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George Komis
Jozef Šamaj *Editors*

Plant MAP Kinases

Methods and Protocols

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Cover illustration: The cover image is a collage showing comparison of whole seedlings of *Arabidopsis thaliana* Columbia wild type (left image) and a mitogen activated protein kinase 6-2 mutant (*mpk6-2*; middle image). Contrasting of the roots with toluidine blue histochemical stain allows better visualization and documentation of differences in root patterning between wild type and mutant using binocular optics. The image on the right illustrates a part of living *Arabidopsis thaliana* root stably overexpressing the heterologous SIMKK of *Medicago sativa* tagged with yellow fluorescent protein (YFP; pseudocolored dark red). The root was counterstained with FM4-64 (pseudocolored blue) to delineate root cell borders and visualized with confocal laser scanning microscope. In this way two successive and juxtaposed lateral root primordia are visible, reflecting the impact of SIMKK-YFP overexpression on lateral root formation. All images were kindly provided by Dr. Miroslav Ovečka (Department of Cell Biology, Centre of the Region Haná for Biotechnological and Agricultural Research, Palacký University Olomouc, Olomouc, Czech Republic).

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Preface

The highly expanded family of mitogen-activated protein kinases (MAPKs) allows remarkable versatility in the adaptation and tolerance of plants to abiotic and biotic stresses when compared to other eukaryotes. Furthermore, MAPK signaling modules in model plants such as *Arabidopsis thaliana* are densely interconnected, often function redundantly, while different MAPK pathways may cross talk during a single signaling event. The above complexity as well as the central role of MAPK signaling in the reaction and adaptation to often unfavorable abiotic and biotic conditions have prompted the vigorous research necessary to uncover MAPK signaling networks, not only in the model plant Arabidopsis but also in important crops. Ever since the first description of plant MAPKs, the number of publications related to the topic increases in an exponential manner. For the above reasons, we believe that the time is ripe to provide a central source of proofread and exhaustively troubleshot protocols that will encompass the entire array of experimental resources necessary for either the novice or the expert researcher.

The present book entitled "Plant MAP Kinases: Methods and Protocols" from the Methods in Molecular Biology™ series addresses the complexity of conditional and developmentally important plant MAPKs at many levels. Technically, the contents cover a wide array of techniques and methods used in MAPK research as these were contributed by experts of each method described.

The experimental survey of the plant MAPK world in the Part 1 is devoted to the collection of protocols aiming to interrogate MAPK function, and it starts with a robust transient expression system using Arabidopsis mesophyll protoplasts (Chapter 1). The following chapter (Chapter 2) addresses the MAPK transcriptional regulation during abiotic and biotic stresses with quantitative real-time PCR explained to thorough detail. Next three chapters (Chapters 3–5) are dedicated to the assessment of MAPK phosphorylation/activation and function by nonradioactive means. Chapter 3 demonstrates the efficient use of phospho-specific antibodies that were originally raised against mammalian MAPKs, in order to follow temporal and dose-dependent activation of MAPKs. The other two chapters employ two different electrophoretic approaches which allow the efficient discrimination of phosphorylated and non-phosphorylated protein forms in one dimension. This can be achieved by either one-dimensional isoelectric focusing (Chapter 4) or phospho-affinity-based denaturing SDS-PAGE (Chapter 5). Part 2 encompasses protocols discovering function of MAPK signaling by genetic tools including the engineering of constitutively active MAPKs (Chapter 6), the silencing of MAPKs by either virus-induced silencing (Chapter 7) or RNA interference (Chapter 8). In Part 3 effort is made to put MAPK signaling at the cellular context. Thus MAPKs are immunolocalized in either root whole-mount samples (Chapter 9) or Steedman wax sections (Chapter 10), while strategies for their in vivo imaging as well as for the subcellular visualization of their interactions are presented in following respective chapters (Chapters 11 and 12). Part IV surveys approaches to identify and study MAPK substrates. Thus, Chapter 13 shows the design of experimental work necessary to identify phosphorylation sites in putative MAPK substrates using as an example the microtubule-associated protein MAP65-1. Next, a strategy to generate phospho-specific

antibodies against verified substrates such as the WRKY transcription factors is presented (Chapter 14). Finally, a mutational approach towards the identification of MAPK substrates is aimed to uncover previously unknown targets of MAPK signaling (Chapter 15). The last part of the book tops up MAPK research and brings into light large-scale protocols. These will provide strategies for high-throughput screening of MAPK interactors by yeast two-hybrid technique (Chapter 16) or by protein microarrays (Chapter 17). Chapter 18 provides the protocol for tandem affinity purification of MAPK complexes. Finally, Chapter 19 describes iTRAQ for the enrichment of phosphoproteins which will allow the mass identification of MAPK targets.

On the side of the "Plant MAP Kinases: Methods and Protocols" the reader will find classical protocols that accompany MAPK research, including immunocomplex and in-gel kinase assays as well as redundant information of thoroughly described workflows including plant handling, work with transgenes and standard biochemical techniques such as co-immunoprecipitation, polyacrylamide gel electrophoresis, and western blotting to name a few. We trust therefore that all individual chapters are autonomous and can be used as a bench-side aid to researchers irrespective of the level of experience.

We are grateful to all 54 authors who contributed to the content of the present volume. Each author disclosed his/her experience in each respective chapter, but also provided critical troubleshooting—Notes—representing important sections for the novice reader. After all even the most ambitious experiment may fail due to the tiniest detail. We warmly acknowledge Professor John M. Walker, series editor, who apart from honoring us with the invitation to host the present volume also provided enthusiastic support throughout the entire editing procedure. Finally, we extend our thanks to the members of the Methods in Molecular Biology™ Springer editorial team for guiding us through the assembly of a useful book.

Olomouc, Czech Republic

George Komis
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Part I

Expression and Activity of Plant MAPKs

Chapter 1

Transient Expression in *Arabidopsis* Leaf Mesophyll Protoplast System for Cell-Based Functional Analysis of MAPK Cascades Signaling

Jong Hee Im and Sang-Dong Yoo

Abstract

Mitogen-Activated Protein Kinase (MAPK) cascade is one of the main signaling components mediating abiotic and biotic stress and hormone information in plants. Plant MAPK study has been impeded with a genetic approach using a long-term phenotypic analysis in spite of the transient nature of the protein kinase signaling. *Arabidopsis* leaf mesophyll protoplasts provide a versatile resource for diverse cell-based assays to acquire immediate molecular and biochemical responses with transient expression of MAPK cascade components of interests. Thus, it is an attractive tool for a high-throughput functional analysis of *Arabidopsis* MAPK cascade signaling. However, transient expression in *Arabidopsis* mesophyll protoplast (TEAMP) system requires mastered skills for protoplast preparation and handling to achieve steady and stable data. Here, we have described two analytical methods for MAPK cascade signaling using TEAMP system.

Key words *Arabidopsis* leaf mesophyll protoplasts, Transient expression, Immunocomplex kinase assay, Protein blot analysis, Mitogen-Activated Protein Kinase cascade

1 Introduction

The evolutionarily conserved mitogen-activated protein kinase (MAPK) is one of the well-characterized information processing modules that can mediate various internal and external signals in microbes, plants and animals [1–3]. Upon signaling, MAPK is activated by the dual phosphorylation of threonine (T) and tyrosine (Y) residues of the TXY motif in a typical MAPK catalytic domain [4]. Mammalian MAPKs belonging to the Extracellular Signal-Regulated Protein Kinase (ERK) group have a TEY motif, whereas cJUN N-terminal kinases (JNK) and p38 MAPK have TPY and TGY motifs, respectively [5]. These different classes of MAPKs are activated by specific extracellular stresses and involve in distinct cellular responses in mammalian cells. In plant genomes MAPKs

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have been identified and characterized as those containing only a TEY motif so far. Thus, plant MAPK may have phosphorylation mechanisms similar to ERKs rather than JNKs and p38 MAPKs in mammalian genomes.

To constitute a phosphorylation cascade MAPK is activated by upstream kinase, MAPK kinase (MAPKK) that is activated by MAPKK kinase (MAPKKK) [6]. The MAPK cascade is found in most eukaryotic signaling pathways. A plant model system *Arabidopsis* (*Arabidopsis thaliana*) contains genes encoding putative 20 MAPKs, 10 MAPKKs and more than 80 MAPKKKs in the genome [7], although biochemically wired complete MAPK signaling cascades for a specific stimulus are rare [8].

Arabidopsis has many advantages with respect to genetic studies. It contains a small size genome (Mb), and produces a large number of offspring with self-pollination. Moreover, *Arabidopsis* can be easily transformed with a well-established protocol using *Agrobacterium tumefaciens.*

Nevertheless, *Arabidopsis* has a major disadvantage concerning genetic analysis as the genome has recently endo-duplicated and shown characteristics of a primitive tetraploid. For a genetic study relying on a specific mutant defective in a gene of interest, a typical mutational effect is expected to be observed after deleting four copies of the gene in *Arabidopsis* genome in theory. This is obviously time-consuming and cost-ineffective.

