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bio-protocol www.bio-protocol.org/e2880 Vol 8, Iss 12, Jun 20, 2018 DOI:10.21769/BioProtoc.2880

Analysis of Autophagic Activity Using ATG8 Lipidation Assay in *Arabidopsis thaliana*

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[Abstract] As a fundamental metabolic pathway to degrade and recycle cellular cargos, autophagy is highly induced upon stress, starvation and senescence conditions in plants. A double-membrane structure named autophagosome will form during this process for cargo sequestration and delivery into the vacuole.

A number of regulators have been characterized in plants, including the autophagy-related (ATG) proteins and other plant-specific proteins. Among them, ATG8 will undergo a lipidation process to become a membrane-bound ATG8-phosphatidylethanolamine form and mark the growing autophagosomal membrane as well as the completed autophagosome. Therefore, ATG8 has been regarded as a marker for autophagosomes; and biochemical detection of the membrane-associated form of ATG8 is used as one of the principal methods for measurement of autophagic activity. Here, we describe an ATG8 lipidation assay for detection of the ATG8-PE form using *Arabidopsis thaliana* seedlings.

Keywords: ATG8, ATG8-PE, Lipidation, Autophagy, Autophagosome

[Background] Autophagy is an essential metabolic process which mediates the bulk degradation of the damaged organelles and unwanted cellular contents. During autophagy, a double-membrane structure called autophagosome will form and deliver the cargos into the vacuole for degradation. The autophagy-related (ATG) proteins are required to regulate the autophagic activity (Liu and Bassham, 2012). Among them, two conjugation systems, including ATG8 conjugate and ATG5-ATG12 conjugate, are involved for autophagosome formation. Upon autophagic induction, the ATG5-ATG12 conjugate forms and functions as an E3-like enzyme to promote ATG8 lipidation for binding to the phosphatidylethanolamine (PE) on the autophagosome membrane (Ohsumi, 2001). Although ATG8-PE on the outer membrane will be recycled before the autophagosome fusion with the vacuole, ATG8-PE on inner membrane will traffic together with the cargo into the vacuole for degradation. Thus, the amount of ATG8-PE usually correlates with the number of punctate ATG8-positive structures as well as autophagic activity (Mizushima *et al.*, 2010). Particularly, due to the high hydrophobicity of ATG8-PE, ATG8-PE migrates faster than ATG8 in SDS-PAGE gel, though the actual molecular weight of ATG8-PE is larger than the unconjugated ATG8 (Mizushima and Yoshimori, 2007). Accordingly, the amount of ATG8-PE from cell membrane fraction (CM) can be detected by immunoblotting with ATG8 antibodies. For example, in *Arabidopsis atg5* mutant, the level of ATG8-PE is severely impaired upon autophagic induction, whereas no autophagosome structures labeled by ATG8 are formed (Chung *et al.*, 2010). Therefore, biochemical

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detection of the ATG8 lipidation can serve as a useful method to access the autophagic activity when combined with different treatments, which has been applied in our previous study as well as others related to plant autophagy (Chung *et al.*, 2010; Suttangkakul *et al.*, 2011; Li *et al.*, 2014; Zhuang *et al.*, 2017). Here, we describe the protocol for ATG8 lipidation detection by ultracentrifuge separation of the membrane and cytosol fractions using acibenzolar-S-methyl (BTH)-treated seedlings (Zhuang *et al.*, 2017).

Materials and Reagents

1. 10 µl pipette tips (Thermo Fisher Scientific, catalog number: 3510)
2. 200 µl pipette tips (Wolf Laboratories, catalog number: 2100.YN)
3. 1,000 µl pipette tips (Thermo Fisher Scientific, catalog number: 3580)
4. 1.5 ml microcentrifuge tubes (Corning, Axygen®, catalog number: MCT-150-C)
5. PVDF membrane
6. X-ray film (Advansta, catalog number: L-07014-100)
7. 5-day-old seedlings
8. MS salt (Caisson, catalog number: MSP01-50LT)
9. UltraPure™ Sucrose (Thermo Fisher Scientific, Invitrogen™, catalog number: 15503022)
10. BTH (Acibenzolar-S-methyl) (Sigma-Aldrich, catalog number: 32820)
11. Methanol (VWR, BDH, catalog number: 10158)
12. Liquid nitrogen
13. PIC (Protease Inhibitor Mixture) (Roche Diagnostics, catalog number: 11873580001)
14. Tris Base (Caisson, catalog number: T041-1KG)
15. NaCl (Alfa Aesar, USB, catalog number: J21618)
16. EDTA (Alfa Aesar, USB, catalog number: J15701)
17. SDS (Sodium dodecyl sulfate) (Alfa Aesar, USB, catalog number: J75819)
18. Triton X-100 (GE Healthcare, Amerrsham, catalog number: 17-1315-01)
19. Glycerol ultrapure (Alfa Aesar, USB, catalog number: J16374)
20. Bromophenol Blue (Sigma-Aldrich, catalog number: B5525)
21. β-mercaptoethanol (Sigma-Aldrich, catalog number: M3148)
22. Urea (Alfa Aesar, USB, catalog number: J75826)
23. 40% Acrylamide/Bis Solution (Bio-Rad Laboratories, catalog number: 161-0148)
24. TEMED (Bio-Rad Laboratories, catalog number: 161-0801)
25. APS (Ammonium persulfate) (USB, catalog number: US76322)
26. Non-fat milk powder
27. ATG8 antibody (Agrisera, catalog number: AS14 2769)
28. cFBPase (Agrisera, catalog number: AS04 043)
29. Secondary antibody (Anti-rabbit IgG peroxidase conjugate, Sigma-Aldrich, catalog number: A6154)

