Using *Arabidopsis* Mesophyll Protoplasts to Study Unfolded Protein Response Signaling

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[Abstract] Various environmental stresses or artificial reagents can trigger unfolded protein accumulation in the endoplasmic reticulum (ER) due to the folding capacity of the ER being exceeded. This is termed ER stress, and triggers the unfolded protein response (UPR). Assays for activation of the UPR in plants include Tunicamycin (Tm)- or dithiothreitol (DTT)-mediated root growth inhibition, analysis of splicing of the UPR-responsive transcription factor bZIP60 (basic Leucine Zipper Domain 60), and upregulation of relevant UPR genes. We provide here a quick and robust method to detect UPR signaling in *Arabidopsis thaliana* protoplasts. This assay can also be applied to other plant species for which protoplasts can be isolated.

Keywords: *Arabidopsis*, bZIP60 splicing, DTT, ER stress, Protoplasts, Tm, UPR

[Background] Substantial progress has been made over the last two decades in our understanding of the UPR in plants. In addition to its important role in stress responses (Howell, 2013; Bao *et al.*, 2017; Bao and Howell, 2017), the UPR also participates in plant reproductive development, vegetative growth and root development (Deng *et al.*, 2013; Deng *et al.*, 2016; Kim *et al.*, 2018). There are two arms to the UPR in plants that employ three transcription factors: bZIP60, which is activated by IRE1 (Inositol Requiring 1) -mediated splicing of its mRNA (Deng *et al.*, 2011), and bZIP17 and bZIP28, which are activated by S1P- and S2P-mediated cleavage of their membrane-localized proteins (Liu *et al.*, 2007; Srivastava *et al.*, 2013). All three transcription factors induce expression of downstream genes, including molecular chaperones such as BIP3 that assist in protein folding.

Protoplasts can be easily isolated from various plant species, allowing transient expression in protoplasts of potential factors involved in UPR signaling, in place of the time-consuming generation of transgenic plants. In our recent paper on ER stress-regulated autophagy mediated by IRE1b, we successfully detected bZIP60 splicing and BIP3 expression in *Arabidopsis* protoplasts (Bao *et al.*, 2018) as hallmarks of the UPR (Iwata and Koizumi, 2005; Iwata *et al.*, 2008; Deng *et al.*, 2011), and used this assay to determine the effect of expression of IRE1b mutants. Here, we describe this assay in detail; we believe the robustness and reproducibility of this assay will facilitate plant UPR research.

Materials and Reagents

1. Aluminum Foil (Reynolds Kitchens, REYNOLDS WRAP, catalog number: 353224)
2. 6-well Plate (Corning, Falcon, catalog number: 353224)
3. 12-well Plate (Corning, Falcon, catalog number: 353225)
4. 100 x 15 mm Square Petri Dish (Corning, Falcon, catalog number: 351112)
5. 14 ml Polypropylene Round-Bottom Tube (Corning, Falcon, catalog number: 352059)
6. Electrical tape (ULINE, Hyper Tough, catalog number: 34366)
7. Rosette leaves of wild-type *Arabidopsis thaliana* (WT, Columbia-0 ecotype)
8. *ire1a ire1b* mutant *Arabidopsis* plants
9. IRE1b plasmids
10. *bZIP60s*, *BIP3* and *Actin2* specific primers
 Primers for detecting bZIP60s (spliced form of bZIP60: bZIP60F4 and bZIP60SB2), BIP3 and Actin2:
 bZIP60F4: GAAGGAGACGATGATGCTGTGGCT
 bZIP60SB2: AGCAGGGAACCCAACAGCAGACT
 BIP3F: TTCGACCCGAAACGTCTGATTGGA
 BIP3R: GCTTGCCTCTGCGCATCATTGAAA
 Actin2F: GGAAGGATCTGTACGGTAAC
 Actin2R: GGACCTGCCTCATCATACT
11. Maxiprep Kit (Gene Elute HP Plasmid) (Sigma-Aldrich, catalog number: NA310-1KT)
12. RNA Extraction Kit (DNeasy Plant Mini Kit) (QIAGEN, catalog number: 69104)
13. cDNA kit (iScript™ cDNA Synthesis Kit) (Bio-Rad, catalog number: 1708890)
14. Tunicamycin (Tm) (Sigma-Aldrich, catalog number: T7765)
15. Dithiothreitol (DTT) (Sigma-Aldrich, catalog number: 3483-12-3)
16. PEG4000 (PEG) (Sigma-Aldrich, catalog number: 25322-68-3)
17. Cellulose R-10 (Yakult, CELLULASE “ONOUKAR-10”)
18. Macerozyme R-10 (Yakult, MACEROZYME R-10)
19. BSA (Sigma-Aldrich, Bovine Serum Albumin, catalog number: 9048-46-8)
20. DMSO (Sigma-Aldrich, dimethyl sulfoxide, catalog number: 67-68-5)
21. Mannitol (Sigma-Aldrich, catalog number: 69-65-8)
22. β-mercaptoethanol (Sigma-Aldrich, catalog number: 60-24-2)
23. MgCl₂ (Sigma-Aldrich, Magnesium chloride, catalog number: 7791-18-6)
24. MES (Sigma-Aldrich, catalog number: 4432-31-9)
25. NaCl (Sigma-Aldrich, Sodium chloride, catalog number: 7647-14-5)
26. CaCl₂ (Sigma-Aldrich, Calcium chloride, catalog number: 10043-52-4)
27. KCl (Sigma-Aldrich, Potassium chloride, catalog number: 7447-40-7)
28. KOH (Sigma-Aldrich, Potassium hydroxide, catalog number: 1310-58-3)
29. 2x YT media (see Recipes)