Arabidopsis MAPK study has been particularly hindered with genetic analysis that is popular in plant biology. With a little over hundred genetic components of *Arabidopsis* MAPK cascades in its genome only a handful MAPKKK mutants have been identified and characterized for their signaling functions with mutant-based phenotype analysis [9]. Again gene functional redundancy is a major drawback in genetic analysis of MAPK cascades as intracellular signaling often diverges and/or converges via multiple components in MAPK cascades. In addition, oversaturated screens of T-DNA insertion lines still miss some knock out mutants for MAPK components [10]. Furthermore, an instant nature of MAPK activity makes it conceptually difficult to study the PK signaling function with a long-term phenotypic analysis of a mutant.

A transient expression in a versatile *Arabidopsis* mesophyll protoplast (TEAMP) system is an alternative simple and fast solution for plant MAPK signaling study. TEAMP system is a powerful tool for various types of cell-based assays for enzyme (in)activation, protein quantitation, protein subcellular localization, and protein–protein interaction [11]. TEAMP system has numerous merits in molecular and biochemical analysis of *Arabidopsis* MAPK signaling. First MAPK cascade component expression and its cellular response can be literally acquired in TEAMP system within 12 h [12]. Eventually whole experimental data can be achieved in several days.

Although constitutive overexpression of protein coding genes is routinely used in TEAMP system, inducible and/or controlled expression can be achieved using a chemical inducible promoter activity and/or a calculated level of effector constructs for protoplasts transfection. Moreover, protein functional analysis can be designed in a multidimensional means as protoplasts can be treated with potent chemicals and conditions with anticipated cellular responses.

However, TEAMP system also has some limitation. CsCl–DNA preparation is desirable for efficient transfection to protoplasts, which needs extra facility and efforts. Protoplast generation is currently limited to a few cell types that can provide authentic cellular contexts for protein behaviors. Interpretation of data for proteins that are ectopically expressed in mesophyll protoplasts has to be considered together with other complementary experimental data. Moreover, to gain reliable data using TEAMP system, skills on protoplast preparation and handling should be mastered through repeated practices with positive and negative control experiments.

As learned enough about protoplasts for cell-based protein functional assays, we have described two methods for MAPK cascade signaling analysis using TEAMP system.

2 Materials

2.1 Plant Growth and AMP Isolation

1. *Arabidopsis* seeds: Col-0 (*Arabidopsis* Biological Resource Center; http://abrc.osu.edu).
2. Plant growth facility: with programmable light (12 h 50–70 μE) and dark (12 h) regime, temperature (24 °C), and humidity (ca. 50 %).
3. Professional Growth Mix (Sun Gro, USA).
4. Generic razor blades.
5. Arabidopsis Mesophyll protoplast (AMP) enzyme mixture solution: Add 1.5 % (w/v) of Cellulase R10 and 0.8 % (w/v) of macerozyme R10 (Yakult Pharmaceutical) to preheated 20 mM MES–KOH pH 5.7, 0.4 M D-mannitol, and 20 mM KCl solution. Incubate the solution at 55 °C for 10 min and cool down on ice. After cooling down to room temperature, add 10 mM $CaCl_2$ and 0.1 % (w/v) BSA to final concentrations.
6. 100 × 25 mm petri dish.
7. Bell-shaped desiccators (e.g., Thermo-Fisher Scientific Inc.).
8. 70 μm nylon mesh (e.g., Carolina Biological Supplies Co).
9. 30 mL round bottom centrifuge tube (e.g., Kinesis LTD).
10. Benchtop centrifuge (e.g., Vision Scientific LTD).

11. W5 solution: 2 mM MES–KOH pH 5.7, 0.4 M D-mannitol, 15 mM $MgCl_2$.
12. MMG solution: 4 mM MES–KOH pH 5.7, 0.4 M D-mannitol, 15 mM $MgCl_2$.

2.2 Transient Expression of MAPK in Arabidopsis Leaf Mesophyll Protoplasts

1. Neubauer 0.1 mm-deep hemocytometer (Hausser Scientific Co.).
2. Round bottom 2 mL microfuge tube.
3. Calcium-polyethylene glycol (PEG) solution: 40 % (w/v) PEG 4000, 0.2 M D-mannitol, and 100 mM $CaCl_2$.
4. WI solution: 4 mM MES–KOH pH 5.7, 0.5 M D-mannitol, 20 mM KCl.
5. 6-well cell culture plate.
6. 1.5 mL of microfuge tube.

2.3 MAPK Assay (Immunocomplex Kinase Assay)

2.3.1 Immunoprecipitation and In Vitro Kinase Assay

1. Kinase buffer: 20 mM Tris–HC1 (pH 7.5), 40 mM $MgCl_2$, 5 mM EDTA, and 1 mM DTT.
2. Vortexor (e.g., Vision Scientific Ltd).
3. Tabletop Centrifuge (e.g., Eppendorf Ltd).
4. Protein A-agarose (e.g., F. Hoffmann-La Roche Ltd).
5. Angled rotary shaker (e.g., Thermo-Fisher Scientific Inc., USA).
6. Anti-HA and Anti-Myc antibody (e.g., Covance Inc., USA).
7. IP buffer: 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 50 mM EDTA, 1 mM DTT, 1× protease inhibitor cocktail, 1 % (v/v) Triton X-100, 10 mM NaF, and 10 mM Na_3VO_4.
8. Phosphorylation buffer: 20 mM Tris–HC1 (pH 7.5), 40 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 0.1 mM ATP, 0.25 mg/mL MBP, and 50 μM [γ^{32}P]ATP (or 50 μM ATP).
9. 5× SDS Sample buffer: 225 mM Tris–HCl pH 6.8, 50 % (v/v) glycerol, 5 % (w/v) SDS, 225 mM DTT, and 0.5 % (w/v) bromophenol blue.
10. Heat block (e.g., Bioer technology Co. Ltd, CHN).
11. Protein gel electrophoresis system (e.g., Bio-Rad, USA).
12. Running buffer: 100 mM Tris, 400 mM glycine, and 0.1 % (w/v) SDS.
13. Washing buffer: 10 % (v/v) ethanol and 3 % (v/v) acetic acid.
14. Vacuum-heat drier (e.g., Major Science, USA).
15. 3MM paper (Whatman, USA).

2.3.2 MAPK Activity Detection with Western Blot Analysis

1. Polyvinyldifluoride (PVDF) membrane (e.g., Millipore, USA).
2. Generic 100 × 40 × 20 mm square box.
3. Absolute methanol (e.g., SAMCHUN Co. LTD, USA).

4. Solution I: 300 mM Tris and 10 % (v/v) methanol.
5. Solution II: 25 mM Tris and 10 % (v/v) methanol.
6. Solution III: 25 mM Tris, 40 mM glycine, and 10 % (v/v) methanol (adjust pH 9.4 with 2 M Tris).
7. TBS: 30 mM Tris–HCl (pH 7.5) and 150 mM NaCl.
8. TBST: 30 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.5 % (v/v) Tween 20.
9. Blocking buffer: 5 % (w/v) nonfat dry milk in TBST.
10. Anti-pTEpY antibody (e.g., Cell signaling, USA).
11. IR-800 conjugated anti-mouse antibody (e.g., LI-COR, USA).

3 Methods

3.1 Plant Growth and AMP Isolation

1. Incubate *Arabidopsis* seeds at 4 °C for 3–4 days in water for cold treatment and grow on soil in growth room with 12 h/12 h light at constant temperature of 24 °C. Adjust the light intensity to 50–70 μE and keep the relative humidity to around 50 %. In this condition, 3.5–4-week-old plants are useful for generating *Arabidopsis* Mesophyll Protoplasts (AMP).
2. Collect two to four rosette leaves from each plant and slice the leaves as thin as possible with a razor blade. It is desirable not to exceed 1 mm thickness to increase cell wall-digesting enzyme accessibility.
3. Submerge the leaf slices in the AMP enzyme mixture solution prepared in a petri dish.
4. Place the petri dish in a bell-shaped desiccator and apply vacuum at room temperature for 30 min in the dark to infiltrate the enzyme solution into the leaf slices. After vacuum infiltration, incubate the cell wall digesting solution in the dark at room temperature for another 2–3 h.
5. Filter AMP enzyme solution through a layer of 70 μm nylon mesh into a 30 mL round bottom tube. Centrifuge at $100 \times g$ for 2 min and remove supernatant of enzyme solution.
6. Resuspend the pellet with 5 mL of cold W5 solution by gentle shaking and stand straight the W5 resuspension on ice for 30 min.
7. Remove cell debris in W5 solution by pipetting out and resuspend the pellet with MMG solution and keep at room temperature (*see* **Note 1**).

3.2 Transient Expression of MAPK in Arabidopsis Mesophyll Protoplasts

1. Determine AMP number by using hemocytometer and adjust to $2–4 \times 10^4$ in 200 μL of MMG.
2. Combine 10 μL of *MAPK-HA* and 10 μL of *MKK-Myc* DNA constructs (2 μg/μL each) in a round bottom 2 mL microfuge

tube with 200 μL of protoplast, followed by mix them with gentle tapping 2–3 times (*see* **Note 2**).

