Chapter 16

Transient Expression Assays for Quantifying Signaling Output

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Abstract

The protoplast transient expression system has become a powerful and popular tool for studying molecular mechanisms underlying various plant signal transduction pathways. *Arabidopsis* mesophyll protoplasts display intact and active physiological responses and are easy to isolate and transfect, which facilitate high-throughput screening and systematic and genome-wide characterization of gene functions. The system is suitable for most *Arabidopsis* accessions and mutant plants. Genetic complementation of mutant defective in sensor functions, gene expression, enzymatic activities, protein interactions, and protein trafficking can be easily designed and explored in cell-based assays. Here, we describe the detailed protocols for protoplast isolation, polyethylene glycol-calcium transfection, and different assays for quantifying the output of various signaling pathways.

Key words: Arabidopsis, Mesophyll protoplast, Transient expression, Signal transduction, Reporter assay, Genetic complementation, Genomics

1. Introduction

As sessile organisms, plants have evolved sophisticated mechanisms to regulate their growth and development as well as to adjust their physiological state for survival in response to environmental changes and challenges. Perception of the environmental cues and subsequent signal transduction lead to the transcriptional and physiological reprogramming that are central to these adaptive responses. Although genetic approach has been extensively used to identify signal transduction components (1–4), the functional redundancy or overlap in the *Arabidopsis* genome and the dynamics and complexity of intertwined signaling mechanisms make it difficult to understand the role of a particular gene, often with multiple unrelated functions. Gain-of-function approaches, such as generating overexpression transgenic plants, can help to elucidate the function of the gene of interest; however, they are more time consuming and

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unpredictable or yield puzzling and complex results due to long-term gene expression consequences that are remote from the original gene activity. Transient gene expression in isolated plant cells provides a versatile and rapid alternative approach, which is a relatively simple way to explore new ideas and investigate the role of a candidate gene.

Since the isolation of plant protoplasts was reported 50 years ago (5), protoplasts from various plant species have been widely employed in physiological, biochemical, and molecular studies, such as signal transduction processes, ion transport, cell wall synthesis, protein trafficking, viral replication, and programmed cell death (1). *Arabidopsis* mesophyll protoplasts isolated from fresh leaves have been proven to be ideal for studying signal transduction pathways (1, 6, 7), as they respond sensitively and specifically to diverse signals, such as heat, cold, darkness, light, elicitors, H_2O_2, auxin, abscisic acid (ABA), and cytokinin (Fig. 1). Moreover, fast and simple procedures

