Chapter 12

MAPK Assays in *Arabidopsis* MAMP-PRR Signal Transduction

Hoo Sun Chung and Jen Sheen

Abstract

Activation of MAPK (Mitogen-Activated Protein Kinase) cascades after MAMP (Microbe-Associated Molecular Pattern) perception through PRR (Pattern Recognition Receptor) is one of the first conserved responses when plants encounter microbial organisms. Phosphorylation of various cellular factors in the MAMP-PRR pathway by MAPK cascades is critical for broad-spectrum plant innate immunity. Measurement of MAPK activation and identification of MAPK phosphorylation targets in the MAMP-PRR signal transduction pathway are essential to understand how plants reprogram their cellular processes to cope with unfavorable microbial attack. Here, we describe detailed protocols of three assays measuring MAPK activity after MAMP perception: (1) immune-blotting analysis with anti-phospho ERK1/2 antibody; (2) in-gel kinase assay using a general substrate myelin basic protein (MBP); (3) an in vitro kinase assay to evaluate phosphorylation of MAPK substrate candidates during MAMP-PRR signaling based on a protoplast expression system.

Key words *Arabidopsis*, Signal transduction, MAMP, PRR, MAPK cascades, Protoplast transient expression system, Anti-phospho-ERK1/2 antibody, Myelin basic protein (MBP), Kinase assay

1 Introduction

In the immediate environment, plants are able to recognize highly conserved features of microbial organisms known as MAMPs such as flagellin, elongation factor Tu (EF-Tu), and oligosaccharides from microbial cell walls [1–4]. Remarkably sensitive recognition of those non-self-molecules by plants is achieved by specific PRRs such as FLS2 and EFR for flagellin and EF-Tu, respectively, located on the plasma membrane [1, 2]. As this self-surveillance system gets the alert, one of the immediate cellular processes triggered by MAMP-PRR signaling is the activation of MAPK cascades composed of three-tiered protein kinases, MAPKKK (MTK), MAPKK (MKK), and MAPK (MPK) ([4, 5], Fig. 1a). In both plants and animals, MAPK cascades play central roles in innate immune responses by modulating functions of downstream targets critical for broad-spectrum immunity [3–6]. Current knowledge in the

Libo Shan and Ping He (eds.), *Plant Pattern Recognition Receptors: Methods and Protocols*, Methods in Molecular Biology, vol. 1578, DOI 10.1007/978-1-4939-6859-6_12,

