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Chapter 14

Detection of MAPK3/6 Phosphorylation During Hypersensitive Response (HR)-Associated Programmed Cell Death in Plants

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Abstract

Programmed cell death (PCD) is an essential component of development, biotic and abiotic responses. Hypersensitive response (HR)-associated cell death activated under pathogen attack is one of the most dramatic manifestations of PCD in plants. Signal transduction through mitogen-activated protein kinase (MAPK) cascades, a very conserved signaling pathway across eukaryotes, is a core mediator for HR-associated PCD. Therefore, monitoring MAPK activation enables the mechanisms underlying HR-associated PCD to be elucidated. Here, we describe the use of a phosphorylation-specific MAPK3/6 antibody to monitor the activation of MAPK3/6 during HR-associated PCD. The technique may be adapted for use in other types of PCD.

Key words Programmed cell death, MAPK, Hypersensitive response, Phosphorylation

1 Introduction

Programmed cell death (PCD), a sequence of events that leads to the controlled and organized destruction of the cell [1], is an essential component of development, biotic and abiotic responses in plants [2]. A number of biological processes, including gametophyte development, fertilization, embryo formation, seed germination, and xylem formation depend on PCD [3]. In addition, hypersensitive response (HR)-associated cell death activated under pathogen attack is one of the most dramatic manifestations of PCD in plants [4]. While PCD is well studied in animals, despite recent advances, our knowledge of the mechanisms underlying PCD in plants is still in its infancy [5]. Recent biochemical and molecular genetic studies have revealed that a number of signaling modules, such as reactive oxygen species (ROS) and MAPK, are involved in the plant PCD and have broadened our understanding of the mechanisms [2, 5].

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MAPKs have been recognized as central regulators of PCD [6–9]. MAPK cascades consist of three kinase modules: MAP kinase kinase kinase (MAP3K/MEKK), MAP kinase kinase (MAPKK/MAP2K/MEK/MKK), and MAP kinase (MAPK/MPK) [10]. The cascades amplify signals via reversibly phosphorylated kinases, leading to the phosphorylation of substrate proteins, whose altered activities mediate many biological processes [11]. Increasing evidence suggests that the plant MAPK cascade is one of the convergence points to mediate HR-associated PCD [12]. During the tobacco HR, a MAPK cascade shuts down carbon fixation in chloroplasts, and hydrogen peroxide, generated by light in the chloroplasts, triggers PCD [12]. In addition, MAPK cascades activate PCD transcriptional reprogramming via cell death-specific transcription factors [5]. For example, MEKK1 interacts with WRKY53, a transcription factor involved in PCD, and *mekk1* mutants accumulate high levels of ROS and develop local lesions reminiscent of PCD [13–16]. MAPK3 and MAPK6 are the two of the best-studied MAP kinases. The activation of these kinases is a critical step and an early marker for full induction of defense responses and HR-associated PCD under pathogen attack. Because they are highly conserved across eukaryotes, the commercially available, anti-phosphorylated MAPK3/6 antibodies that are generated for mammalian studies also recognize plant orthologs, allowing us to track the activation of MAPKs in plants.

Here, we describe a simple and robust method to detect MAPK3/6 phosphorylation during HR-associated PCD. We use a phosphor-p44/42 MAPK(Erk1/2)(Thr202/Tyr204) antibody, which specifically reacts with phosphorylated MAPK3/6 (Erk1/2), to track the phosphorylation of MAPK3/6 in HR-associated PCD tissues. This tool facilitates the elucidation of mechanisms underlying HR-associated PCD, and has the potential to be adapted for use in other types of PCD or other pathways involving MAPK activation, such as those functioning during development [17].

2 Materials

2.1 Protein Extraction and Quantification

1. Protein extraction buffer: 0.35 M Tris–HCl pH 6.8, 30% (v/v) glycerol, 10% SDS, 0.6 M DTT, 0.012% (w/v) bromophenol blue (*see* **Note 1**).
2. cOmplete, Mini, EDTA-free protease inhibitor cocktail (Roche).
3. PhosSTOP phosphatase inhibitor tablets (Roche).