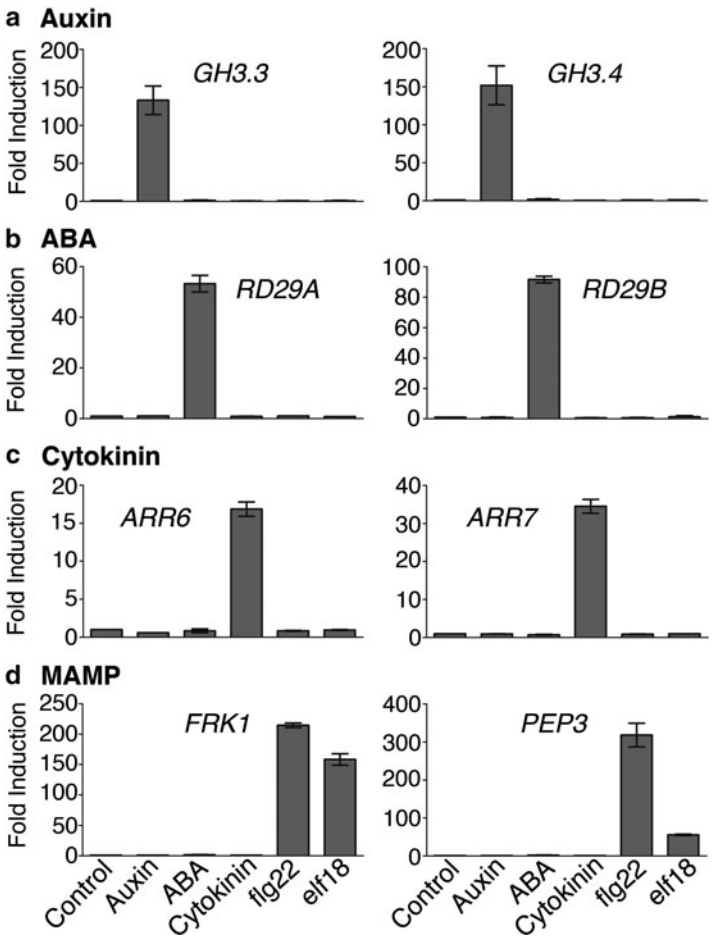

Fig. 1. Quantitative real-time RT-PCR analysis of marker gene expression in response to different signals. The protoplasts were treated for 1 h with 10 μM NAA (auxin), 10 μM ABA, 100 nM 2-iP (cytokinin), 10 nM flg22, or 100 nM elf18. Protoplasts without treatment were used as control. Error bars, SD ($n = 2$).

Table 1
List of reporter constructs useful for quantifying signal transduction in the *Arabidopsis* protoplast transient expression system (the reporters used in Fig. 2 are labeled with asterisks)

Name	Locus	GenBank accession	Use	ABRC[a] stock number	References
HBT-sGFP (S65T)	35S derivative	EF090408	Efficiency/ internal control	CD3-911	(17)
**RD29A-LUC*	At5g52310	EF090409	ABA/stress	CD3-912	(10, 18)
**AtGH3-LUC*	At2g23710	EF090410	Auxin	CD3-913	(10, 18)
WRKY29-LUC	At4g23550	EF090411	Innate immunity	CD3-914	(8)
GST6-LUC	At2g47730	EF090412	H_2O_2/ stress	CD3-915	(18)
HSP18.2-LUC	At5g59720	EF090413	Heat/H_2O_2	CD3-916	(10)
**ARR6-LUC*	At5g62690	EF090414	Cytokinin	CD3-917	(10)
GCC1-LUC	8× GCC-Box synthetic promoter	EF090415	Ethylene/ stress	CD3-918	(12)
**FRK1-LUC*	At2g19190	EF090416	Innate immunity	CD3-919	(9)

[a] *Arabidopsis* Biological Resource Center (http://abrc.osu.edu/index.html)

of isolation and high transfection efficiency make *Arabidopsis* mesophyll protoplasts a good system for transient expression of candidate genes to investigate their functions. We have successfully used luciferase (LUC) and β-glucuronidase (GUS) as reporters for quantitatively measuring the effect of regulators in different signal transduction pathways (8–14). A set of LUC reporters controlled by promoters that respond to different signals have been donated to the *Arabidopsis* Biological Resource Center (ABRC) for public use (Table 1). One drawback of the reporter assays is that same as other gain-of-function approaches transgene overexpression may cause an ectopic effect on the expression of reporter gene. However, the results of reporter assays can be validated by quantitative measurement of endogenous gene expression (e.g., marker genes for each signal pathway; Fig. 1) and further investigated in appropriate mutants. The assays can be applied for more ambitious high-throughput screening and systematic and genome-wide characterization of

gene functions in most *Arabidopsis* accessions and mutant plants (8, 11, 13, 14).

2. Materials

2.1. Plant Material and Growth Conditions

Grow Arabidopsis plants in soil (Metro-Mix 360 or Jiffy-7) in a greenhouse or growth chamber with a photoperiod of 12 h light/12 h dark under low light (75 mmol/m^2/s) at a 23°C/20°C light/dark temperature regime and 65% relative humidity for 4 weeks.

2.2. Protoplast Isolation

1. Enzyme solution: 0.4 M mannitol, 20 mM KCl, 20 mM MES, pH 5.7, 1.5% cellulase R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan), 0.4% macerozyme R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan). Heat the enzyme solution at 55°C for 10 min to inactivate proteases and DNase and enhance enzyme solubility (see Note 1). Cool the solution to room temperature and add 10 mM $CaCl_2$, 1 mM β-mercaptoethanol, and 0.1% BSA. Pass the solution through a 0.45-μm nylon membrane syringe filter into a Petri dish.
2. Razor blades.
3. Petri dish (100 × 25 mm).
4. Desiccator.
5. Nylon mesh (35–75 μm).
6. 30-ml Round-bottom polypropylene tube (Sarstedt).
7. Hemacytometer.