30. Yeast Extract (BD Biosciences, BD Bacto, catalog number: 212750)
31. Tryptone (Neogen Food Safety, Acumedia, catalog number: 7351A)
32. Enzyme solution (see Recipes)
33. MMg buffer (see Recipes)
34. 40% PEG (see Recipes)
35. W5 solution (see Recipes)
36. Stock solutions (see Recipes)

Equipment

1. Pipette (Eppendorf, Eppendorf Research Plus, catalog number: 2231000222)
2. Incubator (New Brunswick Scientific, Innova42, catalog number: M1335-0000)
3. Hemocytometer (Hauserscientific, Bright-Line Counting Chambers, catalog number: 3100)
4. Microscope (Nikon, Eclipse TS100)
5. High Speed Centrifuge (Thermo Scientific, model: Sorvall™ RC 6 Plus, catalog number: 36-101-0816)
6. Low Speed Centrifuge (Thermo Scientific, model: Hereus™ Multifuge™ X3R, catalog number: 75004516)
7. Microcentrifuge (Thermo Scientific, model: Sorvall™ Legend™ Micro 17, catalog number: 75002403)
8. NanoDrop Spectrophotometer (Scientific Industries, model: Vortex-Genie 2, catalog number: SI-0236)
9. Thermocycler (S1000 Thermal Cycler) (Bio-Rad, catalog number: 1852196)
10. DNA Gel electrophoresis cell (Bio-Rad, Sub Cell GT Cell, catalog number: 1640302, or similar)
11. DNA Gel scanner (FOTODYNE, FOTO/Analyst ImageTech, catalog number: 6-9030S2DL, or similar)

Software

1. ImageJ
2. Adobe Photoshop

Procedure

1. Prepare plasmids for expression of genes of interest (IRE1b is used as an example in this protocol). For each extraction, grow *E. coli* in 500 ml 2x YT media overnight and extract using a maxiprep kit (QIAGEN product) according to the manufacture's protocol. Set the DNA concentration to 1 µg/µl.

2. All buffers and chemicals should be prepared at least one day before protoplast transformation (Yoo *et al.*, 2007).
3. Make fresh enzyme solution with Cellulose R-10 and Macerozyme R-10 (Products of Yakult).
4. Prepare protoplasts from rosette leaves of wild-type (WT) and/or mutant *Arabidopsis* plants (Yoo *et al.*, 2007) (*ire1a ire1b* is used as an example in this protocol) using the tape method as described by Wu *et al.* (2009) (Figures 1A-1C).
5. Load 10 μ l of cell suspension in a hemocytometer and count cells under a light microscope; resuspend protoplasts in MMg buffer to a density of 2.5×10^6 /ml.
6. For each reaction, in a 2 ml round bottom tube, mix 400 μ l protoplasts with 30 μ l IRE1b plasmid (1 μ g/ μ l), add an equal volume of 40% PEG (430 μ l, see Recipes for making 40% PEG), mix and incubate at room temperature for 10 min (Figure 1D). Spin down at 60 x g and wash twice with W5 to remove PEG.
7. Resuspend with 1 ml W5 and transfer the transformed protoplasts into a 6-well culture plate (or 12-well culture plate if more than 6 samples), add W5 to a final volume of 5 ml (Figure 1E), and incubate overnight in the dark by covering with foil.
8. Carefully discard the supernatant by gentle pipetting (protoplasts will stay on the bottom due to gravity, no need to centrifuge), and re-suspend with W5 to a final volume of 3 ml.
9. Divide the 3 ml protoplasts equally into two 2 ml round bottom tubes; add Tm to 5 μ g/ml or DTT to 2 mM, mix the protoplasts by gentle inversion, and incubate for 6 h, mixing every 2 h.
10. Spin at 60 x g for 1 min in a low speed centrifuge, discard the supernatant.
11. Add RNA extraction buffer (QIAGEN, Plant Mini RNA kit) and vortex thoroughly for 30 s. Extract RNA according to the manufacturer's protocol.
12. Test the concentration and quality of RNA using a NanoDrop Spectrophotometer; if desired, RNA can be stored at -80 °C for later use.
13. Use 0.5 μ g RNA to synthesize cDNA according to the manufacturer's protocol (Bio-Rad cDNA kit).
14. Detect the *bZIP60* spliced form (named *bZIP60s*) by RT-PCR using bZIP60F4 and bZIP60SB2 primers, and *BIP3* expression using BIP3F and BIP3 primers. *Actin2* (using Actin2F and Actin2R primers) is used as a loading control.
15. Run the PCR products on 1.5% agarose gel, stain with Ethidium Bromide and visualize using a gel scanner under UV light (Figure 1F). qRT-PCR can be used for quantification if needed, normalized against *Actin2*.

