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Analysis of Unfolded Protein Response in Arabidopsis

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Abstract

The unfolded protein response (UPR) is fundamental for development and adaption in eukaryotic cells. *Arabidopsis* has become one of the best model systems to uncover conserved mechanisms of the UPR in multicellular eukaryotes as well as organism-specific regulation of the UPR in plants. Monitoring the UPR *in planta* is an elemental approach to identifying regulatory components and to revealing molecular mechanisms of the plant UPR. In this chapter, we provide protocols for the induction and analyses of plant UPR at a molecular level in *Arabidopsis*. Three kinds of ER stress treatment methods and quantitation of the plant UPR activation are described here.

Keywords

Unfolded protein response; UPR; Endoplasmic reticulum stress; Tunicamycin; Protein folding; *Arabidopsis*

1 Introduction

The unfolded protein response (UPR) is a collection of signaling pathways aiming to maintain endoplasmic reticulum (ER) protein folding homeostasis in eukaryotic cells [1, 2]. There is approximately one-third of total protein folded and modified in the ER. Environmental or physiological factors that cause an imbalance between demand and capability of ER protein folding lead to ER stress. To relieve the ER stress, increase in the ER protein folding ability is one of the most instant and central regulation in the UPR. Diverse stimuli from exogenous or endogenous signals trigger the activation of the UPR. To experimentally examine the UPR, chemicals disturbing the ER protein folding homeostasis are applied to induce the UPR. One of the most frequently used UPR inducers is a glycosylation inhibitor, tunicamycin (Tm). The majority of secretory proteins are glycosylated in the ER as the glycosylation is crucial for protein structure formation and for protein targeting to cellular compartments. As Tm blocks the first step of N-linked glycosylation, it can efficiently lead to accumulation of unfolded protein in the ER lumen and therefore activate the UPR [3 – 6].

To observe the plant UPR at different growth stages, we describe three experimental approaches to perform ER stress treatment. To investigate long-term ER stress tolerance, seeds are directly germinated on medium containing a relatively low concentration of Tm. Tm can also been infiltrated into leaves to monitor the UPR specifically on ground tissues.

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Finally, to examine the early outputs of the plant UPR, a short-term Tm treatment using a liquid method is conducted to observe a more instant and direct response to ER stress.

To cope with dynamic ER protein folding demands, the UPR adjusts the transcription of genes function in assembling protein structure, degrading mis-folded protein, and determining cell fates [7, 8]. Hence, the upregulation of well-established UPR target genes, such as *BiP3* in Arabidopsis [9], is considered a molecular indicator of UPR activation. To introduce the quantitative method of reading UPR outputs, real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis of UPR target genes induction is included in this chapter.

2 Materials

1. Basic reagents and equipment for plant sterile tissue culture and RNA work handling.
2. Plant growth medium: Linsmaier and Skoog (LS) with Buffer and Sucrose (Caisson LSP04); Phytagel (Sigma P8169).
3. Growth chamber: temperature set to 21 °C, 16 h light/8 h dark cycle, 100 mEinstein/m^2 s, and 65 % humidity.
4. Tunicamycin (Sigma T7765).
5. Dimethyl sulfoxide (DMSO) solvent.
6. 1 ml needleless syringes.
7. Liquid nitrogen.
8. RNeasy plant mini kit (Qiagen 74904).
9. RNase-Free DNase Set (Qiagen 79254).
10. SuperScript® VILO™ Master Mix (Invitrogen 11755500).
11. Reagents for qRT-PCR: MicroAmp® Fast optical 96-well reaction plate (ABI 4346936); optical adhesive cover (ABI 4311971); FAST SYBR Master Mix (ABI 4385612).

3 Methods

3.1 Germination Under the ER Stress

To examine the tolerant ability of plants in coping with different intensities of ER stress, seeds are directly germinated on medium containing Tm concentrations ranging from 10 to 50 ng/ml. Comparison of phenotype between wild-type plants and mutants of interest can reveal whether the mutants display over-sensitive or resistant growth phenotype under ER stress conditions. The Tm infiltration assay enables the observation of the plant UPR using adult plants.

1. Sterilize seeds and store at 4 °C for 2 days (*see* Note 1).
2. Prepare ½ LS with 0.4 % Phytagel medium.

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3. Autoclave the ½ LS medium on liquid cycle program for 25–40 min.

4. Dissolve Tm powder in DMSO to prepare 10 mg/ml Tm stock solution (*see* Note 2).

5. Prepare 10, 20, 30, 40, and 50 μg/ml Tm stock solutions by 1,000, 500, 333, 250, and 200× dilution of 10 mg/ml Tm stock solution respectively using ½ LS liquid medium.