2.3. Protoplast Transfection

1. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7.
2. WI solution: 0.5 M mannitol, 4 mM MES, pH 5.7, 20 mM KCl.
3. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7.
4. Polyethylene glycol (PEG) solution: Add 4 g of PEG 4000 (Sigma-Aldrich), 2.5 ml of 0.8 M mannitol, and 1 ml of 1 M $CaCl_2$ to 3 ml of ddH_2O for a total volume of 10 ml.
5. 2-ml Round-bottomed natural microcentrifuge tubes.
6. Tissue culture plates (6-, 12-, or 24-well).
7. Bench-top centrifuge (IEC Centra CL2; International Equipment Company).

8. Chemicals: 1-naphthaleneacetic acid (NAA; Sigma-Aldrich), (±)-abscisic acid (ABA; Sigma-Aldrich), 6-(γ,γ-dimethylallylamino)-purine (2-iP; Sigma-Aldrich), flg22 (the conserved 22 amino acids of flagellin, chemically synthesized according to the published peptide sequence (15)), and elf18 (N-terminal, acetylated 18 amino acid fragment of bacterial elongation factor Tu, synthesized according to the published peptide sequence (16)).

2.4. Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Assay

1. TRIzol reagent (Invitrogen).
2. Oligo$(dT)_{15}$ primer (Promega).
3. dNTP (mix of dATP, dTTP, dGTP, and dCTP).
4. ImProm-II 5× Reaction Buffer (Promega).
5. 25 mM $MgCl_2$.
6. Protector RNase Inhibitor (Roche).
7. ImProm-II Reverse Transcriptase (Promega).
8. iQ SYBR Green Supermix (Bio-Rad).
9. CFX96 Real-Time polymerase chain reaction (PCR) Detection System (Bio-Rad).

2.5. Reporter Assays

1. Lysis buffer: 25 mM Tris-phosphate, pH 7.8, 2 mM 1, 2-diaminocyclohexane *N*,*N*,*N*,*N*-tetraacetic acid, 10% glycerol, 1% Triton X-100, 2 mM dithiothreitol (DTT).
2. Luciferase assay system (Promega).
3. MUG solution for GUS assay: 1 mM 4-methylumbelliferyl-β-D-glucuronide (MUG; Sigma-Aldrich, Cat. No. M9130), 10 mM Tris–HCl, pH 8, 2 mM $MgCl_2$.
4. Luminometer and fluorometer (Modulus microplate multimode reader; Promega).

2.6. In Vitro Kinase Assay

1. Immunoprecipitation (IP) buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 2 mM NaF, 2 mM Na_3VO_4, 1× protease inhibitor cocktail (Roche), 1% Triton X-100.
2. Kinase buffer: 20 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 50 μM ATP and 2 μCi ATP, [γ-^{32}P] or [γ-^{33}P] (PerkinElmer).
3. Protein G Sepharose beads (GE Healthcare).
4. Fixing solution: 10% ethanol and 10% acetic acid.
5. Gel dryer (Bio-Rad).
6. Typhoon imaging system (GE Healthcare).

3. Methods

3.1. Protoplast Isolation

1. Select well-expanded leaves from 3- to 4-week-old plants (see Note 2).
2. Remove the leaf tip (3 mm), cut the middle part of a leaf into 0.5- to 1-mm strips with a clean and sharp razor blade without crushing the edge, and immediately transfer the leaf strips into the solution and submerge the leaf strips (see Note 3).
3. Cover the Petri dish with aluminum foil and vacuum infiltrate for 30 min using a desiccator.
4. Continue the digestion without vacuum or shaking for another 2.5–3 h. The digestion time may vary depending on the material and experimental goals.
5. Release the protoplasts by gently shaking the Petri dish (either by hand or using a shaker at 50 rpm). Under the ideal conditions, the leaf strips turn transparent.
6. Add equal volume of W5 solution and filter the solution containing protoplasts with a 35- to 75-μm nylon mesh into a 30-ml round-bottom tube.
7. Pellet the protoplasts by spinning for 2 min at 100 × *g* or speed 3 using an IEC clinical centrifuge.
8. Remove the supernatant as much as possible and resuspend the protoplasts in 0.5 ml of W5 solution by gentle swirling.
9. Count protoplasts using a hemacytometer under a light microscope and adjust the concentration to 2×10^5/ml by adding W5 solution.
10. Keep the protoplasts on ice for at least 30 min for recovery from isolation stress.
11. The protoplasts should settle at the bottom of the tube after 5–10 min. Right before PEG-Ca^{2+} transfection, pipette the W5 solution out and resuspend the protoplast pellet in MMg solution to 2×10^5/ml (see Note 4).