4. Pierce 660 nm protein assay kit (ThermoFisher Scientific).
5. Ionic detergent compatibility reagent for Pierce 660 nm protein assay reagent (ThermoFisher Scientific) (*see* **Note 2**).

2.2 SDS–Polyacrylamide Gel

1. 1.5 M Tris–HCl, pH 8.8, autoclave.
2. 1 M Tris–HCl, pH 6.8, autoclave.
3. 10% (w/v) sodium dodecyl sulfate (SDS).
4. 10% ammonium persulfate (APS) (*see* **Note 3**).
5. 30% acrylamide–Bis (29:1) solution (Bio-Rad).
6. *N*,*N*,*N*′,*N*′-Tetramethyl-ethylenediamine (TEMED) (Bio-Rad) (*see* **Note 4**).
7. PageRuler prestained protein ladder, 10–180 kDa (ThermoFisher Scientific).
8. 10× SDS running buffer: 250 mM Tris, 1.92 M glycine, 1% SDS. Store at 4 °C. Dilute 1:10 before using.
9. 10% resolving gel: 4.0 mL H_2O, 3.3 mL 30% acylamide–bis, 2.5 mL 1.5 M Tris–HCl, pH 8.8, 100 µL 10% SDS, 100 µL 10% APS, 4 µL TEMED. This is enough to cast two mini resolving gels (e.g., 8.3 × 6.4 cm Bio-Rad mini gel). Leave 1 cm space below the top of the short glass plate for a stacking gel and overlay with H_2O. Remove H_2O after the resolving gel has polymerized.
10. 5% stacking gel: 2.7 mL H_2O, 0.67 mL 30% acylamide–bis, 0.5 mL 1 M Tris–HCl, pH 6.8, 40 µL 10% SDS, 40 µL 10% APS, 4 µL TEMED. Mix well and pour the mixture onto the polymerized resolving gels, then immediately insert the comb without introducing air bubbles.

2.3 Immunoblotting

1. PVDF membranes (Millipore).
2. Methanol (Sigma-Aldrich).
3. 10× Western blot transfer buffer: 250 mM Tris, 1.92 M glycine. Store at 4 °C.
4. 1× Western blot transfer buffer: add 700 mL H_2O and 200 mL methanol to 100 mL 10× Western blot transfer buffer.
5. 10× Tris-buffered saline (TBS) buffer: 500 mM Tris, 1.5 M NaCl, adjust pH to 7.6 with HCl.
6. 1× TBST buffer: Add 900 mL H_2O to 100 mL 10× TBS buffer, then add 500 µL Tween 20.
7. Blocking solution: 5% nonfat milk powder (w/v) in 1× TBST.
8. Filter paper (Bio-Rad).
9. Ponceau S solution for electrophoresis (Sigma-Aldrich).

10. Amersham ECL western blotting detection reagent (GE Healthcare Life Sciences).
11. Restore Western blot stripping buffer (ThermoFisher Scientific).

2.4 Antigens and Conjugates

1. Phospho-p44/42 MAPK(Erk1/2)(Thr202/Tyr204) Rabbit mAB #4370 (Cell Signaling Technology).
2. Anti-Tubulin alpha chain #AS10680 (Agrisera).
3. Amersham ECL Rabbit IgG, HRP-linked whole Ab (from donkey) (GE Healthcare Life Sciences).

2.5 Equipment and Supplies

1. 1.5 mL Eppendorf tubes.
2. Benchtop centrifuge.
3. Equipment for SDS-PAGE and Western blot.
4. Spectrophotometer or plate reader.
5. Platform rocker.
6. X-ray film cassette.
7. Amersham Hyperfilm ECL (GE Healthcare Life Sciences).