6. Cool the autoclaved ½ LS medium to 50 °C.

7. Add 10, 20, 30, 40, and 50 μg/ml Tm stock solutions respectively to cooled ½ LS medium (50 °C) by 1,000× dilution to make ½ LS medium containing 10, 20, 30, 40, and 50 ng/ml Tm (see Note 3).

8. Swirl to mix and use a pipette to pour equal amount of Tm-containing medium per plate in the sterile tissue culture hood. Prepare Tm-containing medium freshly right before the Tm germination assay (*see* Note 4).

9. For Mock control, the same preparation procedure is carried out with the exception of replacing the Tm in the ½ LS medium with 0.0005 % DMSO.

10. Germinate *Arabidopsis* seeds on ½ LS medium containing 0.0005 % DMSO, 10, 20, 30, 40, and 50 ng/ml Tm. Place a single seed on the medium in an equally spaced manner (*see* Note 5). Perform the germination at least in triplicate with minimal three individual plates for each Tm concentration and mock control.

11. Grow the plants under these conditions: 21 °C, 16 h light/8 h dark cycle, 100 mEinstein/m^2 s, and 65 % humidity.

12. Observe the growth phenotype 7–14 days after germination (*see* Note 6).

13. Using Col-0 ecotype wild-type Arabidopsis, the plants shows more pronounced growth defects starting from 30 ng/ml Tm (*see* Fig. 1).

3.2 Tm Infiltration into Leaf Tissues

1. Dissolve Tm powder in DMSO to prepare 10 mg/ml Tm stock solution (*see* Note 2).

2. Prepare 15 μg/ml Tm working solution by 666× dilution of 10 mg/ml Tm stock solution using ½ LS liquid medium. Prepare Tm-containing medium freshly right before the Tm infiltration assay (*see* Note 4).

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[1]The quality of seed stock is extremely important for ER stress related assays. Using seeds freshly harvested from healthy plants is one of key points to get dependable and consistent results.

[2]Aliquot Tm stock solution (10 mg/ml) into relatively small amount and store in a −20 °C freezer. Avoid freezing and thawing.

[3]High temperature destabilizes Tm.

[4]Tm-containing medium is unstable if it is not freshly prepared.

[5]For a fair comparison, the distance between seeds should be consistent. Square petri dish with Grid (Fisher 08-757-11A) is useful as a single seed can be placed on the center of each small square area on the plates (*see* Fig. 1).

[6]The growth phenotype can be more obvious at a relatively early stage (within 1 week) or vice versa. To detect stage-specific phenotypes, the growth of Tm-treated seedlings should be observed every day during the assay.

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3. Use a needleless syringe to infiltrate ½ LS liquid medium containing 15 μg/ml Tm into abaxial sides of 5-week-old rosette leaves (*see* Note 7).
4. For Mock control, the same treatment procedure is performed with the exception of replacing the Tm in the ½ LS liquid medium with 0.0015 % DMSO (*see* Note 8).
5. Observe the leaves phenotype 1–4 days after infiltration.

3.3 Short Period of ER Stress Treatment

While the ER stress tolerance assay can examine whether mutants of interest show a plant phenotype under ER stress, even if the mutants display a comparably visible plant phenotype to wild-type plants, it is possible that the defects of UPR in mutants of interest do not reflect on the plant growth morphology. For instance, a mutant of *AtbZIP60* shows compromised UPR activation phenotype at a molecular level but displays a similar tolerant plant phenotype when germinated under ER stress [10, 11]. To verify whether genes of interest are involved in the UPR, short-term ER stress treatment coupled with analyses of UPR target genes induction are performed to monitor the UPR at a molecular level.

1. Sterilize seeds and store at 4 °C for 2 days (*see* Note 1).
2. Germinate seeds in vertical plates for 10 days. Medium: ½ LS with 0.4 % Phytagel. Place ten seeds evenly spaced per small round plate (100 × 15 mm) or square plate. Seal the bottom part of plates with parafilm and the upper part of plates with 3M surgical tape (*see* Fig. 2 and Note 9).
3. Dissolve Tm powder in DMSO to prepare 10 mg/ml Tm stock solution (*see* Note 2).
4. Prepare 5 μg/ml Tm-containing medium by 2,000× dilution of 10 mg/ml Tm stock solution using ½ LS liquid medium. Prepare Tm-containing medium freshly right before the Tm treatment (*see* Note 2).
5. Gently transfer 10-day-old vertically grown seedlings to 5 μg/ml Tm-containing medium for an appropriate time period (*see* Notes 10 and [11]).
6. Collect 10–20 individual Tm-treated seedlings per biological replicate using liquid nitrogen (*see* Notes 12 and [13]).
7. For Mock control, the same treatment procedure is performed with the exception of replacing the Tm in the ½ LS liquid medium with 0.05 % DMSO.