3.2. Protoplast PEG-Ca^{2+} Transfection

1. Prepare fresh 40% (*w*/*v*) PEG solution.
2. Add 10 μl (10–20 μg) of the plasmids DNA into a 2-ml round-bottom tube (see Note 5).
3. Add 100 μl of protoplasts in MMg solution into the tube (see Note 6).
4. Add 110 μl of PEG solution and then mix completely by gently tapping the tube.
5. Incubate at room temperature for up to 10 min (5 min is sufficient).

6. Add 440 µl of W5 solution and mix by gently inverting to stop the transfection.
7. Spin at 100 × *g* for 1 min and remove the supernatant.
8. Resuspend the protoplasts gently with 500 µl of WI solution in each well of a 12-well tissue culture plate (see Note 7).
9. Treat the protoplasts with plant hormones or microbe-associated molecular patterns (MAMPs) (optional; see Note 8).
10. Incubate the protoplasts under desirable conditions (see Note 9).
11. After incubation, resuspend and harvest protoplasts by centrifugation at 100 × *g* for 2 min.
12. Remove the supernatant and freeze the samples on dry ice. Samples can be stored at –80°C until further analysis.

3.3. Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Assay

1. Isolate total RNA by using TRIzol Reagent according to the manufacturer's instructions.
2. Add 0.8 ml of TRIzol for 2 × 10^5 protoplasts (see Note 10).
3. Combine 1 µg of total RNA and 1 µl of Oligo$(dT)_{15}$ (500 ng/µl) in nuclease-free water for a final volume of 10 µl per RT reaction.
4. Heat the mixture at 70°C for 10 min and then chill on ice.
5. Add 10 µl of the reverse transcription reaction mix (4 µl of ImProm-II 5× reaction buffer, 2 µl of 25 mM $MgCl_2$, 2 mM dNTP, 0.5 µl of Protector RNase Inhibitor, and 0.5 µl of ImProm-II Reverse Transcriptase) to each reaction tube.
6. Incubate the tubes at 25°C for 5 min and then at 42°C for 1 h.
7. Inactivate the reverse transcriptase at 70°C for 15 min.
8. Add 80 µl of nuclease-free water to each RT product.
9. Take 1 µl of the diluted RT product for 10-µl quantitative real-time reverse transcription-PCR (qRT-PCR) with primers for genes of interest or control genes, such as "housekeeping" genes (e.g., Actin, Ubiquitin, or Tubulin genes). An example of the results produced is shown in Fig. 1.

3.4. Reporter Assays

3.4.1. Luciferase Activity Assay

1. Add 50 µl of cell lysis buffer to the frozen protoplast samples and thaw on ice (see Note 11).
2. Vortex vigorously for 10 s to break up the protoplasts and then incubate on ice for 5–10 min.
3. Spin down cell debris at 8,000–10,000 × *g* for 1 min.
4. Mix 2–20 µl of the lysate with 100 µl of LUC assay reagent to measure LUC activity with a luminometer (see Note 12).

3.4.2. GUS Activity Assay

1. Add 2 µl of the protoplast lysate prepared from Subheading 3.4.1, step 3, into 25 µl of MUG substrate solution and mix well.

