologen in a 6-well plate. Wrap the 6-well plate with aluminum foil and gently shake for 1–2 h.

3.3 Labeling of Autophagosomes in Seedlings by MDC Staining

1. Dilute the 20× MDC stock solution to 1× MDC solution with PBS buffer.
2. Carefully transfer 5–10 seedlings from the solid medium to 6-well plates with 2 mL MDC solution in each well. If seedlings are in 6-well plates with liquid medium, carefully remove liquid medium from seedlings by pipetting. Gently dispense 2 mL MDC solution into each well. Immerse seedlings in the MDC solution and shake gently for 10 min in the dark.
3. Wash the seedlings twice with PBS buffer for 5 min each. Be sure to remove any visible remains of MDC solution. Leave the seedlings in PBS buffer and wrap the plate with aluminum foil until observation by microscopy (*see Note 9*).

3.4 Labeling of Autophagosomes in Seedlings with GFP-ATG8 Fusion Protein (See Note 10)

1. To detect autophagy using GFP-ATG8 fusion proteins, grow GFP-ATG8e transgenic seedlings under the same conditions as described in Subheading 3.1, and induce autophagy using stress conditions as in Subheading 3.2 (*see Note 11*).
2. To detect autophagy using a GFP-ATG8 fusion protein in mutant lines or other genetic backgrounds, cross the desired lines with GFP-ATG8e transgenic plants or use *Agrobacterium*-mediated transformation to generate transgenic plants with both the desired genotype and GFP-ATG8e.
3. To facilitate visualization of autophagosomes by inhibiting autophagic body degradation with Concanamycin A (optional), transfer 5–10 seedlings to a 6-well plate with 2 mL liquid half-strength MS medium with sucrose plus 1 μM Concanamycin A. Incubate the plate with shaking for 6–8 h (*see Note 12*).

3.5 Visualization of MDC-Stained or GFP-Labeled Autophagosomes by Fluorescence Microscopy

Procedure described is for both epifluorescence microscopy and confocal microscopy unless otherwise specified.

1. Add a drop of PBS buffer to a slide and gently lay out 5–8 seedlings onto the slide with roots submerged in buffer. Cover the roots with a cover slip. Carefully place the slide onto the stage of the microscope.
2. Adjust the focus of the eyepiece (10×). Set the objective lens to 10× or 20× to find the root tips under bright field illumination. From the root tips, move up along the root to the elongation zone, where autophagy can be observed most easily.
3. Switch the objective lens to 40×/0.75 for epifluorescence microscopy or 63×/1.4 oil for confocal microscopy and adjust focus.
4. For MDC detection, select filter sets specific for imaging DAPI, UV, or with an excitation wavelength of 335 nm and emission wavelength of 508 nm. For GFP fluorescence detection,

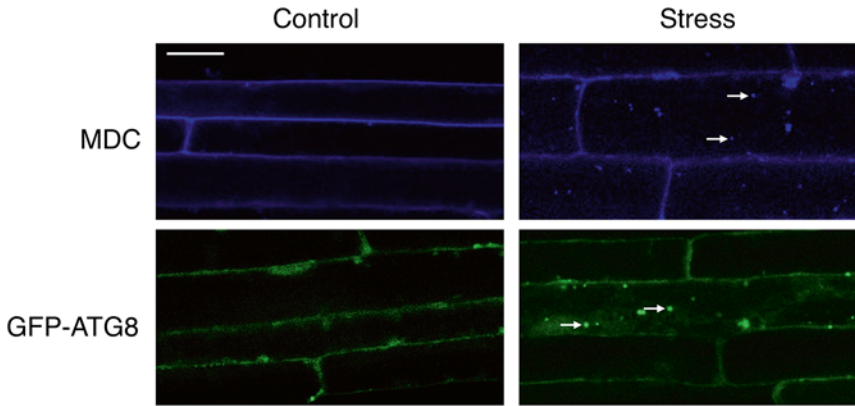


Fig. 1 Imaging of autophagosomes labeled with MDC or GFP-ATG8 in Arabidopsis root cells. Arabidopsis seedlings stained with MDC (*upper panels*) or expressing GFP-ATG8e (*lower panels*) were incubated in stress or control conditions as described in this protocol. Root cells in the elongation zone were observed using confocal microscopy. MDC-stained or GFP-ATG8e labeled autophagosomes are indicated by *white arrows*, showing autophagy induction in Arabidopsis root cells upon stress treatment. Scale bar = 20 μm

select filter sets specific for imaging Fluorescein Isothiocyanate (FITC), GFP, or with excitation wavelength of 488 nm and emission wavelength of 525 nm.