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30. Precision Plus Protein™ Dual Color Standards (Bio-Rad Laboratories, catalog number: 161-0374)
31. Sodium hydrogen carbonate ($NaHCO_3$) (VWR, catalog number: 144-55-8)
32. Sodium carbonate (Na_2CO_3) (USB, catalog number: 21602)
33. Sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$) (USB, catalog number: 20233)
34. Sodium phosphate dibasic dihydrate ($Na_2HPO_4 \cdot 2H_2O$) (Sigma-Aldrich, catalog number: 04272)
35. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: 31248)
36. Tween-20 (Sigma-Aldrich, catalog number: 63158)
37. MS liquid medium (see Recipes)
38. 10 mM BTH stock (see Recipes)
39. 25x PIC (see Recipes)
40. 10% (v/v) Triton X-100 (see Recipes)
41. 1 M Tris-HCl stock (pH 6.8 or pH 7.4) (see Recipes)
42. 0.5 M EDTA (pH 8.0) (see Recipes)
43. 5x extraction buffer (see Recipes)
44. 1x extraction buffer containing 1x PIC and 1% (v/v) Triton X-100 (see Recipes)
45. 5x sample loading dye (see Recipes)
46. 30% APS (see Recipes)
47. 3x separation buffer (see Recipes)
48. 5x stacking buffer (see Recipes)
49. 15% SDS-PAGE gel with 6 M urea (see Recipes)
    a. 15% Urea separating gel
    b. 5% Stacking gel
50. Running buffer (see Recipes)
51. Transfer buffer (see Recipes)
52. PBS (see Recipes)
53. PBS-T (see Recipes)

Equipment

1. Eppendorf Research® plus Pipette 0.5-10 µl (Eppendorf, catalog number: 3120000020)
2. Eppendorf Research® plus Pipette 10-100 µl (Eppendorf, catalog number: 3120000046)
3. Eppendorf Research® plus Pipette 100-1,000 µl (Eppendorf, catalog number: 3120000062)
4. Mortar and pestle
5. Centrifuge (Eppendorf, model: 5430)
6. X-ray film cassette (Amersham Biosciences Hypercassette™)
7. Ultracentrifuge tube (7 x 20 mm)
8. Ultracentrifuge (Beckman Coulter, model: Optima™ MAX-XP)
9. Western Blotting apparatus (Bio-Rad)

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10. Developer machine (Fujifilm FPM100A)

Procedure

1. Incubate 0.2 g 5-day *Arabidopsis thaliana* seedlings in MS liquid medium with and without 100 μM BTH (10 mM stock in methanol) for 8 h (see Notes 1 and 2).
2. Freeze the seedlings in the mortar using liquid nitrogen and grind the plants thoroughly.
3. Add 2x extraction buffer containing PIC without detergent into the mortar on ice.
4. After defrost slowly on ice, transfer the liquid to a 1.5 ml tube and centrifuge at 1,000 *x g* for 5 min, 4 °C.
5. Transfer the supernatant to an ultracentrifuge tube (7 x 20 mm) and centrifuge at 100,000 *x g* for 45 min, 4 °C.
6. Transfer the supernatant (Cell Soluble fraction, CS) into a labeled new Eppendorf tube and keep on ice.
7. Wash the pallet (Cell Membrane fraction, CM) slightly with the 1x extraction buffer containing 1x PIC for 2-3 times.
   Note: In this step, add the 1x extraction buffer slightly without touching the surface of the pallet, and the centrifuge and re-suspend are not required. The main purpose of this step is washing out the remaining CS liquid on the CM surface.
8. Resuspend the pallet using 1x extraction buffer containing 1x PIC and 1% (v/v) Triton X-100 to solubilize the membranes.
9. Add the 5x sample loading dye to both of the CS and CM samples.
10. Boil the samples at 100 °C for 10 min.
11. Prepare the 15% SDS-PAGE gel with 6 M urea.
    Note: Only add the urea in the separating gel.
12. Load the CM samples and run at 90 V for 3 h with 1x running buffer. Run for additional 30 min at 90 V after the blue band reaches the bottom of the gel (see Notes 3 and 4).
13. Transfer the proteins from the gel to PVDF membrane at 55 V for 2.5 h with 1x transfer buffer.
14. After blotting, soak the blotted membrane in 1x PBS with 5% milk powder for 1 h. Wash with PBS-T for 3 times.
15. Incubate with ATG8 specific primary antibody (4 μg/μl in PBS-T) for 1 h. Wash with PBST for 3 times.
16. Incubate with the secondary antibody (1:5,000 in PBS-T) for 1 h. Wash with PBS-T for 3 times.
17. Add Western blotting detecting reagents onto the membrane, and expose it to the X-ray film or use an imaging machine. (Figure1)