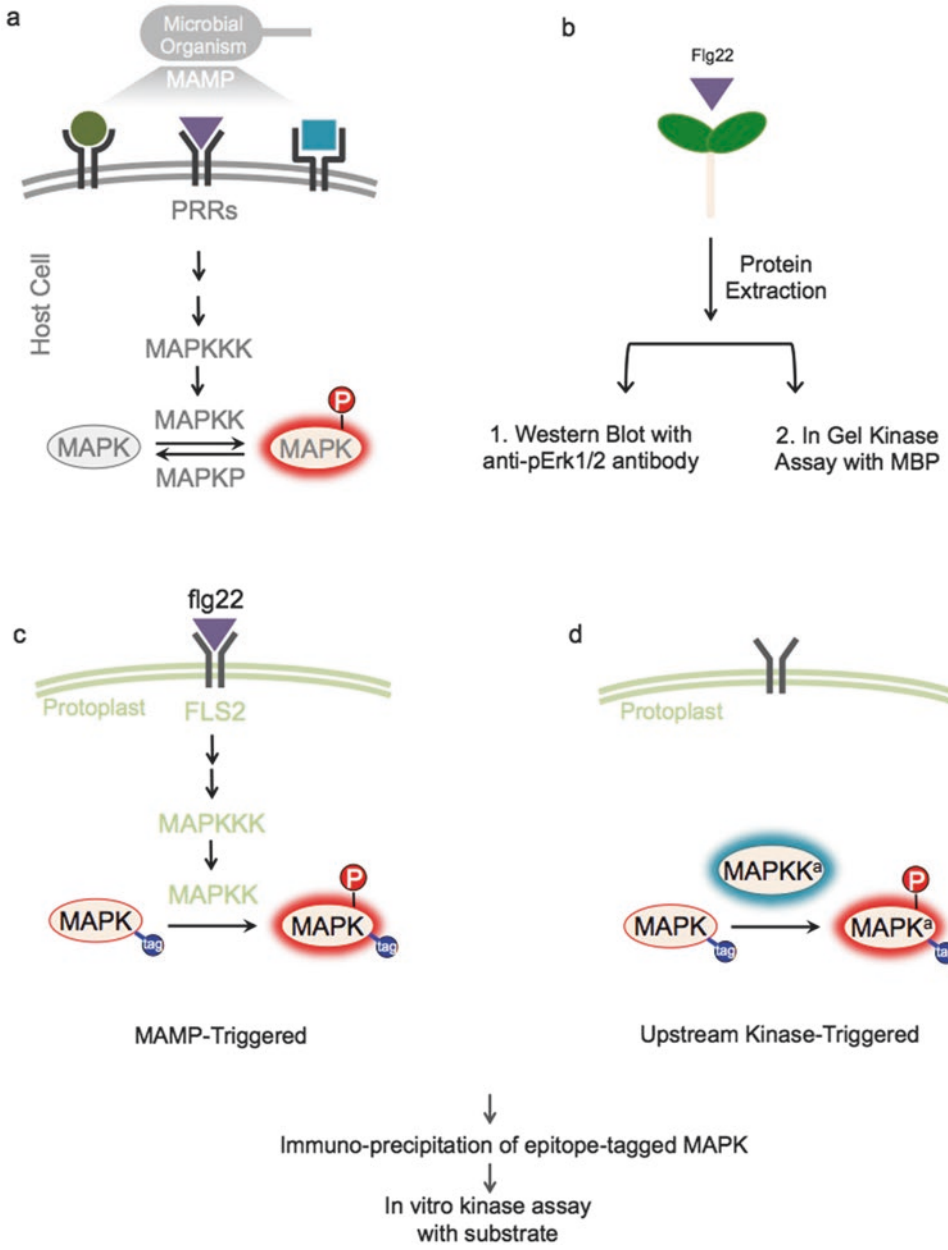

Fig. 1 Scheme of MAPK activation assays in MAMP-PRR signaling. (**a**) Activation of MAPK cascade in MAMP-PRR signal transduction pathway. Various MAMPs from microbial organism are recognized by specific PRRs at the cell membrane and triggers immune signal transduction pathway including activation of MAPK cascade. MAPK phosphatase (MAPKP) reverses MAPK activity by de-phosphorylation. (**b**) Experimental scheme of measuring endogenous MAPK activity after MAMP perception in Arabidopsis seedlings. (**c**, **d**) Underlying molecular basis of testing target phosphorylation by active MAPK using protoplast system. Ectopically expressed epitope-tagged MAPK in protoplasts is activated after MAMP perception through the PRR at the cell membrane (**c**). Co-expression of constitutively active upstream kinase (MKK^a) triggers MAPK activation without MAMP treatment (**d**)

field indicates that different MAMPs and bacterial proteases may trigger different members of MAPKs through distinct mechanisms, exemplified by flg22, LPS, and PrpL [7, 8]. It is also essential to study the signaling consequences of differential dynamics, amplitude, duration, and specificity of MAPK activation triggered by individual MAMP of various microbial organisms to understand sophisticated mechanisms that plants develop for their immune system.

All plant MAPKs identified so far share the highest sequence similarity and general phosphorylation and activation features to the ERK1/2 type of MAPKs in mammals. The development of an antibody specific to phosphorylated ERK1/2 (p42/p44) and identification MBP as a general substrate of ERK1/2, established valuable and convenient assays to study MAPK activities in plants for various signaling pathways including the MAMP-PRR pathway [9, 10]. In this chapter, we first describe experimental details of two protocols for the analysis of endogenous MAPK activation in the MAMP-PRR pathway, including immuno-blot analysis using an anti-phospho ERK1/2 antibody and in-gel kinase assay with MBP as a general substrate (Fig. 1b).