3. Add 220 μL of PEG solution and again mix them thoroughly by gentle tapping several times.
4. Place the mixture at room temperature for 4 min and add 800 μL of WI solution, followed by inverting them with more than two times.
5. Immediately centrifuge at 200×*g* for 2 min at room temperature.
6. Remove the supernatant and resuspend the pellet in 1 mL of WI with gentle tapings.
7. Transfer the AMP in WI to each well of 6-well cell culture plate.
8. Incubate under dim light (30–40 μE) at room temperature for 6 h.
9. After incubation, gently shake and transfer AMP contained WI to 1.5 mL of microfuge tube, followed by centrifugation at 360×*g* for 2 min at room temperature.
10. Remove the supernatant, flash-freeze, and keep the protoplasts in −80 °C until further use.

3.3 MAPK Assay (Immunocomplex Kinase Assay)

3.3.1 Immunoprecipitation and In Vitro Kinase Assay

1. Add 200 μL of kinase buffer to frozen AMP samples, mix them by tapping several times and, incubate on ice for 5 min. Briefly vortex the samples and incubate on ice for another 5 min, followed by centrifugation at 15,800×*g* for 5 min at 4 °C.
2. Transfer whole supernatant to new tubes and add 10 μL of 50 % slurry protein A-agarose and incubate on an angled rotary shaker at 50 rpm for 2 h at 4 °C.
3. Centrifuge at 15,800×*g* for 1 min at 4 °C and transfer the supernatant to a new tube.
4. Add 1 μL of anti-HA antibody (0.5 μg/μL) to each sample and incubate on an angled rotary shaker at 50 rpm for 4 h at 4 °C.
5. Spin briefly and add 10 μL of 50 % slurry protein A-agarose to each sample and incubate 50 rpm for 4 h at 4 °C on the shaker.
6. Precipitate immunocomplexed MAPK-agarose with centrifugation at 15,800×*g* for 1 min at 4 °C.
7. Remove supernatant and wash the agarose pellet with 1 mL of the IP buffer. Repeat the washing step three times.
8. For MAPK assay in vitro, add 10 μL of phosphorylation buffer to each sample and incubate at room temperature for 30 min and apply vigorous shaking every 10 min.

9. Add 5 μL of 5× SDS sample buffer to stop the reaction and mix well by tapping several times (*see* **Note 3**).
10. Heat up the sample with 95 °C for 3 min for protein denaturation and then cool down on ice for 5 min.
11. Centrifuge at 15,800 × *g* for 1 min and transfer supernatant to a new tube and keep it on ice.
12. Separate proteins by sodium dodecyl sulfate–polyacrylamide gel (15 %) electrophoresis (SDS-PAGE) system (110–130 V, ca. 90 min) until the Coomassie dye in the sample reaches to near the bottom of the gel.
13. Cut the stacking gel and the Coomassie dye-stained separation gel. Incubate separating gel in washing buffer on a rotary shaker at 50 rpm for 10 min. Repeat the washing step three times.
14. Put the gel on a layer of plastic wrap and then put three layers of 3MM papers on the gel. Flip over of the assembled layers on a gel drier. Dry gel by applying vacuum at 70 °C for 30 min.
15. Detect MAPK activity using X-ray film or image analyzer (Fig. 1).

3.3.2 MAPK Activity Detection by Protein Blot Analysis

1. Conduct the processes to **step 12** in Subheading 3.3.1, except **step 8**.
2. Cut the stacking gel off and transfer the separating gel to Solution III in a 100 × 40 × 20 mm square box. Wash out SDS by gentle shaking at 50 rpm with changing solution every five minutes for three times.
3. Soak the PVDF membrane in absolute methanol for 30 s and wash three times with Solution II and keep it in Solution II.
4. Cut six pieces of 8 × 4 cm 3MM papers, soak three pieces in Solution I and the other three pieces in Solution III.
5. Put three pieces of 3MM papers soaked in Solution I on an electrotransfer machine, put the PVDF membrane on the paper, stack the gel on the membrane, and put the other three pieces of 3MM papers soaked in Solution I on the gel (*see* **Note 4**). Transfer proteins with appropriate voltage and time (at 10 V for 30 min or at 15 V for 15 min).
6. After transferring, remove gel and 3MM papers and then submerge the membrane in TBS with gentle shaking.
7. Decant the buffer and incubate the membrane with blocking buffer by gentle shaking at 50 rpm for 60 min.
8. Prepare anti-pTEpY antibody with TBS (1:1,000–3,000) in 5 mL and keep on ice or in 4 °C refrigerator.
9. Decant the blocking buffer and wash with 5 mL of TBST for 5 min. Repeat this step three times.
10. Add antibody solution and incubate on a rotary shaker at 50 rpm for 1 h at room temperature.

11. Wash the membrane with 5 mL of TBST with incubation on a rotary shaker at 50 rpm for 5 min at room temperature. Repeat this step three times.
12. Place the membrane in a dark box, add 10 mL of anti-mouse antibody conjugated with infrared-800 (1:100,000), and incubate on a rotary shaker at 50 rpm for 1 h at room temperature.
13. Wash the membrane as in **step 9**.
14. Analyze MAPK activity with an IR-image detector (Odyssey, LI-COR, USA; *see* Fig. 1).

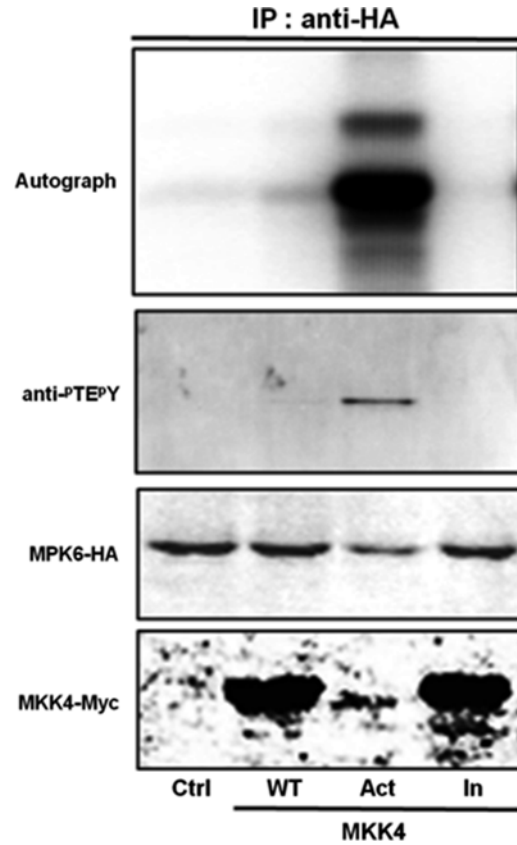

Fig. 1 MPK6 activation by an active form of MKK4. Individual designated *MKK4-Myc* were co-transfected with *MPK6-HA* to *Arabidopsis* mesophyll protoplasts. Transfected protoplasts were incubated for 6 h and MPK6-HA was immunoprecipitated using anti-HA antibody. MPK6 activity was measured with either in vitro kinase assay with myelin basic protein as a general substrate or protein blot analysis with anti-pTEpY antibody. (Ctrl control, WT MKK4 wild-type MKK4, Act MKK4 active form of MKK4, In MKK4 inactive form of MKK4). MPK6 and MKK4 expression was shown by protein blot analysis using anti-HA and anti-Myc antibodies, respectively

4 Notes

1. You can observe two types of separated areas in W5 solution after incubating on ice for 30 min. Dark-green area is comprised of AMPs at the lower part, and light green area is cell debris. It is recommended that you remove the light green cell debris as much as you can to harvest intact leaf mesophyll protoplasts.
2. In order to transfect DNA to protoplasts, DNA purity is an important factor determining transfection efficiency. DNA preparation is recommended to use a method using a CsCl density gradient [13].
3. Until you perform immunoprecipitation and in vitro assay for MAPK activity, you can keep the samples in −20 °C for several days after Subheading 3.3.1, **step 9**.
4. Solution should be sufficiently applied to each layer, and air bubbles should be removed between layers.

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Chapter 2

Quantification of Stress-Induced Mitogen-Activated Protein Kinase Expressional Dynamic Using Reverse Transcription Quantitative Real-Time PCR

Pavel Křenek and Veronika Smékalová

Abstract

Although it is generally accepted that signal transduction in plant mitogen-activated protein kinase signaling cascades is regulated via rapid posttranslational modifications, there are also several compelling examples of swift stress induced transcriptional activation of plant MAP kinase genes. A possible function of these fast and transient events is to compensate for protein losses caused by degradation of phosphorylated MAP kinases within stimulated pathways. Nevertheless, there is still need for additional evidence to precisely describe the regulatory role of plant MAP kinase transcriptional dynamics, especially in the context of whole stress stimulated pathways including also other signaling molecules and transcription factors. During the last two decades a reverse transcription quantitative real-time PCR became a golden choice for the accurate and fast quantification of the gene expression and gene expression dynamic. In here, we provide a robust, cost-effective SYBR Green-based RT-qPCR protocol that is suitable for the quantification of stress induced plant MAP kinase transcriptional dynamics in various plant species.