3 Methods

3.1 Protein Extraction

1. Add one tablet cOmplete, Mini, EDTA-free protease inhibitor cocktail and PhosSTOP phosphatase inhibitor to 10 mL protein extraction buffer right before the experiment. Vortex to dissolve the tablets (*see* **Note 5**).
2. Collect the healthy and PCD tissues and freeze in liquid nitrogen.
3. Grind tissue to fine powder using a pre-chilled mortar and pestle with liquid nitrogen. Transfer the frozen powder to a 1.5 mL pre-chilled Eppendorf tube with a spatula.
4. Add 2 volume extraction buffer. For example, for 100 mg of tissue, add 200 μL buffer.
5. Vortex until the samples are completely thawed. Keep at room temperature and continue with further samples.
6. Short spin all samples for 5 s at 11,000 × *g* to remove the sample from the tube lid.
7. Boil for 5 min.
8. Centrifuge at 11,000 × *g* for 5 min to precipitate debris.
9. Transfer the supernatant liquid to a fresh 1.5 mL Eppendorf tube.

3.2 SDS–Polyacrylamide Gel Electrophoresis

1. Add ionic detergent compatibility reagent to Pierce 660 nm protein assay solution then determine the protein concentration using a spectrophotometer or plate reader.
2. Adjust all the protein samples to the same concentration.
3. Load 5 μL Prestained protein ladder and 15 μL of each protein sample on the SDS-PAGE gel.
4. Run the SDS-PAGE gel with a constant voltage at 80 V as the dye front moves through the stacking gel then 120 V through the resolving gel, until the dye front reaches the bottom of the glass plates.

3.3 Western Blotting for Phosphor-MAPK3/6 and Tubulin

1. Soak the PVDF membrane in methanol.
2. Equilibrate the resolving gel and soak the PVDF membrane, filter paper, and fiber pads in 1× Western blot transfer buffer.
3. Assemble "sandwich" for membrane transfer. Place one prewetted fiber pad on the black side of the cassette. Place two sheets of filter papers on the fiber pad. Then place the gel on the filter paper. Place the equilibrated PVDF membrane on the gel. Gently roll the PVDF membrane using a test tube to remove air bubbles, then place another piece of filter paper on the membrane. Complete the "sandwich" by placing the second fiber pad and closing the cassette firmly.
4. Place the blotting module containing the "sandwich" into the tank and fill up with 1× prechilled Western blot transfer buffer.
5. Run the transfer at 100 V for 1 h in cold room.
6. Upon completion, disassemble the "sandwich" and place the PVDF membrane with the protein side up into 1× TBST buffer for 5 min at room temperature on a platform rocker.
7. Stain the PVDF membrane with Ponceau S solution for 1 min. Then destain in H_2O until the desired signal–background ratio is reached. Take a picture to make a record at this point to confirm all the protein samples are equally transferred onto the membrane (*see* **Note 6**).
8. Wash the membrane with 1× TBST for 5 min to remove the remaining Ponceau S solution.
9. Block the membrane in 10 mL blocking buffer by incubating on a rocking platform for 0.5–1 h at room temperature.
10. Discard the blocking buffer and replaced with 1: 2500 diluted anti-phosphor MAPK3/6 antibody (4 μL antibody in 10 mL blocking buffer). Rotate on a rocking platform for 2 h at room temperature.

11. Remove the primary antibody and wash four times for 10 min each with 1× TBST buffer on a rocking platform.
12. Incubate with the anti-rabbit HRP conjugated secondary antibody diluted 1:10,000 (1 μL antibody in 10 mL blocking buffer) on a rocking platform for 1 h at room temperature.
13. Discard the secondary antibody and wash four times for 10 min each with 1× TBST buffer on a rocking platform at room temperature.
14. Drain off the excess 1× TBST buffer from the washed membrane and place on a plate with protein-side-up.
15. Load 2 mL ECL detection reagents on the top of the membrane and incubate for 5 min.
16. Drain off excess detection reagents and place the membrane into a transparent plastic wrap.
17. Place the wrapped blot protein-side-up in an X-ray film cassette.
18. In a darkroom, place a sheet of Amersham Hyperfilm on the top of the wrapped blot, close the cassette, and expose for 30 s.
19. Remove the film from the cassette and develop it immediately. Adjust the exposure time until the desired signal–noise ratio is reached (*see* **Note 7**).

Below steps are for probing for tubulin as a loading control.