[7]To fairly compare the ER tolerance between wild-type and mutant plants, choose the same stage, size, and condition of leaves for both varieties of plants.

[8]The infiltration process needs be performed carefully and should not lead to any damage of plants. Leaves infiltrated with DMSO (Mock control) should appear comparable to leaves without infiltration after 1 day.

[9]To allow proper ventilation, do not wrap plates completely with parafilm.

[10]Select well-grown and unstressed seedlings as well as similar growth morphology for all plants.

[11]Using 10-day-old seedlings coupled with a qRT-PCR system, the induction of UPR target genes can be detected starting from 0.5 h. Prolonged treatment is not recommended using this liquid system.

[12]Using more seedlings per biological sample can reduce the standard deviation of fold change of UPR target genes induction between biological replicates.

[13]Sample collection should be done carefully and timely to avoid additional stress before seedlings are frozen by liquid nitrogen.

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3.4 Quantitative Measurement of UPR Activation

The regulation of UPR target genes transcription is one of the major outputs of the plant UPR. Hence, measurement of UPR target genes induction under ER stress is a classical method to quantify the plant UPR activation.

1. Extract RNA from Tm-treated seedlings using an RNeasy plant mini kit and RNase-Free DNase Set.
2. Synthesize CDNA from RNA using a SuperScript® VILO™ Master Mix.
3. Perform qRT-PCR with SYBR Green detection in triplicate using the Applied Biosystems 7500 fast real-time PCR system. The primer sequence of UPR target genes is listed in Table 1 [11].
4. Analyze Data by the DDCT method.

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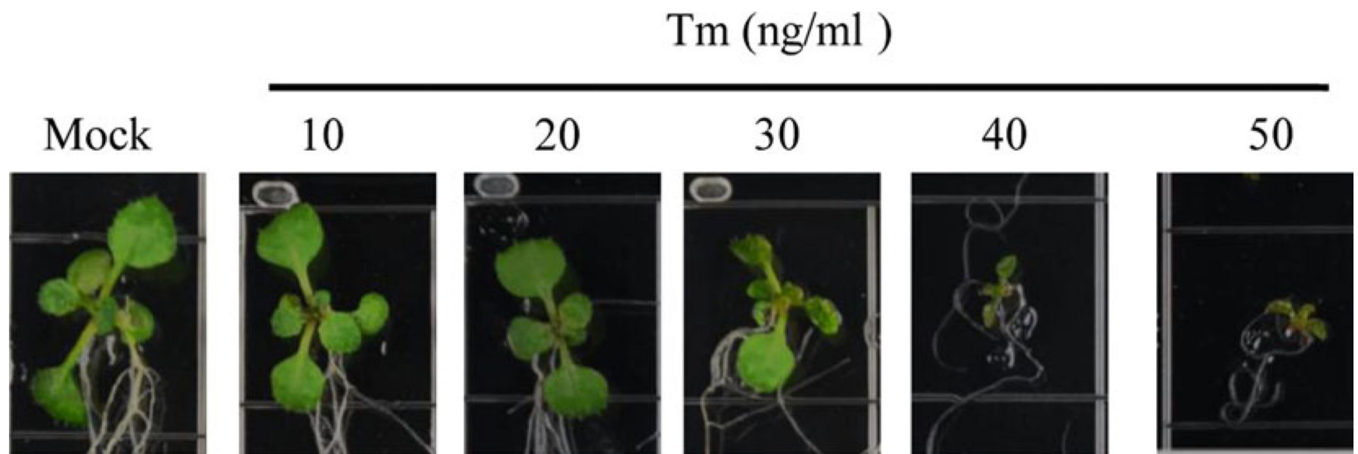

Fig. 1.

Wild-type Col-0 plants were germinated on ½ LS medium containing DMSO, 10, 20, 30, 40, or 50 ng/ml Tm for 2 weeks