Key words Plant mitogen-activated protein kinase, Abiotic stress, Biotic stress, Tri reagent, Reverse transcription quantitative real-time PCR, SYBR green

1 Introduction

Plant mitogen-activated protein kinase (MAPK) cascades are complex signaling pathways that transduce myriads of extracellular environmental and developmental signals into cellular responses. Signal transduction in a MAP kinase pathway is mediated via consecutive phosphorylation of key pathway components in which the most upstream MAPKKK phosphorylates MAPKK which in turn phosphorylates downstream MAPK. Once activated MAPK transmits phosphorylation signal into variety of substrates like transcription factors, protein kinases, and cytoskeleton-associated proteins [1]. In many well documented cases, especially those addressing abiotic and biotic stress stimulation, the phosphorylation of MAP

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kinase pathway components is extremely fast, occurring within minutes post-stress. Such a fast signal transduction enables plants to quickly reprogram cellular metabolism resulting into a timely adaptation to stress. Nevertheless, not only are plant MAP kinases swiftly activated, but also the gene expression of MAP kinases might be induced by stress in short time periods.

There are several compelling examples of rapid stress induced transcriptional activation of plant MAP kinase genes. We summarize here and compare the expression data mainly for the well-studied *Arabidopsis* MAP kinase genes *AtMPK3* and *AtMPK6* together with their orthologues in other plant species. In one of the pilot studies, the expression levels of *AtMPK3* and *AtMEKK1* (*Arabidopsis* MAPKKK) were shown to be markedly increased within 1 h following cold and salt exposure [2]. The same work shows in parallel rapid increase in mRNA levels of these two MAP kinase genes occurring within 10 min after touch stimulation of rosette leaves. Interestingly, at least transcriptional activation of *AtMPK3* appears to be of transient nature as evidenced by the resumption of gene expression basal levels within 40 min only. The transient nature of the rapid transcriptional activation of *AtMPK3* was also documented in hydroponically grown *Arabidopsis* seedlings elicited with chitin and in soil grown *Arabidopsis* plants exposed to ozone [3, 4]. On the other hand, the expression of *AtMPK6* was indifferent to chitin treatment for up to 4 h while ozone induced weakly only but more persistently the expression of *AtMPK6* in the same experimental frameworks. Other stimuli including low temperature, wounding, touch and low humidity induced not any or rather weak transcriptional activation of *AtMPK6* and *AtMPK4* in the 6–8 weeks old *Arabidopsis* plants [5]. Interestingly, these contrasting expression patterns of *AtMPK3* and *AtMPK6* induced by various stress factors were also observed in the case of *Nicotiana WIPK* orthologue of *AtMPK3* and *Nicotiana SIPK* orthologue of *AtMPK6*. While WIPK gene is rapidly and transiently transcriptionally activated within minutes post wounding and this activation is even more pronounced post wounding when oral secretions of *Manduca sexta* are co-supplied, the expression of *SIPK* is rather weakly but more persistently induced under the same experimental regime [6, 7]. *WIPK* gene is also transiently induced within 20–30 min after infiltration with water, cutting and abrasion, conditions against which *SIPK* expression is non-responsive for as long as 6 h [8]. In addition to this, no changes in *SIPK* mRNA levels were detected for nearly 1 day after salicylic acid treatment or fungal elicitation of tobacco cell suspension culture [9]. Another example concerns the transcriptional activation of an *AtMPK3* orthologue from alfalfa, namely, *MMK4*, which was shown to be rapidly induced by cold, drought, and wounding [10, 11].

A possible explanation for the rapid and transient transcriptional activation of plant MAP kinases was exemplified in the case of wound induced alfalfa MAP kinase pathway (*reviewed in* [12]). In this work, it is mentioned that wound induced transcriptional upregulation of the MAPK gene is not correlated with an increase in the amounts of the MAPK protein. To explain this discrepancy, evidence is stated there for the degradation of wound-activated MAP kinase, followed by the compensation of MAPK protein lost by the rapid transient activation of MAPK gene. Thus, the rapid transcriptional activations of MAPK gene are to reset the MAP kinase pathway. The MAP kinase pathway is switched off by both rapid degradation of activated MAPK protein and the transcriptional induction of *MP2C* encoding a protein phosphatase type 2C, a protein that degrades wound induced MAP kinase cascade (*reviewed in* ref. 12). In many cases described in the previous paragraph, the rapid transient increase in the activity is provided as an evidence for the involvement of MPK6 kinase or its functional protein homologue SIPK in a particular stress signaling [3–5, 7–9]. However, in those occasions, the expression of *AtMPK6* or *SIPK* is either not induced or induced rather weakly as already mentioned. Conversely, the rapid transient increase in the activity of AtMPK3, WIPK, or MMK4 is accompanied by the rapid, pronounced transient transcriptional induction of *AtMPK3* or its orthologues [3–7, 10, 11]. Hypothetically the differential transcriptional regulation of *AtMPK3* and orthologues (*WIPK* or *MMK4*) and *MPK6* or *SIPK*, may relate to the need for compensation at the protein level.

The occurrence, the mechanisms and the rationale behind plant MAP kinase transcriptional regulation and its role in MAP kinase signaling needs to be further substantiated. Future experiments intended for the characterization of stress induced expression of MAP kinase gene pathways should be designed carefully by combining short and long term stress treatments. Wherever possible, protein abundance and kinase activity data should be comparatively studied together with transcriptional dynamics. Moreover, the experiments should be performed in an organ or tissue specific manner and should involve not only proved or putative MAP kinase gene pathways but also other signaling components and transcription factors. An excellent framework for such analyses was recently provided in *Arabidopsis* [13].

Since its appearance in mid-1990s [14–16], quantitative real time PCR (qPCR) was quickly commercialized and became an essential tool for the quantification of nucleic acids. The basic principle of the method relies on the usage of fluorescent dyes for the labeling of PCR products that arise during PCR cycling. The reaction is performed in real-time PCR devices that in addition to the rapid thermal cycling simultaneously measure the accumulation of

fluorescent signal during the PCR exponential phase. The online measurement of fluorescence accumulation enables the accurate quantification of PCR product and further objective data analysis. The reverse transcription quantitative real-time PCR (RT-qPCR) based quantification of the gene expression offers several advantages [17] with its high sensitivity being the most important one. It was demonstrated that the detection limit of qPCR could be as low as two copies of gene [18]. The SYBR Green or TaqMan assays were shown to provide 4- to 5-log dynamic range of amplification, while being much more sensitive than semiquantitative RT-PCR with subsequent densitometric analysis or ribonuclease protection assay [19, 20]. On the other hand, the high sensitivity of RT-qPCR is also its major disadvantage. Therefore, good laboratory practice precautions must be taken to prevent cross-contamination of samples, chemicals, consumables, and devices with plasmid DNA, PCR products, or genomic DNA.

Herein we describe a robust RT-qPCR method which is routinely used in our laboratory for the quantification of stress induced plant MAP kinase transcriptional dynamics. Our approach relies on TRI reagent RNA extraction which proved to be efficient in various plant organs and tissues from different plant species such *Arabidopsis thaliana*, *Medicago* sp., and *Hordeum vulgare*, using of M-MLV reverse transcriptase for cDNA synthesis and SYBR Green based amplicon detection. As an example of the method application, quantification of MAP kinase transcriptional dynamics in the roots of *Arabidopsis* exposed to osmotic stress (NaCl) is presented.

2 Materials

All standard chemicals should be of analytical grade. Except for sterilization of seeds, growing and treatment of *Arabidopsis* plants, solutions should be prepared in RNase and DNase-free ultrapure water (18.2 MΩ × cm specific resistivity). Toxic compounds should be handled appropriately under fume hood and according to institutional safety regulations. Always wear gloves and protective clothing.

2.1 Growth Media, Sterilization of Plants and Their Treatment

1. ½ Murashige–Skoog medium (1/2 MS; solid/1 L): 2.2 g MS medium (Duchefa) without vitamins, 10 g sucrose, 0.8 % (v/v) Phytagel, pH 5.8 (KOH).
2. ½ Murashige–Skoog medium (liquid/1 L): 2.2 g MS medium without vitamins, 10 g sucrose, pH 5.8 (KOH).
3. 70 % (v/v) ethanol, 96 % (v/v) ethanol, 150 mM NaCl.

2.2 RNA Isolation

1. TRI Reagent solution (Sigma-Aldrich).
2. 1-Bromo-3-chloropropane (BCP) (Molecular Research Center).
3. Micropestle (Eppendorf).
4. Isopropanol.
5. 75 % (v/v) ethanol.
6. Filtered tips.

2.3 DNase I Treatment

1. DNase I (Thermo Scientific).
2. 10× DNase I reaction buffer (Thermo Scientific).
3. EDTA (Thermo Scientific).

2.4 cDNA Synthesis

1. 100 μM PAGE purified 18 bp oligo dT primers.
2. M-MLV Reverse Transcriptase 5× reaction buffer (Promega).
3. Deoxynucleotide mix (dNTPs) (Fermentas).
4. RNasin® Plus RNase inhibitor (40 U/μL) (Promega).
5. M-MLV Reverse Transcriptase (100 U/μL) (Promega).

2.5 Quantitative Real-Time PCR

1. Power SYBR® Green PCR Master Mix (Life Technologies).
2. 96-well microtiter plates (Life Technologies).
3. MicroAmpTM Optical Adhesive Film (Life Technologies).