A critical question in studying the MAMP-PRR signaling pathway is to identify downstream targets that are phosphorylated by MAPKs, which may directly control reprograming of various cellular processes to support plant immunity [3, 11]. Validating phosphorylation of target candidates of a specific MAPK is crucial to further understand detailed molecular mechanisms of plant immune responses. A relatively straightforward and high-throughput way to answer the question would be the use of a biochemical assay to directly determine protein phosphorylation with a signal-activated kinase in the tube. *Arabidopsis* mesophyll cell protoplasts have been proved to serve as a great tool to study signal transduction pathways [12–14]. Combined with a facile and efficient DNA transfection technique, it could be an excellent system to express epitope-tagged signaling proteins such as kinases, activate them by desired signals such as MAMPs, and immunoprecipitate the proteins for further biochemical and functional assays. We introduce the third detailed protocol for using *Arabidopsis* protoplast system in a biochemical assay to evaluate substrate phosphorylation by the MAMP-PRR activated MAPKs (Fig. 1c, d).

2 Materials

2.1 Plant Growth and MAMP Treatment

1. Seeds of *Arabidopsis thaliana* Columbia-0.
2. Jiffy-7 peat soil pellets (Jiffy group) or Fafard soil mix (Fafard).
3. Liquid growth media: 0.5× Murashige and Skoog basal salt mixture, 0.5% Sucrose, pH 5.8 with KOH.

4. Falcon™ 6–12 well tissue culture plates.
5. Growth chamber: 12 h light/12 h dark under low light (75 μmol/m².s) at a 23/20 °C light/dark temperature, 65% relative humidity.
6. Flg22 peptide: the conserved 22 amino acids of flagellin, chemically synthesized according to the published peptide sequence [1].

2.2 Immuno-Detection of MAPK Activity

1. Protein Isolation (PI) 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, protease inhibitor (Roche, one tablet of Complete™ Mini for 10 mL of PI buffer, prepare fresh), 1% v/v Triton X-100.
2. 10% SDS-polyacrylamide gel.
3. 10× Tris-Glycine SDS-PAGE running buffer.
4. 10× Tris buffered saline with 0.05%? Tween®20 (TBST).
5. Blocking buffer: 1× TBST, 5% w/v nonfat dry milk.
6. Primary antibody incubation buffer: 1× TBST, 5% w/v Bovine Serum Albumin (BSA), 1:1000 dilution of anti-phospho ERK1/2 antibody (Cell Signaling, #9101).
7. Secondary antibody incubation buffer: 1× TBST, 5% w/v nonfat dry milk, 1:10,000 dilution of HRP-conjugated anti-rabbit IgG (Pierce, #31460).

2.3 In-Gel MAPK Assay

1. Protein Isolation buffer: same as in Subheading 2.4.
2. Myelin basic protein (MBP from Invitrogen).
3. Wash buffer: 25 mM Tris–HCl, pH 7.5, 0.5 mM DTT, 5 mM NaF, 0.1 mM Na_3VO_4, 0.5% w/v BSA (Sigma, A7906), 0.1% v/v Triton X-100.
4. Renaturation buffer: 25 mM Tris–HCl, pH 7.5, 0.5 mM DTT, 5 mM NaF, 0.1 mM Na_3VO_4.
5. Reaction buffer: 25 mM Tris–HCl, pH 7.5, 2 mM EDTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4.
6. Stop/Wash buffer: 5% w/v trichloroacetic acid, 1% w/v sodium pyrophosphate.
7. 50 μCi γ-^{32}P-ATP per gel (Perkin Elmer).
8. Gel dryer (Bio-Rad).

2.4 Isolation of Protoplasts and DNA Transfection

1. Enzyme solution: 0.4 M mannitol. 20 mM KCl. 20 mM MES, pH 5.7, 1% w/v cellulase R10 (Yakult Pharmaceutical Industry Co., Ltd, Japan), 0.2% w/v macerozyme R10 (Yakult Pharmaceutical Industry Co., Ltd, Japan), 10 mM $CaCl_2$, 1 mM β-mercaptoethanol, 0.1% w/v BSA. Heat the enzyme solution including everything except $CaCl_2$ and BSA at 55 °C

for 10 min to dissolve enzymes and inactivate proteases and DNases. Cool the solution to room temperature and then add $CaCl_2$ and BSA. Filter the solution through a 0.45 μm filter. Prepare the enzyme solution fresh.

2. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7.
3. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7.
4. Polyethylene glycol (PEG) solution: 40% w/v PEG 4000 (Sigma-Aldrich, #81240), 0.2 M mannitol, 100 mM $CaCl_2$. Prepare the PEG solution fresh.
5. WI solution: 0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7.
6. Bovine calf serum (Sigma, A7906).
7. Nylon mesh (35–70 μm, Lab-Line Instruments, #190-158-00).
8. Hemocytometer (Hausser Scientific, #1492).
9. Falcon™ tissue culture plates, 6–12 wells.
10. DNA plasmid of epitope tagged MAPKs and the constitutively activated version of MKK[a].

2.5 In Vitro MAPK Assay

1. Protein Isolation buffer: same as in Subheading 2.4.
2. Monoclonal anti-FLAG M2 antibody (Sigma, F3165).
3. Protein G Sepharose beads (GE Healthcare, #17-0618-02).
4. MAPK phosphorylation buffer: 20 mM Tris–HCl, pH 7.5, 20 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM EDTA, 1 mM DTT.
5. MAPK phosphorylation mix (15 μL per sample): MAPK phosphorylation buffer, 1–5 μg of desired substrate (e.g., MBP), 2 μCi of γ-^{32}P-ATP.
6. Fixation buffer: 10% v/v ethanol, 10% v/v acetic acid.
7. Gel dryer (Bio-Rad).

3 Methods

3.1 Arabidopsis Seedling Growth and MAMP Treatment

1. Sterilize *Arabidopsis* seeds with 30% v/v bleach solution and stratify them at 4 °C for 3 days.
2. Add 0.5 mL of 0.5× MS media into each well of the 12-well Falcon™ Tissue Culture Plate.
3. Place six seeds in each well of the plate.
4. Seal the plate with 3 M micropore™ tape.
5. Grow plants for 7 days (*see* Subheading 2.1 for growth condition).

6. Prepare 10 nM flg22 in 0.5× MS liquid media.
7. Carefully take out liquid medium remaining in the plate and add 0.5 mL of fresh media containing 10 nM flg22 (*see* **Note 1**).
8. Incubate seedlings at room temperature for desirable periods (*see* **Note 2**).
9. Collect seedlings into the 1.5-mL microcentrifuge tube after gently blot them on Kimwipes® to remove residual liquid, and immediately freeze them with liquid nitrogen (*see* **Note 3**).
10. Store samples at −80 °C or proceed to the next step immediately.

3.2 Protein Isolation from Whole Seedlings (see Note 4*)*

1. Grind seedlings to a fine powder using a pellet pestle.
2. Add 100 μL of PI buffer and then homogenize well by vortexing vigorously for 30 s.
3. Centrifuge the samples at 18,000 × *g* at 4 °C for 5 min.
4. Transfer 80 μL of the clear supernatant to the new tubes.
5. Quantify total protein extract.
6. Store samples at −80 °C or proceed to the next step immediately.

3.3 Western Blot Analysis with Anti-Phospho ERK1/2 Antibody

1. Prepare 10% SDS-polyacrylamide gel (*see* **Note 5**).
2. Take 20 μg of total protein prepared from Subheading 3.2, and add appropriate amount of 4× sample buffer.
3. Boil the samples for 5 min and ice bath them.
4. After brief spin down, load the samples and run a gel until 20- to 100-kDa protein markers are well resolved.
5. Electrotransfer to PVDF membrane.
6. Wash membrane with 1× TBST for 5 min.
7. Incubate membrane in 10 mL of blocking buffer for 1 h at room temperature with gentle shaking.
8. Wash membrane three times for 5 min each with 15 mL of 1× TBST at room temperature.
9. Incubate membrane with of 10 mL of primary antibody incubation buffer at 4 °C for overnight with gentle shaking.
10. Wash membrane three times for 5 min each with 15 mL of 1× TBST three times at room temperature.
11. Incubate membrane with 10 mL of secondary antibody incubation buffer for 1 h at room temperature.
12. Wash membrane three times for 5 min each with 15 mL of 1× TBST at room temperature.
13. Proceed detection with ECL reagent (Fig. 2).