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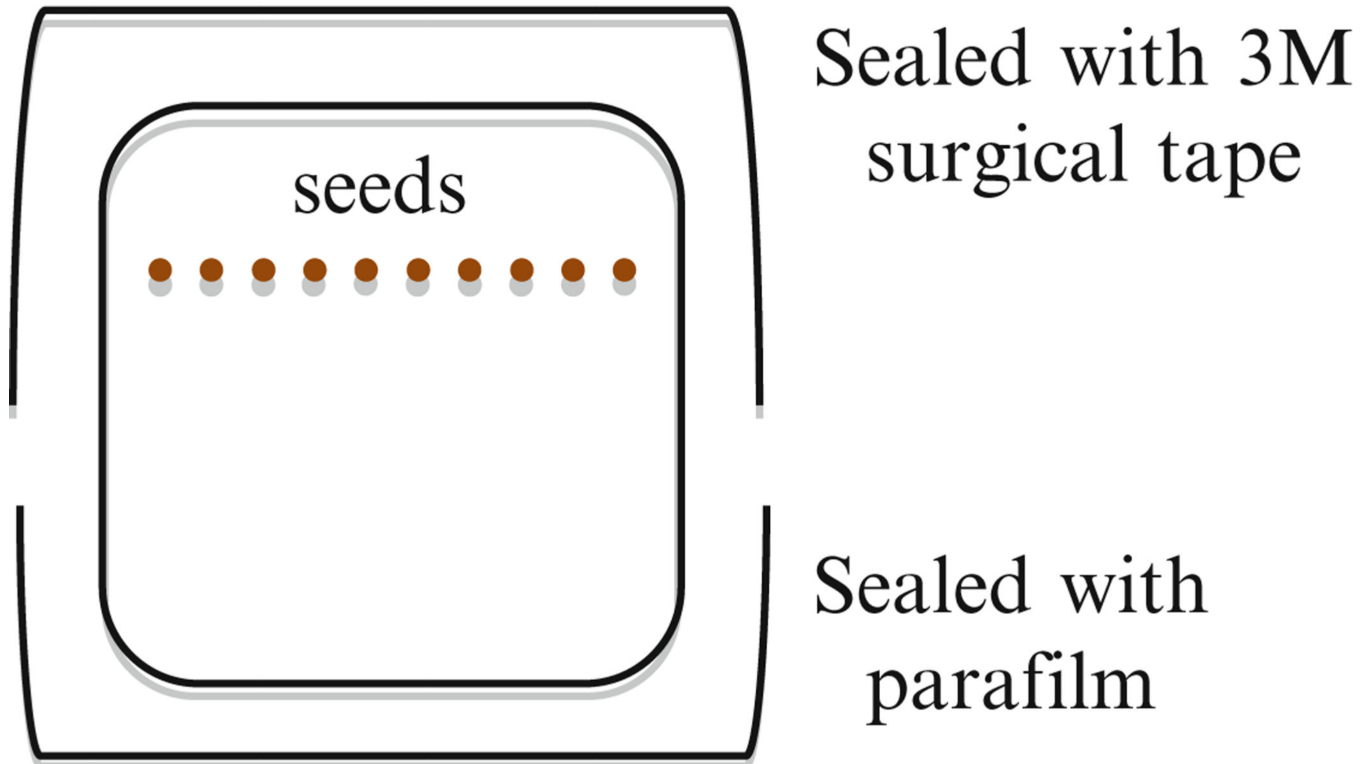

Fig. 2.
The 3M surgical tapes and parafi lm are used respectively to seal the upper and bottom part of vertical plates

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Table 1

Primers of UPR target genes for the Applied Biosystems 7500 fast real-time PCR system

Primers	Sequence (5#x02032;–3′)	Gene
BiP1/2-qP For	ccaccggccccaagag	AT5G28540/AT5G42020
BiP1/2-qP Rev	ggcgtccacttcgaatgtg	AT5G28540/AT5G42020
BiP3-qP For	aaccgcgagcttggaaaat	AT1G09080
BiP3-qP Rev	tcccctgggtgcaggaa	AT1G09080
AtERdj3A-qP For	tcaagtggtggtggtttcaact	AT3G0890
AtERdj3A-qP Rev	cccaccgcccatattttg	AT3G0890
AtERdj3B-qP For	gaggaggcggcatgaatatg	AT3G62600
AtERdj3B-qP Rev	ccatcgaacctccaccaaaa	AT3G62600
PDI6-qP For	cgaagtggctttgtcattcca	AT1G77510
PDI6-qP Rev	gcggttgcgtccaatttt	AT1G77510
PDI9-qP For	ggccctgttgaagtgactgaa	AT2G32920
PDI9-qP Rev	cagcagaaccacacttcttttcc	AT2G32920
CNX1-qP For	gtgtcctcgtcgccattgt	AT5G61790
CNX1-qP Rev	ttgccaccaaagataagcttga	AT5G61790
CRT1-qP For	gatcaagaaggaggtcccatgt	AT1G56340
CRT1-qP Rev	gacggaggacgaaggtgtaca	AT1G56340
AtERdj2A-qP For	tgggcttgtaggcgctctt	AT1G79940
AtERdj2A-qP Rev	aacccaatagttttcctccttgtg	AT1G79940
AtERdj2B-qP For	tgaaacgtcccaatggactca	AT4G21180
AtERdj2B-qP Rev	cctctttgtggaaaggaaagtaagg	AT4G21180
AtP58IPK-qP For	gcgttatagtgatgccctcgat	AT5G03160
AtP58IPK-qP Rev	gaaagcgcagggtctgctt	AT5G03160