20. Strip the membrane in the stripping buffer for 30 min by rotating on a rocking platform (*see* **Note 8**).
21. Wash the membrane with 1× TBST three times for 5 min each.
22. Incubate the stripped membrane with 5000-fold diluted anti-tubulin antibody (2 μL in 10 mL blocking buffer) for 1 h at room temperature on a rocking platform.
23. Repeat **steps 11–19**.
24. The example results for phosphor-MAPK3/6 and tubulin western blots in healthy and PCD tissues are shown in Fig. 1. Here, the PCD tissues were from a maize mutant with autoimmune phenotype. One or two bands in phosphor-MAPK3/6 western blot will be shown depends on the organisms and tissues used.

3.4 Analyzing the Western Blots with ImageJ

1. Scan the phosphor-MAPK3/6 and tubulin films and save as. JPEG format.
2. Open the images using ImageJ and convert to a gray-scale image by going to *Image* > *Type* > *8-bit*.
3. Choose the "Rectangular Selections" tool from the ImageJ toolbar and draw a rectangle around the first band. Go to *Analyze* > *Gels* > *Select First Lane* to set the first band.
4. Click and hold in the middle of the rectangle on the first band and drag it over to the next band. Then go to

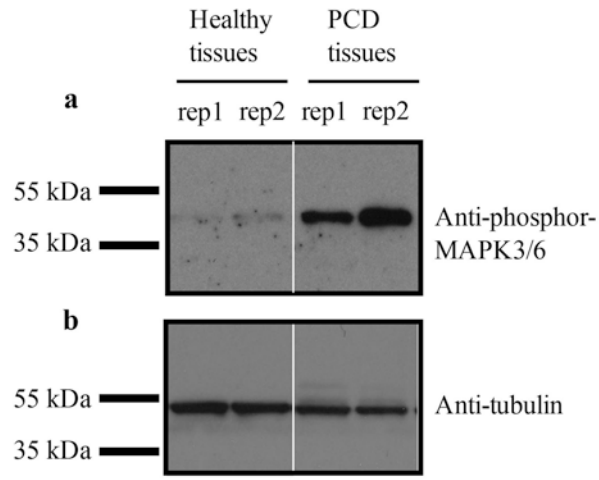

Fig. 1 Western blot results for phosphor-MAPK3/6 (**a**) and tubulin (**b**). There were duplicates for both healthy and PCD tissues from 10-day-old maize seedlings. The MAPK3/6 was highly phosphorylated in the PCD tissues

Analyze > *Gels* > *Select Next Lane* to set the second band. Repeat **steps 3** and **4** until all the bands are selected.

5. After all the bands are selected, go to *Analyze* > *Gels* > *Plot Lanes* to draw a profile plot for each lane.
6. Choose the "Straight Line" selection tool from the ImageJ toolbar. For each band that you want to analyze in the profile plot, draw a line across the base of the peak to enclose the peak.
7. Select the "Wand" tool from the ImageJ toolbar, then click inside the peak. The area of the peak will be returned. Record the peak area number for the band. Repeat this step until the peak areas of all the bands are collected.
8. Place the data in a spreadsheet. The first column should be the peak areas of the phosphor-MAPK3/6 bands, and the second column should be the peak areas of the tubulin bands.
9. The relative density of the phosphor-MAPK3/6 band is calculated by dividing its peak area by that of the tubulin band from the same sample. Then the phosphor-MAPK3/6 levels across different samples can be compared and the statistical analysis can be conducted using the relative density value.

4 Notes

1. DTT needs to be added freshly.
2. The ionic detergent compatibility reagent is stable in solution up to 1 day.

3. The freshly prepared APS can be aliquoted and stored at −20 °C for up to 1 month. The SDS-PAGE gel will not polymerize if using old APS.
4. TEMED is very toxic. Always open the bottle in the hood.
5. The protease inhibitor cocktail and phosphatase inhibitor tablets need to be added immediately before the protein extraction.
6. The Ponceau S staining should be performed before milk blocking, otherwise, it will result in a strong background.
7. Do not overexpose the film as it will affect the protein quantification.
8. It is very important to probe with the anti-phosphor MAPK antibody first, followed by the anti-tubulin antibody for loading control because the stripping buffer can remove the phosphor-groups of the proteins.

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