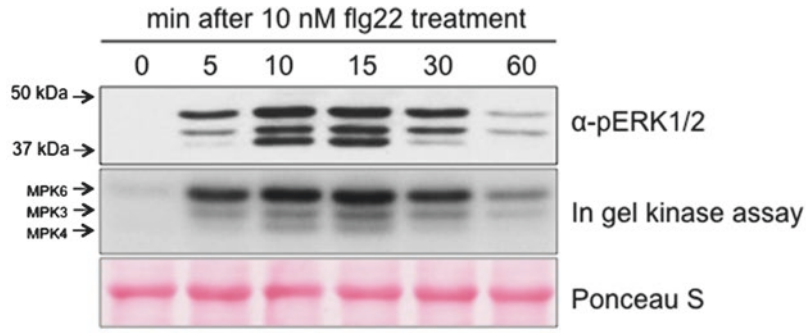

Fig. 2 Activation of endogenous MAPK after MAMP perception. Seven-day-old seedlings grown in liquid medium were treated with 10 nM flg22 peptide for indicated time length (0–60 min) and activation of endogenous MAPK proteins was measured by western blot analysis using anti-phospho Erk1/2 antibody (*top panel*) and by in-gel kinase assay using a general substrate MBP (*middle panel*). Twenty microgram of total protein was loaded in each well. Ponceau S staining of Rubisco large subunit (rbcL) shows equal amount of protein loading

3.4 In-Gel MAPK Assay

1. Prepare 10% SDS-polyacrylamide gel containing 0.25 mg/mL of MBP.
2. Take 20 μg of total protein prepared from Subheading 3.2, and add appropriate amount of 4× sample buffer.
3. Boil the samples for 5 min and ice bath them.
4. After brief spin down, load samples and run a gel until 20- to 100-kDa protein markers are well resolved.
5. Wash the gel three times for 30 min each with 100 mL of the wash buffer at room temperature with gentle agitation.
6. Incubate the gel in renaturation buffer at 4 °C overnight with three changes of the buffer during the period.
7. Equilibrate the gel with 25 mL of reaction buffer (without ATP) at room temperature for 1 h with gentle agitation.
8. Bring the gel to the designated radioactive work area.
9. Add 50 μCi of γ-^{32}P-ATP to the remaining 25 mL of reaction buffer.
10. Replace buffer with ATP-containing reaction buffer and incubate the gel for 1 h with gentle agitation at room temperature.
11. Stop reaction by replacing buffer with 100 mL of stop/wash buffer and wash the gel with gentle agitation at room temperature for 6 h. Change buffer at least six times (*see* **Note 6**).
12. Vacuum dry the gel at 80 °C for 1 h.
13. Proceed detection with autoradiography film or any phosphor imaging system.

3.5 Isolation of Protoplasts

1. Prepare fresh enzyme solution into a 100 × 20 mm petri dish (*see* **Note 7**).
2. Select well-expanded leaves from 4-week-old *Arabidopsis* plants.
3. Cut the middle part of the leaves into 0.5–1 mm strips with a clean and sharp razor blade and immediately submerge them in the enzyme solution.
4. Cover the petri dish with aluminum foil and incubate for 3 h at room temperature.
5. Gently agitate the petri dish to release protoplasts.
6. Add 10 mL of W5 solution and filter the slurry through a 35–70 μm nylon mesh into a 30-mL round-bottom tube.
7. Centrifuge the flow-through at 100 × *g* and remove the supernatant.
8. Gently resuspend protoplasts with 10 mL of W5 solution and rest the slurry on ice for at least 30 min.
9. The protoplasts should settle at the bottom of the tube. Pipet out W5 solution and resuspend the pellet in MMg solution to 2×10^5 cells/ml.