Data analysis

As shown in Figure 1, in the wild-type (WT) background, the ATG8-PE from the CM sample

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increases after BTH induction, with a size about 12-15 kDa, indicating that autophagy is induced with the formation of autophagosomes. However, ATG8-PE is not detected in the ATG5 deficient mutant after autophagic induction, implying that autophagosome formation is inhibited. Differently, a higher level of ATG8-PE was detected in *atg9* mutant, suggesting that autophagosome formation might be interrupted at a certain stage. There might be non-specific bands in the ATG8 antibody detection. Also, it should be pointed out that there are multiple isoforms of ATG8 in the *Arabidopsis* genome with different SDS-PAGE mobilities, resulting the detection of cross-reacting species with similar size to the ATG8-PE adducts and making the results contradictory (Chung *et al.*, 2010). Therefore, it is critical to include both the WT and *atg5* samples as the positive and negative controls respectively to identify the correct size of ATG8-PE, as *atg5* mutant lacks ATG8-PE but accumulates a large amount of non-lipidated ATG8. Our further examinations under confocal and electron microscopy identified that abnormal tubules labeled by ATG8 accumulated in the *atg9* mutant, thus demonstrating that ATG9 is required for autophagosome progression (Zhuang *et al.*, 2017). Therefore, the ATG8 lipidation assay should combine with other approaches such as microscopy analysis to further assess autophagic activity.

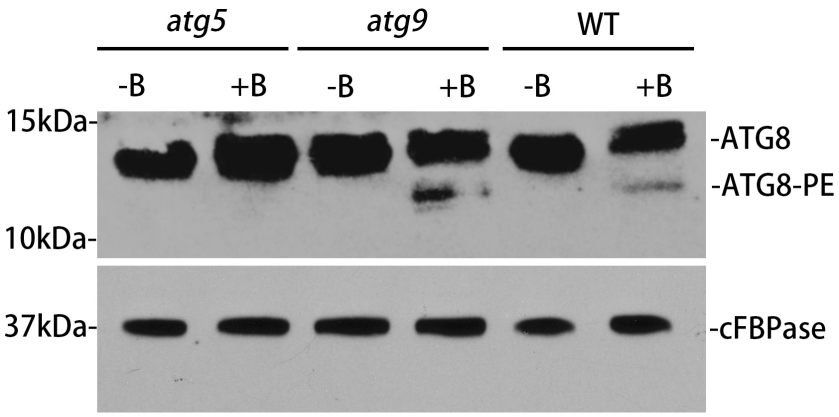

Figure 1. ATG8 lipidation detection in *Arabidopsis thaliana* wild-type and *atg* mutants. The 5-day-old wild-type, *atg5* and *atg9* seedlings were incubated in the MS liquid medium with or without BTH treatment (+B and -B) for 8 hours respectively. Crude extracts were subjected to ultracentrifuge to collect the cell membrane fraction (CM), followed by immunoblotting with plant ATG8 antibody. cFBPase represents the loading control with immunoblotting cFBPase antibodies.

Notes

1. It is critical to make sure that the seedlings are in good condition without any stress before the autophagic induction, otherwise stressed seedlings will have induced autophagic activity, which may obscure the results.
2. It is recommended to include known *atg* mutants defective in ATG8 lipidation (*e.g.*, *atg5* or *atg7*) as negative controls in the lipidation assay.
3. When running the SDS-PAGE, the urea gel will generate a lot of heat and it should keep at a

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low voltage, so that the two forms of ATG8 can be well separated.

4. ATG8-PE only occurs on the membrane fraction, therefore the CM samples are used for detecting the ATG8-PE adducts. The CS fraction with non-lipidated ATG8 could be included in the western blot as a control to distinguish the non-lipidated ATG8 and lipidated ATG8.