5. Observe the elongation zone, focusing on different layers of the root to get an initial overview of the level of autophagy in the seedling. In seedlings without stress treatment, GFP fluorescence signal should be diffuse in the cytoplasm, and MDC weakly stains cell walls. Upon stress treatment, small spherical puncta form and move around rapidly in the cytoplasm, indicating autophagosome accumulation due to autophagy activation (Fig. 1). If Concanamycin A is used, the majority of GFP fluorescence will be associated with autophagic bodies inside the vacuole (*see Note 13*).
6. For quantification, epifluorescence microscopy is most convenient for imaging large numbers of autophagosomes. Take 2–3 representative images for each seedling at different places of the elongation zone of the root, with at least ten images for all seedlings of a certain genotype or treatment. The corresponding bright field images are used as a reference. A differential interference contrast (DIC) filter may also be used for a bright field reference as it provides better contrast. Save and export the images including the scale bar using ZEN or other appropriate software for future quantification and statistical analysis.

For higher quality images, take representative images of autophagosomes in each seedling using confocal microscopy, again with bright field images as reference. Save and export the fluorescence images, bright field images, and merged images

for qualitative presentation of autophagy in a certain genotype or treatment (*see* **Note 14**).

7. For quantitative analysis of autophagy, count the number of autophagosomes in each frame and calculate the average number of autophagosomes per frame for all images for each genotype or treatment. The average number of autophagosomes in each image indicates the level of autophagy. At a minimum, calculate the standard deviation to indicate the variation for each data set, and determine the statistical significance of any differences seen using a Student's *t*-test or other appropriate analysis. Statistical analysis can be performed using EXCEL (Microsoft Corporation, Redmond, WA, USA), SAS (SAS Institute Inc. Cary, NC, USA), JMP (JMP Group Inc. San Francisco, CA, USA), or other similar software.

4 Notes

1. For sucrose starvation induction of autophagy, prepare standard half-strength MS medium, but do not add sucrose. For liquid medium, prepare as for solid medium but without addition of Phytoblend agar. Store liquid medium at room temperature after autoclaving. Addition of other chemicals to the medium should be performed when the medium has cooled to 45–50 °C (when the bottle can be held with hands). Chemicals should be dissolved into solution and sterilized by autoclaving or using a 0.22 µm syringe filter before addition to the medium.
2. MDC is difficult to dissolve into a 20× stock solution and precipitation may occur in the stock solution. Use pipet tips to grind the MDC powder in PBS buffer, and aliquot to 1 mL or 500 µL with regular vortexing to assure equal distribution. Since MDC is light-sensitive, fast preparation and dark storage is necessary to maintain its activity.
3. Any microscope with fluorescence capability and attached camera can be used for basic detection and imaging of autophagosomes.
4. Any confocal microscopy system with fluorescence capability and attached camera can be used for detection and imaging of autophagosomes.
5. The ZEN 2012 software is used for image analysis and export of the original images taken from the Zeiss Axio Imager.A2 upright microscope. The software package used will depend on the microscope being used.
6. The Leica Application Suite software is used for image analysis and export from the Leica Sp5×MP confocal/multiphoton

microscope. The software package used will depend on the microscope being used.

7. Besides abiotic stresses as discussed here, autophagy can also be induced by biotic stresses including pathogen infection, and is activated during leaf senescence.
8. When immersing seedlings in liquid medium, 2 mL medium is typically sufficient for 5–10 7-day-old seedlings. For treatment of more seedlings, increase the volume accordingly to allow all seedlings to be fully immersed in liquid. The speed of shaking should be around 50–100 rpm to avoid root damage caused by excessive agitation.
9. Detection of autophagy by microscopy should begin immediately after sample preparation and staining. In the interval between staining and microscopy, avoid exposure of the seedlings to high temperatures, as heat stress may induce autophagy.
10. Fluorescent proteins other than GFP can also be used for detection of autophagosomes by fusion with ATG8. Fluorescent protein fusions should be designed with the fluorescent protein at the N-terminus of ATG8, as ATG8 lipidation occurs at the C-terminus during autophagosome formation. The fusion protein can be expressed either transiently or in transgenic plants. In this protocol, only detection of autophagosomes in transgenic plants is discussed.
11. The GFP-ATG8e transgenic plants and constructs are as described in Contento et al. [13] and can be obtained from the Arabidopsis Biological Resource Center (stock # CS66943). GFP-ATG8a transgenic Arabidopsis lines have also been commonly used to detect autophagosomes (ABRC stock # CS39996) [16]. There are nine isoforms of ATG8 in *Arabidopsis thaliana*, all of which are thought to associate with the autophagosome membrane. The detection of autophagosomes can therefore be performed by fusion of GFP with any isoform of ATG8.
12. The addition of Concanamycin A is optional, for the purpose of increasing the number of autophagosomes to be visualized, as it inhibits the degradation of autophagic bodies in the vacuole by raising the vacuolar pH. However, this precludes its use with acidotropic dye staining methods such as MDC staining. Be careful and use personal protective equipment when dealing with Concanamycin A or treated samples since Concanamycin A is a carcinogen.
13. Occasionally, small puncta are also visible in seedlings without stress treatment, suggesting the presence of a basal level of autophagy functioning as a housekeeping mechanism.

14. Representative images should be taken randomly in different regions of the elongation zone with similar exposure times. The exposure time, cell layer, and region of the root should be kept constant between samples to ensure validity of the results.

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