3 Methods

3.1 General Working Precautions

1. Designate a clean working area, where only RNA and RT-qPCR work is conducted. Ideally, this area should be isolated from laboratory spaces dedicated to cloning, transformation, and gel electrophoresis in order to avoid contamination with plasmids, PCR products, or genomic DNA.
2. Designate pipettes, pipette tips, tubes, and other tools and consumables for RNA and RT-qPCR work only (*see* **Note 1**). Such equipment and consumables should stay only in the RNA and RT-qPCR working area.
3. To avoid RNase contaminations coming from hands always use gloves when working with RNA.
4. Clean the working area with diluted household bleach and let it dry for a while immediately before starting work. Afterwards clean the working area with 70 % (v/v) ethanol. These steps should decontaminate the working area from microorganisms and RNase contamination. Also clean the fume hood, where TRI reagent based RNA isolation will be carried out.

3.2 Growing of Arabidopsis Seedling and Their Treatment

1. Sterilize seeds of *Arabidopsis thaliana* ecotype Col-0 by 70 % (v/v) ethanol for 5 min, then follow with 96 % (v/v) ethanol sterilization for 1 min and finally wash the seeds by sterilized water two times for 5 min. During each step shake gently the tube with the seeds (*see* **Note 2**).
2. Keep the seeds on the sterilized filter paper to dry.
3. Plate the seeds on square petri dishes containing ½ MS medium and place them for germination and growth in environmental chamber for 2 weeks under following conditions: 21 °C, 70 % humidity, 16 h light–8 h dark.
4. Prepare 150 mM NaCl solution, pour it to the dish in horizontal position and incubate plants for 3 h at the root temperature. The dish should be very gently shaken. Collect roots of Arabidopsis plants at 15 min, 30 min, 1 h, 2 h and 3 h post stress exposure. Be careful that all plants are well submerged in the solution (*see* **Note 3**).

3.3 RNA Isolation

Unless indicated otherwise, work at the room temperature.

1. Using sterile scalpel and forceps collect approximately 50 mg fresh weight of *Arabidopsis* roots into a 2 mL round bottom microfuge tube and immediately freeze the samples by immersion into liquid nitrogen (*see* **Note 4**).
2. Disrupt root samples in a 2 mL round bottom tubes using micropestle and liquid nitrogen to a fine powder (*see* **Note 5**).
3. Add 0.7 mL of TRI Reagent solution into liquid nitrogen or dry ice prechilled sample tube, homogenize sample via pipetting up and down several times, and incubate the homogenate for 7 min at room temperature (*see* **Note 6**).
4. Centrifuge sample tubes at 12,000 × *g* for 10 min at 4 °C to get rid of insoluble debris and transfer the resulting supernatant into a clean tube.
5. Add 70 μL of 1-bromo-3-chloropropane, cap sample tubes tightly, and mix by vigorous shaking for at least 15 s. Incubate for 5 min at room temperature.
6. Centrifuge sample tubes at 12,000 × *g* for 15 min at 4 °C. Following centrifugation transfer upper aqueous phase containing RNA into to a clean tube (*see* **Note 7**).
7. Add isopropanol (70 % (v/v) of aqueous phase volume), mix by inverting the tubes several times, and incubate 8 min at room temperature (*see* **Note 8**).
8. Centrifuge at 12,000 × *g* for 8 min at 4 °C and decant the supernatant (*see* **Note 9**).
9. Wash samples with 0.7 mL of 75 % (v/v) ethanol. Do not vortex (*see* **Note 10**).

10. Centrifuge sample tubes at 7,500 × *g* for 5 min, remove ethanol, and briefly dry RNA pellet on laboratory bench (*see* **Note 11**).
11. Dissolve RNA pellet in water by pipetting several times up down and determine the RNA concentration using NanoDrop spectrophotometer. Aliquot each RNA sample into two to three tubes, and store at −80 °C.

3.4 DNase I Treatment (See Note 12)

Unless indicated otherwise, work on ice.

1. Use 2 μg of RNA sample per reaction. Based on the RNA concentration calculate for each RNA sample the volume needed to provide 2 μg of RNA per reaction and transfer this volume into a new tube. Fill up each sample tube containing 2 μg of RNA with water to a final volume of 10 μL (*see* **Note 13**).
2. According to the number of samples processed prepare master mix containing 6 μL of water, 2 μL of 10× DNase I reaction buffer, and 2 μL (2 units) of DNase I per each sample. Mix by gently flicking the tube (*see* **Note 14**). Briefly spin down.
3. Add 10 μL of master mix prepared in **step 2** into each sample tube containing 2 μg of RNA in 12 μL of water. Mix by gently flicking the tubes. Briefly spin down.
4. Incubate sample tubes for 40 min at 37 °C and following incubation briefly spin down.
5. Add 2 μL of EDTA to the sample tube, mix by flicking the tube and briefly spin down. Incubate the reaction mixture for 10 min at 70 °C to inactivate DNase I. Briefly spin down and let cool in a room temperature for a while. Store DNAse I treated samples on ice for further processing.

3.5 cDNA Synthesis

Unless indicated otherwise, work on ice.

1. Transfer 5.5 μL of each DNase I treated RNA sample (500 ng of RNA) into a new tube.
2. According to the number of samples processed prepare master mix containing 4.5 μL of water and 0.5 μL of oligo-dT primer (0.25 μg of oligo-dT primer per reaction) per sample. Mix by shortly vortexing the tube, briefly spin down and add 4.5 μL of master mix into each sample tube containing 5.5 μL of DNase I treated RNA. Incubate the samples for 5 min at 70 °C to denature secondary structures in mRNA and immediately chill on ice. Briefly spin down and keep on ice up to further processing.
3. According to the number of processed samples prepare cDNA synthesis master premix containing 1.2 μL of water, 4 μL of M-MLV Reverse Transcriptase 5× reaction buffer (*see* **Note 15**) 4 μL of dNTPs (2.5 mM), 0.4 μL (40 units) of M-MLV Reverse Transcriptase, and 0.4 μL (16 units) of RNasin® Plus

RNase inhibitor. Mix by gently but thoroughly flicking the tube and briefly spin down.

4. Add 10 μL of cDNA synthesis master mix prepared in **step 3** into each sample tube processed according **step 2**. Mix by gently but thoroughly flicking the tubes, briefly spin down and incubate at 42 °C for 120 min.
5. Inactivate M-MLV Reverse Transcriptase by heating the sample tubes at 70 °C for 10 min. Briefly spin down. Add 60 μL of water into each sample containing synthetized cDNA in 20 μL volume. Mix by flicking the tubes and briefly spin down. Aliquot each cDNA sample into two to three tubes and store at −80 °C (*see* **Note 16**).

3.6 Primer Design

When designing primers for RT-qPCR one should follow certain guidelines in order to obtain reliable results. We present here primer design strategy relaying on the use of Primer-BLAST software [21] and outline primer designing rules as user defined program settings.

1. Use Primer-BLAST web software to design primers (*see* **Note 17**).
2. Insert target mRNA sequence in FASTA format.
3. Set primer melting temperature (T_m) from 58 to 65 °C; however, follow the rule that maximal T_m difference between two primers within a primer pair should be 1 °C. In addition, whenever possible, this rule should be followed also for the T_m differences among different primer pairs.
4. Set PCR product size from 50 bp to 150 bp and size of the primers from 18 bp to 24 bp.
5. If possible primers should be designed to avoid amplification of unintended targets from contaminating genomic contaminating DNA. For this purpose a program option "primer must span an exon-exon junction" might be used. Alternatively primers might be designed to span introns using "intron inclusion" option. In the example provided, primers are designed using "primer must span an exon-exon junction" option (Table 1).
6. Select "nr" database and define "organism" in "Primer Pair Specificity Checking Parameters".
7. Otherwise use program default settings and run program.
8. On the program output select suitable primer pairs primarily based on specificity; further primer selection should be guided by lowest "any" and "3′" complementarity.
9. Check the template secondary structure using mFold server (http://www.bioinfo.rpi.edu/applications/mfold/) to avoid secondary structures that might interfere with the RT-qPCR efficiency.

Table 1
Specific primer properties

Gene and accession number	Primer name	Sequence (5′ → 3′)	Length (bp)	T_m (°C)	Amplicon length (bp)	PCR efficiency (%)
AtMPK4	qMPK4F	TGTCGGCTGGTGCAGTCGATTT	22	65	85	90.4
At4g01370	qMPK4R	TGGCACAACGCCTCATCAACTGT	23	65.29		
AtMPK6	qMPK6F	ACAGCTTCCACCTTATCCTCGCCA	24	65.79	85	92.3
At2g43790	qMPK6R	TGGGCCAATGCGTCTAAAACTGTG	24	64.12		

10. In the Table 1 an example is provided of the primer pairs designed for the RT-qPCR quantification of *Arabidopsis thaliana* MPK4 and MPK6 using Primer-BLAST.

3.7 Set up, Run and Analysis of Quantitative Real-Time PCR

Unless indicated otherwise, work on ice.