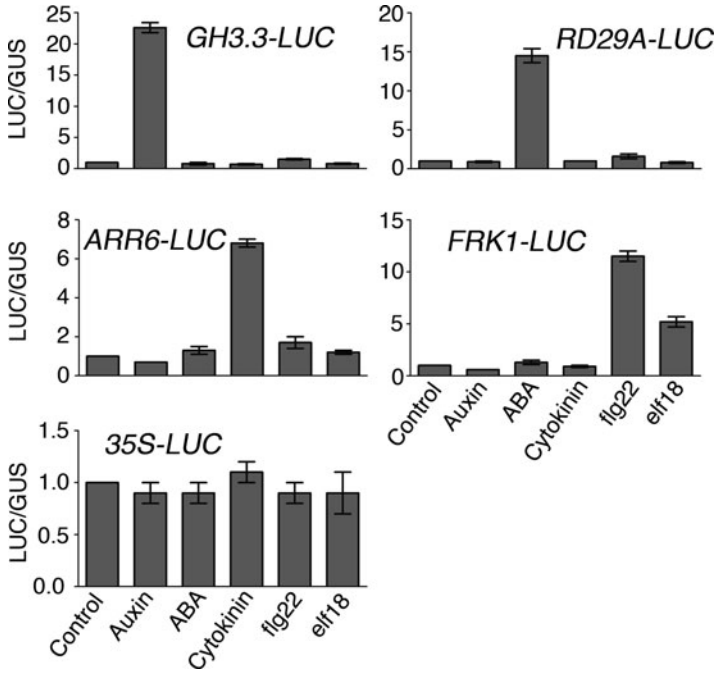

Fig. 2. Specificity of plant hormone or innate immune responses in the *Arabidopsis* protoplast transient expression system. Immediately after transfection, the protoplasts were treated with 10 μM NAA (auxin), 10 μM ABA, 100 nM 2-iP (cytokinin), 10 nM flg22, or 100 nM elf18. Protoplasts were harvested after 4 h of incubation. Promoter activities were normalized to the value obtained from protoplasts without treatment (control) and GUS activity of each sample served as an internal standard. Error bars, SD ($n = 2$).

2. Incubate at 37°C for 30–60 min.
3. Add 100 μl of 0.2 M Na_2CO_3 to stop the reaction.
4. Measure the fluorescence of MU using a fluorometer. Examples of the quantitative measurement of the reporter gene expression in response to specific signals are shown in Fig. 2.

3.5. In Vitro Kinase Assay

1. Add 200 μl of IP buffer to the frozen 200-μl (4×10^4) protoplast sample and thaw on ice.
2. Vortex vigorously for 10 s to break up the protoplasts and then incubate on ice for 5–10 min.
3. Centrifuge at the maximum speed for 5 min at 4°C.
4. Transfer the supernatant to a new tube and add 1 μl of antibody against the protein kinase of interest (see Note 13).
5. Incubate with constant mixing at 4°C for 2 h.
6. During the incubation time, wash Protein G Sepharose beads (5 μl per sample) with 1 ml of IP buffer.
7. Centrifuge at 6,000 rpm for 20 s and carefully remove the supernatant as much as possible.

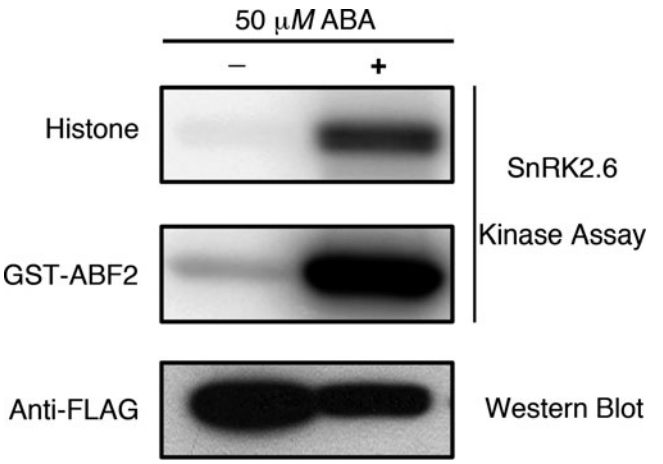

Fig. 3. Activation of transiently expressed SnRK2.6-FLAG by ABA in protoplasts. After transfection, the protoplasts were incubated in WI solution for 5.5 h and then treated with 50 μM ABA for 30 min. The control (−) was the duplicated protoplast sample incubated for 6 h without ABA. Two micrograms of histone and GST-ABF2 (Gly73 to Gln119) were used as substrates in kinase assays, and their phosphorylation by ABA-activated SnRK2.6 was detected by a phosphorimager (the *upper two panels*). The expression level of SnRK2.6-FLAG in protoplasts was detected by Western blot using anti-FLAG antibody (*bottom panel*).