3.6 Protoplast Transfection and MAPK Activation

1. Prepare fresh PEG transfection solution.
2. Add 10 μL of plasmids DNA of MAPK-FLAG (2 μg/μL) into a 2-mL round-bottom tube (*see* **Notes 8** and **9**).
3. If desired, add 5 μL of plasmid DNA of MKK[a] for co-transfection (*see* Fig. 3).
4. Add 200 μL of protoplasts in MMg solution into the tube and mix well by gentle tapping.
5. Add 220 μL of PEG transfection solution and mix by gentle tapping.
6. Incubate at room temperature for 5 min.
7. Add 440 μL of W5 solution and mix gently by inverting tubes several times to stop transfection.
8. Spin at 100 × *g* for 1 min and remove the supernatant.
9. Resuspend the protoplasts with 1 mL of WI solution and place the cell in a 6-well Falcon plate.
10. Incubate protoplasts for 5 h at room temperature.
11. Treat the protoplasts with MAMPs (e.g., flg22 peptide) for desirable period (*see* **Note 10**).
12. Harvest protoplasts by centrifugation at 100 × *g* for 2 min.
13. Remove the supernatant and freeze immediately with liquid nitrogen.
14. Store the samples at −80 °C or proceed to the next step.

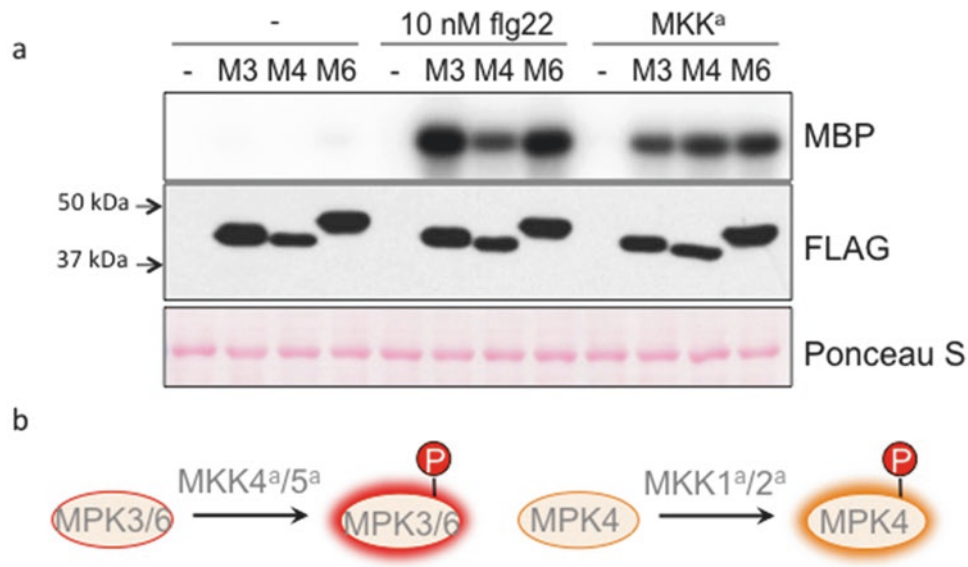

Fig. 3 In vitro phosphorylation of MBP by MAPK triggered by MAMP or upstream kinase in protoplast system. (**a**) Isolated protoplasts from 4-week-old *Arabidopsis* were transfected with indicated DNA plasmid to express epitope-tagged MAPK (e.g., MPK3-FLAG, MPK4-FLAG, and MPK6-FLAG). Protoplast with no DNA transfection (–) was served as a negative control. To activate MAPK, cells were treated with 10 nM flg22 for 10 min after 5 h of transfection, or co-transfected with constitutively active upstream kinase (MKK5^a for MPK3 and MPK6, and MKK1^a for MPK4) and incubated for 5 h before harvest. After immuno-purification of individual MAPK-FLAG, kinase activity of each protein was estimated by in vitro kinase assay using a general substrate MBP (*top panel*). Western blot analysis using anti-FLAG antibody indicates expression of individual MAPK-FLAG in protoplast (*middle panel*). Ponceau S staining of Rubisco large subunit (rbcL) shows equal amount of protein loading. (**b**) Different upstream kinases of MPK3/6, and MPK4. Without MAMP, MPK3/6, and MPK4 can be triggered by active MKK4/5 and MKK1/2, respectively