1. Using 10 μM primer stock solutions prepare primer master mix for each tested target that should contain forward primer, reverse primer, and water in 3:3:19 ratio. Vortex briefly twice and spin down.
2. According to the number of samples analyzed prepare for each tested target a RT-qPCR master mix containing 2.5 μL of primer mix (**step 1**, running concentration of each primer = 0.3 μM) and 5 μL of Power SYBR® Green PCR Master Mix per single reaction. Mix reaction by gently but thoroughly flicking the tube and briefly spin down.
3. Design 96-well plate running schedule and pipet 7.5 μL of each RT-qPCR master mix according to this schedule into the bottom of 96-plate wells.
4. Follow 96-well plate running schedule and pipet 2.5 μL of each cDNA sample or control sample to the walls of the wells.
5. Cover plate with optical adhesive film and briefly centrifuge.
6. Run the plate in the StepOne™ Thermal cycler under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.
7. Run the melt curve analysis under the following conditions: 95 °C for 15 s, 60 °C for 1 min and heating from 60 °C to 95 °C with 0.3 °C increments.
8. Analyze obtained data using the StepOne™ software (Applied Biosystem®). Check the negative controls for the amplification

of unintended targets (*see* **Note 18**) and perform melting curve analysis for the targets amplified from cDNA samples (*see* **Note 19**). Calculate relative gene expression using $2^{(-\Delta\Delta Cq)}$ method [25] implemented in StepOne software and verify the calculation in Microsoft Excel spreadsheet (*see* **Note 20**).

3.8 PCR Efficiency Test

1. Determine the efficiency of target amplification for each primer pair that is going to be used in RT-qPCR analyses.
2. Prepare serial dilutions of the appropriate template at ratios of 1, 1/4, 1/16, 1/64 and 1/256 (*see* **Note 21**).
3. Run at least in duplicate RT-qPCR on the dilution series samples using conditions described in Subheading 3.7. Determine PCR efficiency from the slope of the calibration curve. In particular, PCR efficiency = $[10^{(-1/\text{slope})}-1]$, when logarithm of template concentrations is on the *x* axis and Cq values are on the *y* axis. Standard curve analysis can be also performed using StepOne™ software.
4. Good PCR efficiency ranges within 85–115 %; otherwise design new primers (Table 1).

4 Notes

1. We generally find plastic ware to be RNase free and routinely use relatively cheap tubes and tips without any special designations. For RNA isolations (starting from the pipetting of water phase) and cDNA preparations we use filtered tips.
2. Always work in sterile laminar flow box.
3. Dissolve NaCl in liquid ½ MS medium and use liquid ½ MS medium only for time parallel controls.
4. Following freezing in liquid nitrogen, samples should be stored in −80 °C freezer prior to further processing.
5. During the sample disruption procedure 2 mL round bottom tubes bearing samples are maintained on dry ice or floating on liquid nitrogen in a polystyrene box. Samples are disrupted individually in the tubes prechilled in liquid nitrogen by hand using micropestle (also prechilled in liquid nitrogen). During disruption step, samples should be occasionally chilled in liquid nitrogen to avoid thawing of the samples. In general, it is critical to maintain the samples deep frozen during storage and disruption step. After processing of each sample, wash the pestle in 70 % (v/v) ethanol and demi water. Finally, wipe the pestle dry with lint-free tissue.
6. Samples homogenized in TRI reagent solution can be safely kept on ice or in a fridge from several hours up to 1 day.

7. Aqueous phase should be colorless and not viscous, otherwise it might be contaminated with high molecular genomic DNA, proteins, polysaccharides, and phenol. Such contaminants in the aqueous phase might interfere with RT-qPCR, and therefore, they should be avoided. Most often, contaminants in water phase occur when the <1:10 ratio of wet plant material weight to TRI reagent volume is not followed. Aqueous phase could be additionally purified using chlorophorm extraction. Chlorophorm extraction is usually indispensable when working with *H. vulgare* leaves.
8. When working with *Medicago* roots, mix isopropanol in 1:1 ratio with high salt RNA precipitation solution containing 0.8 M sodium citrate and 1.2 M NaCl. Add this mixture (70 % (v/v) of aqueous phase volume) into a tube containing collected aqueous phase.
9. White pellet should be visible at the bottom of 2 mL tube at the end of centrifugation. Remove supernatant using pipet in order to minimize the risk of sample contamination that might occur if supernatant is simply poured out of the tube.
10. It is important to keep the RNA pellet compact. Pellet should be released from the bottom of tube into 75 % (v/v) ethanol simply by carefully inverting the tube.
11. This is the most challenging step. RNA pellet should not be overdried as this might lead to poor RNA solubility and impurity. Initially, ethanol should be removed using pipet and then the pellet is dried out for a few minutes until it becomes clear. No ethanol drops should be visible in the tube before RNA pellet is dissolved.
12. Ideally, especially when processing large number of samples, DNAse I treatment and cDNA synthesis should be done in PCR 8-tube strips and thermocycler dedicated to RNA work only.
13. If the RNA concentration is lower than 200 ng/μL, final volume can be increased up to 16 μL. The volume of water added per single sample should be decreased accordingly in the following DNase I master mix preparation.
14. DNAseI is sensitive to physical denaturation.
15. M-MLV Reverse Transcriptase 5× reaction buffer contains high amounts of salt that may precipitate upon freezing. Therefore, it should be vigorously vortexed to dissolve all salts when thawing.
16. Repeated freeze–thaw cycles might have tremendous effect on cDNA quality and in turn on performance of RT-qPCR. Even two freeze–thaw cycles alone can significantly decrease RT-qPCR sensitivity. When analyzing different batches of cDNA samples, one should consider analyzing batches of similar age only that in addition underwent the same low number of freeze–thaw cycles.

17. Primer3 software [22, 23] is also well suitable for the RT-qPCR primer design. Nevertheless, it bears no primer specificity check option; therefore, prior to primer design, a sequence region specific to the target mRNA should be identified using BLAST [24] searches.
18. Positive but also negative controls should be included into the every plate run. The simplest negative control is the "water", so-called non template control (NTC) control that is run with water template supplied instead of cDNA template. No PCR products of correct size are tolerable in NTC controls. Occasionally, primer dimer products might be detected in NTC control runs. We find those to be usually acceptable when giving late Cq values. However, the identity of amplicons should be always checked using melting curve analysis or in the case of uncertainty, PCR products should be resolved using 2 % (w/v) agarose gel electrophoresis. Additionally, in order to check for genomic DNA contamination, especially when using primer pairs that amplify targets also from genomic DNA templates, no-RT control should be included. This control employs DNAse I treated RNA template that is diluted with water to the exactly same concentration as is putative RNA concentration in standard cDNA sample. To give a numerical example according to our protocol, 5.5 μL of DNAse I treated RNA sample (500 ng of RNA) should be diluted with 74.5 μL of water to get RNA concentration of 34.37 ng/μL. No-RT control should be run for each respective cDNA sample; however, it can be omitted in further runs once it has been validated as free of DNA contamination.
19. Only single "clear" melting curve should be visible. RT-qPCR products can be also resolved in 2 % (w/v) agarose gel electrophoresis in order to verify the specificity of amplification.
20. A reference gene should be always checked for the invariant expression in the running experimental conditions.
21. When working with genes exhibiting sufficient expression (Cq = at least 25–26), cDNA template (undiluted cDNA template corresponds to dilution 1) can be used directly for the preparation of standard curve dilution series. Otherwise, when the gene expression is low (based on initial runs) and cannot be easily induced, plasmid templates should be used.

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Chapter 3

Analysis of MAPK Activities Using MAPK-Specific Antibodies

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Abstract

Phosphorylation of proteins by mitogen-activated protein kinases is central to many cellular processes, including signal transduction after stress encounter. Thus, assays to identify or characterize MAP kinase activities are a key tool for research in this area. While in-gel kinase assays using isotope-labeled ATP are a powerful tool to investigate the general induction of MAPK activities in any organism, alternative methods using phospho-specific MAPK antibodies are now being established for many model organisms. However, both in-gel kinase assay and phospho-specific western blot analysis do not allow for the unambiguous identification of the activated MAPK. To obtain specificity, initial immunoprecipitation purification of the kinase of interest prior to further analysis can be performed.

Key words AtMPK3, AtMPK4, AtMPK6, Immunocomplex kinase assay, Phospho-specific MAPK antibody, Western blot

1 Introduction

The modification of proteins via phosphorylation is a crucial and fundamental step conveying multiple environmental signals into functional cellular responses. A class of well characterized kinases in plant stress responses is the mitogen-activated protein kinases (MAPKs) [1]. Generally, MAPKs have been described as major components of cellular signal transduction pathways mediating various biotic and abiotic stress responses including hormone signaling, cell division, and developmental processes [1–6]. MAPK cascades are universal signal transduction modules in eukaryotes, including yeasts, animals, and plants and are generally composed of three functionally linked kinases, a MAP kinase kinase kinase (MAPKKK or MEKK), a MAPK kinase (MAPKK or MKK), and a MAPK. In response to an extracellular stimulus, a given MAPKKK activates its target MAPKK via phosphorylation of Ser and Ser/Thr residues within the SXXXS/T motif, where X denotes any amino acid. MAPKKs, which are dual-specificity protein kinases, then

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activate their downstream MAPK by dual phosphorylation of the activation loop amino acids threonine and tyrosine within the conserved TXY motif. Once activated, MAPKs phosphorylate a variety of cytoplasmic and nuclear substrates on serine or threonine residues within a minimal S/T-P motif, thereby affecting their localization, activity state, stability, or transcript levels [7–10].