Recipes

1. MS liquid medium (350 ml)

Components	Volume/Quantity
MS salt	1.515 g
Sucrose	3.5 g
ddH_2O	up to 350 ml

Adjust the pH to 5.7 (with KOH)

2. 10 mM BTH stock (10 ml)

Components	Volume/Quantity
BTH	0.021 g
Methanol	10 ml

3. 25x PIC (2 ml)

Components	Volume/Quantity
PIC	one tablet
ddH_2O	2 ml

4. 10% (v/v) Triton X-100 (100ml)

Components	Volume/Quantity
Triton X-100	10 ml
ddH_2O	90 ml

5. 1 M Tris-HCl stock (pH 6.8 or pH 7.4) (100 ml)

Components	Volume/Quantity
Tris Base	12.114 g
ddH_2O	to 100 ml

Adjust pH to 6.8 or 7.4 with conc. HCl

6. 0.5 M EDTA (pH 8.0) (100 ml)

Components	Volume/Quantity
EDTA	18.61 g
ddH_2O	80 ml

Adjust the pH to 8.0 with NaOH (~10 g of NaOH pellets) and add to 100 ml with ddH_2O

7. 5x extraction buffer (10ml)

Components	Volume/Quantity
250 mM Tris-HCl (pH 7.4)	2.5 ml of 1 M Tris-HCl (pH 7.4)
750 mM NaCl	0.438 g

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5 mM EDTA	0.1 ml of 0.5 M stock
ddH_2O	to 10 ml

8. 1x extraction buffer containing 1x PIC and 1% (v/v) Triton X-100 (10 ml)

Components	Volume/Quantity
1x extraction buffer	2.5 ml of 5x extraction buffer
1x PIC	0.40 ml of 25x PIC
1% (v/v) Triton X-100	1 ml of 10% (v/v) Triton X-100
ddH_2O	to 10 ml

9. Sample loading dye (5x, 50 ml)

Components	Volume/Quantity
1 M Tris-HCl (pH 6.8)	12.5 ml
SDS	5 g
Glycerol	25 ml
Bromophenol Blue	0.25 g
β-mercaptoethanol	6.25 ml
ddH_2O	to 50 ml

10. 30% APS (10 ml)

Components	Volume/Quantity
APS	3 g
ddH_2O	10 ml

11. 3x separation buffer (1 L)

Components	Volume/Quantity
Tris base	136.2 g
SDS	3 g
ddH_2O	up to 1 L

Adjust the pH to 8.8 with conc. HCl (~10 ml)

12. 5x stacking buffer (500 ml)

Components	Volume/Quantity
Tris base	37.85 g
SDS	2.5 g
ddH_2O	up to 500 ml

Adjust the pH to 6.8 with conc. HCl (~20 ml)

13. 15% SDS-PAGE gel with 6 M urea
    a. 15% Urea separating gel (10 ml)

Components	Volume/Quantity
Urea	3.6036 g
Acry-bis (40%)	3.75 ml
3x separation buffer	3.33 ml
APS (30%)	20 µl

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TEMED	5 µl
ddH_2O	up to 10 ml

b. 5% Stacking gel (5 ml)

Components	Volume/Quantity
Acry-bis (40%)	0.625 ml
5x stacking buffer	1 ml
APS (30%)	20 µl
TEMED	5 µl
ddH_2O	up to 5 ml

14. Running buffer (10x, 1 L)

Components	Volume/Quantity
Tris Base	30.3 g
Glycine	144 g
SDS	10 g
ddH_2O	up to 1 L

15. Transfer buffer (10x, 1 L)

Components	Volume/Quantity
$NaHCO_3$	8.4 g
Na_2CO_3	3.2 g
ddH_2O	up to 1 L

16. PBS (10x, 2 L)

Components	Volume/Quantity
NaCl	80 g
$NaH_2PO_4 \cdot H_2O$	2.3 g
$Na_2HPO_4 \cdot 2H_2O$	13.9 g
KCl	2 g
ddH_2O	up to 2 L

Adjust the pH to 7.4 with 10 N NaOH

17. PBS-T (1 L)

Components	Volume
1x PBS	1 L
Tween-20	0.5 ml

Acknowledgments

This protocol was adapted from Zhuang *et al*., 2017. Fundings from the Research Grants Council of Hong Kong (G-CUHK402/15, G-CUHK403/17, CUHK14130716, CUHK14102417, C4011-14R, C4012-16E, C4002-17G and AoE/M-05/12) and the National Natural Science Foundation of China (31470294 and 31670179) support this work.

bio-protocol www.bio-protocol.org/e2880 Vol 8, Iss 12, Jun 20, 2018 DOI:10.21769/BioProtoc.2880

Competing interests

The authors declare no conflicts of interests.

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