3.7 In Vitro MAPK Assay

1. Add 200 μL PI buffer to the frozen protoplasts and gently tab the tube to lysate the cells in ice.
2. Centrifuge at 18,000 × *g* at 4 °C for 5 min and transfer all supernatant to the new tube.
3. Add 1 μL of FLAG m2 antibody to the tube and incubate in a rotating wheel for 2 h at 4 °C.
4. Wash Protein G Sepharose beads twice with 1-mL PI buffer.
5. Add 10 μL of Protein G Sepharose beads into the tube and incubate for 1 h at 4 °C.
6. Centrifuge at 3,000 × *g* for 30 s and carefully remove the supernatant.
7. Wash the beads with 1-mL PI buffer and then with 1 mL of MAPK phosphorylation buffer.
8. Bring samples to the designated radioactive work area.
9. Prepare MAPK phosphorylation mix.

10. Add 15 μL of MAPK phosphorylation mix into the tube containing Protein G Sepharose beads associated with MAPK-FLAG protein (*see* **Note 11**).
11. Incubate for 30 min at room temperature on a shaking incubator (or gently tab the tubes every 5 min).
12. Stop the reaction by adding 8 μL of 4× protein sample buffer.
13. Boil the samples for 5 min and then load 10 μL of supernatant on 15% SDS-polyacrylamide gel (*see* **Note 12**).
14. Run a protein gel until the markers are well separated.
15. Wash the gel three times for 10 min each with Fixation buffer at room temperature.
16. Vacuum dry the gel at 80 °C for 1 h.
17. Proceed detection with autoradiography film or any phosphor imaging system.

4 Notes

1. After seedling growth for 7 days, remaining liquid medium amount in each well of the plate is varying. To have identical concentration of MAMP treatment in each well, it is recommended to remove all remaining media and add freshly made MAMP containing media to the plants in each well.
2. Individual MAMP triggers MAPK cascade with specificity. Design experiment accordingly to cover temporal dynamics of MAPK activation.
3. MAPK cascade could be activated by numerous stress signals including mechanical stress. Be cautious with handling of samples to keep the MAMP-specific effect on MAPK activation.
4. It is critical to keep the sample in ice during the whole procedure to keep the proteins intact.
5. 10% SDS-polyacrylamide gel gives you best separation of MPK6, MPK3, and MPK4, as they are resolved between 50 and 37 kDa.
6. First round of used wash buffer is highly radioactive. It must be treated accordingly.
7. Prepare 10 mL of enzyme solution to digest up to 40 leaves with a yield of around 4.0×10^6 protoplasts.
8. The quality of the plasmid DNA is critical to achieve high transfection efficiency. We use CsCl gradient for maxi preparation of plasmid DNA. The protocol is available at the Sheen Lab web site (http://molbio.mgh.harvard.edu/sheenweb/protocols_reg.html).

9. The protoplast system is ideal to prepare proteins for kinase assay when the antibody against endogenous protein is not available. Add an epitope tag sequence (e.g., FLAG, HA, or MYC) to the gene of interest, express in the protoplast, and then immune-precipitate with an appropriate antibody.
10. Dynamics of MAPK activation varies by MAMPs. Incubation time with each MAMP to activate MAPK must be decided based on empirical data. If you coexpress MAPK with constitutively active MKK (MKK^a), MAMP treatment is not necessary.
11. Immuno-purified MAPK proteins prepared from 200 μL protoplast (4×10^4 cells) could be used up to five reactions. After washing the beads, aliquot them into upto five tubes in case to test multiple substrates.
12. Prepare 10–15% SDS-polyacrylamide gel according to the size of the substrate. Size of MBP is 18 kDa. Most of the unbound γ-^{32}P-ATP migrates with blue dye (bromophenol blue) of the loading buffer. Cut out the dye part of the gel before you fix the gel.

Acknowledgment

The authors thank the former and current members of the Sheen Laboratory for their efforts to develop and improve kinase assays and the *Arabidopsis* mesophyll cell transient expression system. This work was supported by the Gordon and Betty Moore Foundation fellowship to H.S.C through Life Science Research Foundation, and the National Science Foundation grant IOS-0618292 and the National Institute of Health grant R01 GM070567 to J.S.

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