The activation of MAPKs can be easily monitored by using myelin basic protein (MBP) as an artificial substrate which is readily accepted by most MAPKs. As MAPK activity can be restored even after denaturing polyacrylamide gel electrophoresis, in-gel kinase assays are a powerful method for the detection of MAPK activities [11]. The assay relies on the co-polymerization of MBP with the SDS polyacrylamide gel matrix. Crude protein extracts are then separated by electrophoresis under denaturing conditions. Afterwards, proteins are renatured in the gel by the removal of SDS, and finally incubated in a kinase buffer supplemented with [γ-^{32}P]-labeled ATP. The in situ MAPK phosphorylation activities can finally be visualized by autoradiography of the dried gel. In-gel kinase assays have been adapted to many plant model systems such as *Arabidopsis thaliana* [5], tobacco (*Nicotiana tabacum*) [12, 13], tomato (*Solanum lycopersicum*) [14], and rice (*Oryza sativa*) [15].

However, alternatives to the work with isotope-labeled ATP have recently been established such as western blot analysis using specific antibodies for phosphorylated MAPKs. Generally, the amino-acid composition in the activation loop of MAPKs is much conserved and at least the motif TXY (with x representing either E or D) can be found in all plant MAPKs similar to animal ERK kinases (Fig. 1). Therefore, an antibody raised against the pTXpY motif should be suitable to detect phosphorylated MAPKs in many different species.

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196-HTGFLTEYVATRWYAR    ERK1
179-HTGFLTEYVATRWYAR    ERK2
190-ENDFMTEYVVTRWYRA    AtMPK3
195-ETDFMTEYVVTRWYRA    AtMPK4
215-ESDFMTEYVVTRWYRA    AtMPK6
215-ETDFMTEYVVTRWYRP    LeMPK1
213-ETDFMTEYVVTRWYRP    LeMPK2
193-ENENMTEYVVTRWYRA    LeMPK3
212-ETDFMTEYVVTRWYRP    NtSIPK
195-ENENMTEYVVTRWYRA    NtWIPK
188-ESDMMTEYVVTRWYRA    OsMPK3
195-ETDLMTEYVVTRWYRA    OsMPK4
219-ETDFMTEYVVTRWYRA    OsMPK6
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Fig. 1 Amino-acid sequence alignment of human ERK1 and ERK2 with plant MAPKs. Sequences obtained from NCBI were aligned using the Clustal V method, the TxY motif is shown in *red, numbers on the left* indicate amino-acid position. Accession numbers are: ERK1 (P27361), ERK2 (P28482), AtMPK3 (Q39023), AtMPK4 (AEE82016), AtMPK6 (Q39026), LeMPK1 (AY261512), LeMPK2 (AY261513), LeMPK3 (AY261514), NtSIPK (BAC53772), NtWIPK (BAA09600), OsMPK3 (AK067339), OsMPK4 (AK111579), OsMPK6 (AK111691)

From recent publications in the model plants *A. thaliana* and *N. benthamiana*, it is evident that the phospho-p44/42 MAPK antibody raised against human p44 and p42 MAP Kinases ERK1 and ERK2 (Extracellular signal-regulated kinase 1 and 2) also detects MAPK activities in plants [16–22]. Similar to plant MAPKs cascades, the human ERK1/2 signaling pathway can be activated in response to a diverse range of extracellular stimuli [23–25] and is an important target in the diagnosis and treatment of cancer [26]. The phospho-specific antibody detects ERK1 and ERK2 when phosphorylated either individually or dually at the TEY motif (Thr202 and Tyr204 in ERK1; Thr185 and Tyr187 in ERK2), but does not cross-react with non-phosphorylated Erk1/2. Polyclonal antibodies are produced by immunizing rabbits with a synthetic phosphopeptide corresponding to residues surrounding Thr202/Tyr204 of human ERK1 (Fig. 1).

We have used this antibody for detection of stress-induced MAPK activities in various model plants such as *Arabidopsis*, rice, tobacco, and tomato. In all cases, at least two different bands were detectable after stimulation of cell suspension cultures with the microbe-associated molecular patterns (MAMPs) flagellin and chitin (Fig. 2).

However, neither the in-gel kinase assay nor the western blot technology allow for an unambiguous identification of the activated MAPK. Comparisons of wild-type protein extracts with that derived from MAPK mutant plants demonstrated that the upper two MAPKs detected in *Arabidopsis* represent the major stress

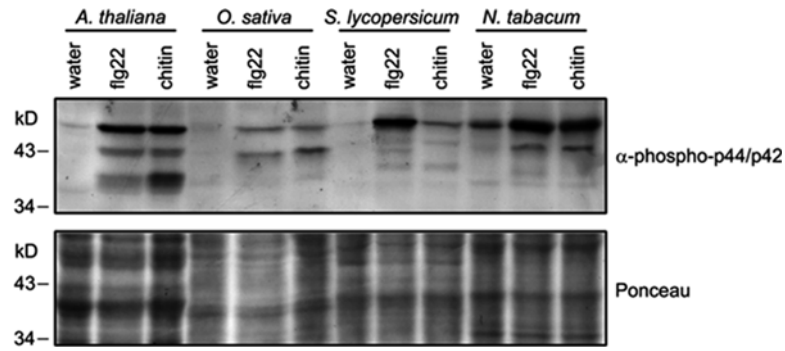

Fig. 2 Detection of MAP kinase activation in plant cell cultures. Suspension cell cultures of *Arabidopsis thaliana*, *Oryza sativa*, *Solanum lycopersicum*, and *Nicotiana tabacum* were treated with 100 nM flg22 (the 22-amino-acid fragment of flagellin sufficient for MAMP activity), 50 μg/mL chitin or with water as control. After 10 min the cells were collected, soluble protein extracts were prepared and subsequently separated by SDS-PAGE. Posttranslational activation of MAP kinases was assayed by immunoblot using anti-phospho p44/p42 antibodies (Cell Signaling Technology). Equal loading was verified by Ponceau S Red staining of the nitrocellulose membrane after blotting

induced MAPKs AtMPK3 and AtMPK6 [17, 18, 27]. Similarly, the upper MAPK band activated in tobacco corresponds to NtSIPK (salicylic acid-induced protein kinase), while the lower band represents NtWIPK (wound-induced protein kinase [20]). However, the third activated MAPK visible on the western blot using anti-phospho-MAPK in *Arabidopsis* is still present in *atmpk4* and *atmpk11* mutants, respectively, and thus might correspond to AtMPK4 or AtMPK11 [27]. The specificity of such assays can be ensured by including an initial immunoprecipitation purification of a kinase of interest prior to further analysis [28]. For this so-called immunocomplex kinase assay isoform-specific antibodies are required which have now been generated for many stress-induced MPKs in several plant species. For instance, immunocomplex kinase assays in *Arabidopsis* using antibodies raised against AtMPK3, AtMPK4, and AtMPK6 demonstrated enzymatic activation of these MAPKs after treatment with ABA [22] and flagellin [29]. Likewise, rice OsMPK3, OsMPK4, and OsMPK6 were shown to be activated upon chitin application and moderately low temperature, respectively [15, 30]. Immunocomplex kinase assays using NtSIPK and NtWIPK-specific antibodies revealed a harpin-induced MAPK activation in tobacco [20] and the tomato MAPKs LeMPK1, LeMPK2, and LeMPK3 are differentially activated upon application of various biotic and abiotic stress stimuli [14, 31, 32].

The protocols described in this chapter will provide the basis for studying MAPK activation in simple western blot analysis and can be combined with immunocomplex kinase assays to ensure the identification of the activated MAPK.

2 Materials

2.1 Maintenance of Arabidopsis Plants and Seedlings

1. *Arabidopsis* soil: steam-sterilized GS90 soil (Gebr. Patzer GmbH, Germany) mixed with vermiculite.
2. Half-strength Murashige–Skoog (1/2 MS) medium, 2.2 g/L (Duchefa).

2.2 Protein Extraction

1. Protein extraction buffer: 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 2 mM DTT, 100 mM ß-glycerophosphate, 10 mM sodium orthovanadate (Na_3VO_4), 10 mM sodium fluoride (NaF), 10 mM Pefablock™ (AEBSF, 4-[2-aminoethyl]-benzenesulfonyl fluoride; Roche), 10 µg/mL Aprotinin, 10 µg/mL Antipain, 10 µg/mL Leupeptin (*see* **Note 1**).
2. Bradford solution (e.g., Bio-Rad) (*see* **Note 2**).

2.3 SDS-PAGE and Western Blotting

1. Loading buffer stock: 33.3 % (v/v) Glycerol, 6 % (w/v) SDS, 1.5 % (w/v) Bromophenol blue, 188 mM Tris–HCl pH 6.8. To obtain the 3× loading buffer freshly mix 200 µL 1 M DTT + 500 µL loading buffer stock.