8. Repeat steps 6 and 7 two more times.
9. After incubation with the antibody, add 5 μl of washed Protein G Sepharose beads into each sample and incubate at 4°C with constant mixing for another hour.
10. Centrifuge at 6,000 rpm for 20 s and carefully remove the supernatant.
11. Wash the beads with 1 ml of IP buffer twice.
12. Wash one more time with 1 ml of the kinase buffer (without cold and hot ATP).
13. Add 15 μl kinase buffer, including 1–3 μg of the desired substrate, into each sample and incubate at room temperature or 30°C for 30–60 min (see Note 14).
14. Stop the reaction by adding 4× SDS-PAGE loading buffer.
15. Boil the sample for 5 min and then load the supernatant of the reaction sample on a SDS-PAGE gel.
16. Run the gel at 30–50 mA until the dye fronts reach the bottom of the gel.
17. Wash the gel in the fixing buffer for 10 min three times.
18. Dry the gel on a gel dryer at 80°C for 1 h and then expose to autoradiography film or phosphorimager (Typhoon imaging system). ABA activation of SnRK2.6 is used as an example for kinase assay in Fig. 3.

4. Notes

1. Prepare 10 ml of the enzyme solution to digest up to 40 leaves with a yield of ~10^6 protoplasts per 10 leaves. After heating, the solution should be clear light brown. A workshop movie for protoplast isolation and transfection can be downloaded on the Sheen Lab Web site (http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html).
2. Younger plants can be used for protoplast isolation. Leaves five to eight are normally used in 4-week-old *Arabidopsis* plants. Leaves three to four are used in 3-week-old plants and leaves one to two are used in 2-week-old plants.
3. This is a critical step for protoplast isolation. Use fresh razor blades to cut the leaves on a piece of clean white paper to evaluate the process. There should not be juicy green stain left on the paper after cutting. In order to get a high yield of the protoplasts, completely submerge the leaf strips into the enzyme solution by gently dipping both sides with an inoculating loop (BD).
4. Be gentle with the protoplasts during all the steps! To resuspend the protoplast pellet, add 0.5–1 ml of the solution first and gently swirl the tube before adding the rest of the solution.
5. The quality of the plasmid DNA is critical for high transfection efficiency. We routinely use CsCl gradient for maxi-plasmid DNA preparation. The protocol is available on the Sheen Lab Web site (http://genetics.mgh.harvard.edu/sheenweb/protocols_reg.html).
6. Based on the purpose of the experiment, the experiments can be scaled up or down with the same DNA/protoplasts ratio. For the reporter assay, use 10–100 µl ($0.2–2 \times 10^4$) protoplasts depending on the promoter activity. Use 100–200 µl ($2–4 \times 10^4$) protoplasts for Western blot, coimmunoprecipitation, and kinase assay. For qRT-PCR analysis, 1 ml (2×10^5) protoplasts are normally required.
7. 24-Well (250 µl of WI), 6-well (1 ml of WI), or 100 × 25-mm Petri dish (5 ml of WI) can also be used depending on the amount of protoplasts (~4×10^4 cells/ml of WI). The plate is coated with 5% sterile calf serum for 1 s to prevent the protoplast to stick on the surface. The depth of the WI solution in the plate is about 0.1 mm in order to prevent hypoxia stress during incubation.
8. Treat the protoplasts immediately if carrying out reporter assays in order to obtain a low basal level of reporter gene expression. To measure the expression level of endogenous genes in

response to a stimulus, it is highly recommended to rest the protoplasts in the WI solution for 3–5 h before signal treatment.

9. The incubation conditions can vary depending on the experiment purposes. We usually incubate the protoplasts at room temperature (22–25°C) under low light (30–35 μmol/m^2/s). Normally, 3–6 h is enough for reporter assay and Western blot. For qRT-PCR analysis, different time points after treatment can be chosen based on the experimental plan. The kinase activity can be detected within minutes to 1 h after treatment.
10. The total RNA yield is about 5 μg for 2 × 10^5 protoplasts.
11. Add fresh DTT to the cell lysis buffer right before use.
12. The amount of the lysate used in LUC assay depends on the promoter activity and the expression level of the reporter. Make sure that the readings of samples are within the linear range. The LUC assay reagent (Promega, Cat. No. E1501) can be diluted five times with water right before use.
13. The protoplast system is an ideal system for coimmunoprecipitation or kinase assays when the antibody against the endogenous protein is not available. Add an epitope tag sequence (e.g., HA, MYC, and FLAG tag) onto the gene of interest and express it in the protoplasts. Then, use the anti-eptitope antibody to immunoprecipitate the protein of interest.
14. The composition of the kinase buffer is specific to the kinase of interest. The kinase buffer used in this protocol is suitable for MAPK and SnRK2.6 kinase assay.

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