Figure 1. An overview of *Arabidopsis* protoplast isolation and transformation with the resulting RT-PCR. A-B. Taped rosette leaves before (A) and after (B) digestion with enzymes; C. Isolated protoplasts in W5 solution; D. Protoplasts during transformation; E. Transformed protoplasts after transfer to a 12-well plate; F. RT-PCR detection of *bZIP60s* and *BIP3* expression in protoplast samples treated with or without 2 mM DTT for 6 h. *IRE1b* was introduced into an *ire1a ire1b* double mutant background and showed restoration of *bZIP60* splicing and *BIP3* induction. *Actin2* was employed as an endogenous loading control.

Data analysis

Three independent transformations are required for each experiment, images are taken and quantified using ImageJ (Reference 15) if needed, and significant differences assessed by Student's *t*-test.

Notes

1. Use round bottom tubes (size: 2 ml and 14 ml) for all experiments.
2. To make sure the protoplast suspension is equally divided into two, pipette slowly and consistently.

3. The product of the spliced form of *bZIP60* (*bZIP60s*) in this assay is small; a 1.5% gel or higher is recommended.
4. If RNA quantities are low, combine two transformations for RNA extraction for each construct.
5. The use of an *ire1a ire1b* double mutant as a control is highly recommended, as splicing of *bZIP60* is completely blocked in this mutant background.
6. For isolation of protoplasts from rosette leaves, four-week-old healthy and well-expanded leaves from plants prior to flowering are preferable.
7. PEG/ Ca^{2+} solution is used to permeabilize the protoplast membrane to allow DNA uptake.
8. Place transformed protoplasts in the dark to minimize potential light stress.

Recipes

1. 2x YT media
 - 16 g/L Tryptone
 - 10 g/L yeast extract
 - 5 g/L NaCl
2. Enzyme solution
 - a. Make enzyme solution fresh with a final concentration of 0.4 M Mannitol, 20 mM KCl, 20 mM MES, 1.5% (w/v) cellulase R-10, 0.3% (w/v) macerozyme R-10
 - b. Heat the enzyme solution in a 55 °C water bath for 10 min, then cool to room temperature before adding 10 mM CaCl_2 , 5 mM β -mercaptoethanol and 0.1% BSA (w/v)
3. MMg buffer
 - 400 mM mannitol
 - 15 mM MgCl_2
 - 4 mM MES (Adjust pH to 5.7 with KOH)
4. 40% PEG
 - 40% PEG4000 (w/v)
 - 200 mM mannitol
 - 100 mM CaCl_2
5. W5 solution
 - 154 mM NaCl
 - 125 mM CaCl_2
 - 5 mM KCl
 - 2 mM MES
 - Adjust pH to 5.7 with KOH
6. Stock solutions
 - a. Make 10 mg/ml Tm (in DMSO) and 1 M DTT (in ddH₂O) stock solutions and store at -20 °C. Do NOT refreeze DTT more than 3 times

- b. Make 1 M mannitol, 0.3 M MgCl₂, 1 M CaCl₂, 0.5 M MES (Adjust pH to 5.7 with KOH), 1 M NaCl, 1 M KCl stock solutions and keep at room temperature

Acknowledgments

This work was supported by the National Science Foundation under Grant IOS 1353867 to DCB.

Competing interests

Authors claim no conflict of interest in this protocol.

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