2. SDS-PAGE running buffer, 10× stock: 250 mM Tris base, 1.92 M Glycine, 1 % (w/v) SDS.
3. Protein marker, pre-stained.
4. Gel running apparatus.
5. Transfer buffer, 10× stock: 250 mM Tris base, 1.92 M Glycine, add 20 % (v/v) Methanol to the diluted 1× buffer prior blotting.
6. Nitrocellulose membrane (e.g., GE Healthcare).
7. Buffer tank-blotting apparatus.
8. Ponceau S Red staining solution: 0.1 % (w/v) Ponceau S Red, 5 % (v/v) glacial acetic acid.
9. TBST: 20 mM Tris–HCI pH 7.5, 150 mM NaCl, 0.1 % (v/v) Tween-20.
10. Nonfat dried milk powder.
11. Bovine serum albumin.
12. Anti-phospho p44/42 MAPK antibody (e.g., Cell Signaling Technology).
13. Secondary anti-rabbit Immunoglobin G from goat, coupled to alkaline phosphatase (e.g., Sigma).
14. Alkaline Phosphatase (AP) buffer: 150 mM Tris–HCl pH 9.5, 5 mM $MgCl_2$, 100 mM NaCl.
15. 200× BCIP: 50 mg/mL 5-Bromo-4-chloro-3-indolyl phosphate in Dimethylformamide, store at –20 °C.
16. 200× NBT: 50 mg/mL nitroblue tetrazolium chloride in 70 % (v/v) Dimethylformamide, store at –20 °C.

2.4 Immunocomplex Kinase Assay

1. AtMPK3, AtMPK4, AtMPK6 isoform-specific MPK-antibodies (e.g., Sigma) (*see* **Note 3**).
2. Protein G-Sepharose (e.g., GE Healthcare).
3. Kinase buffer: 50 mM Tris–HCl pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, and 50 μM ATP.
4. Myelin-basic protein (MBP, e.g., Sigma).
5. [γ^{32}P]ATP, 10 mCi/mL, >6,000 Ci/mmol (e.g., Hartmann Analytic GmbH).

3 Methods

In this section, we describe (a) the maintenance of *Arabidopsis* plants, (b) the isolation of crude protein extracts from *Arabidopsis* tissue, (c) the western blot analysis using phospho-specific MAPK antibodies, and (d) the immunoprecipitation and immunocomplex kinase assay of MAPKs.

3.1 Growth of Arabidopsis Plants

1. *Arabidopsis* seeds are sown on steam-sterilized GS90 soil. After stratification of the seeds for 2 days at 4 °C and in the dark the plants are grown for 4–5 weeks in environmental chambers in short day (8 h light, 16 h darkness) under standard conditions (150 μmol/cm² s light, 40–60 % humidity, 22 °C).
2. In case of using seedlings, seedlings are surface-sterilized with chlorine gas (25 mL NaClO (12 % w/v) + 3 mL HCl (37 % v/v)) for 2 h and then sown on sterile ½ MS plates before the 2 day stratification. After 10 days seedlings are transferred to liquid ½ MS with 1 % (w/v) sucrose (use of microtiter plates, e.g., 24 or 48 wells is advisable) and equilibrated over night while gently shaken. Elicitors are added directly to the liquid MS medium.

3.2 Protein Extraction

1. Freeze 50–100 mg plant tissue (approximately one leaf or five seedlings) in a 2 mL Eppendorf tube in liquid N_2 (*see* **Note 4**). The samples can be stored at −80 °C.
2. Grind the tissue to a fine powder with a metal pestle (pre-cooled in liquid N_2), and then add 100 μL protein extraction buffer (*see* **Note 5**).
3. Collect all samples on ice and then thaw together on ice (*see* **Note 6**).
4. Spin down the cell debris for 20 min at 15,000 × *g* at 4 °C.
5. Transfer supernatant (without any pellet) to a fresh tube and keep samples on ice.
6. Determine the protein concentration after Bradford (e.g., with a commercial Bradford solution from Bio-Rad) in a photometer at 595 nm (*see* **Note 7**) and adjust samples to the same protein concentration. Continue right away with SDS-PAGE (Subheading 3.3) or immunoprecipitation (Subheading 3.5) (*see* **Note 8**).

3.3 SDS-PAGE

1. Mix 40 μL protein with 20 μL 3× loading buffer, heat for 5 min at 95 °C, and spin briefly.
2. Load 10–15 μg protein (or more if possible) on a 10.5 % SDS-PA gel.
3. Run the electrophoresis for 10 min at 130 V and then change to 200 V until the 35 kDa-marker band of the pre-stained protein marker is just approaching the end of the gel (*see* **Note 9**).

3.4 Western Blotting

1. Following electrophoresis, transfer proteins from the polyacrylamide gel to a nitrocellulose membrane in a buffer tank-blotting apparatus for 1 h at 100 V (for gels of 1 mm thickness).
2. After transfer, check transfer efficiency and equal protein loading by staining the proteins on the membrane with Ponceau S-Red

for 30 s, then destain briefly in water before the membrane can be scanned.

3. Block unspecific binding sites by incubating the membrane in TBST containing 5 % (w/v) nonfat dried milk for at least 1 h at room temperature with gentle agitation.
4. Wash the membrane with TBST 3 times, each 5 min at room temperature with gentle agitation.
5. Incubate the membrane with primary antibody raised against p44/42-MAPK (1:2,000 dilution, developed in rabbit) over night at 4 °C in 10 mL TBST containing 5 % (w/v) BSA with gentle agitation.
6. Wash the membrane with TBST 3 times, each 5 min at room temperature with gentle agitation.
7. Incubate the membrane with secondary antibody raised against rabbit Immunoglobulin G (from goat, coupled to alkaline phosphatase, 1:3,000 dilution; *see* **Note 10**) for 1–2 h in 10 mL TBST at room temperature with gentle agitation (*see* **Note 11**).
8. Wash the membrane with TBST 3 times, each 5 min at room temperature with gentle agitation.
9. Equilibrate the membrane in AP-buffer for 2 min at room temperature with gentle agitation.
10. Add BCIP and NBT as a 1:200 dilution in AP-buffer and let the color reaction proceed until clear bands are visible.
11. Wash the membrane with distilled water to stop alkaline phosphatase reaction.

3.5 Immunocomplex Kinase Assay

3.5.1 Immunoprecipitation of MAPKs

1. Prepare protein extracts as described in Subheading 3.2.
2. Incubate 30 µg total proteins in at least a 500 µL volume over night at 4 °C with 10 µL of isoform-specific MPK-antibody with head-over-head rotation.
3. Meanwhile, prepare Protein G-Beads: per sample wash 100 µL 50 % slurry of Protein G-Beads 3 times with each 1 mL water, followed by one wash with 1 mL protein extraction buffer, spin each time 1 min at 400 × *g* in a swing out rotor to collect beads and carefully remove the supernatant by pipetting, finally resuspend beads in 900 µL protein extraction buffer (*see* **Note 12**).
4. Add 50 µL of Protein G-Sepharose (e.g., GE Healthcare) to the protein–antibody mixture and incubate for 2 h at 4 °C with head-over-head rotation.
5. Collect the protein–antibody complex on the beads by centrifugation for 2 min at 400 × *g*.

6. Wash the beads three times with ice-cold protein extraction buffer. Always carefully remove leftover liquid from the beads without disturbing the pellet.
7. Wash the beads once with kinase buffer and carefully remove any leftover liquid.

3.5.2 Kinase Assay

1. Add 20 µL of kinase buffer containing 1 µg of myelin basic protein (MBP) and 2 µCi of [γ^{32}P]ATP to the beads.
2. Incubate the kinase reaction for 30 min at room temperature with gentle agitation.
3. Stop the reaction by adding 3× SDS-PAGE loading buffer.
4. Heat the sample at 95 °C for 3 min and briefly spin.

3.5.3 SDS-PAGE of MBP

1. Load 15 µL of the sample on a 10.5 % SDS-PA gel.
2. Run the electrophoresis as described in Subheading 3.3 until the dye runs out.
3. Transfer the proteins to a nitrocellulose membrane and stain the membrane after electroblotting with Ponceau S Red as described in Subheading 3.4.
4. The phosphorylation of MBP is analyzed by autoradiography exposing the membrane to an X-ray film for overnight or using a Phosphor Imager plate (*see* **Note 13**).

4 Notes

1. Alternatively, a commercial protease inhibitor cocktail and phosphatase inhibitor cocktail from Roche can be used (Complete Mini EASYpack and PhosSTOP EASYpack, each 1 tablet for 10 mL buffer)
2. Instead of a commercial Bradford solution, this solution can be self-made containing in final concentrations 0.01 % (w/v) Coomassie Brilliant Blue G-250, 4.7 % (w/v) ethanol, and 8.5 % (w/v) phosphoric acid [33].
3. Alternatively to the commercial AtMPK antibodies, isoform-specific peptide antibodies have been raised using the following N-terminal peptides: N-MNTGGGQYTDFPAVDTHGG for AtMPK3 and N-MDGGSGQPAADTEMT for AtMPK6 [16, 22]. All self-made antisera were affinity-purified on the corresponding immobilized oligopeptide used to generate the serum.
4. This assay can also easily be performed with plant material derived from seedlings or cell culture; in this case also 50–100 mg of plant material should be used.
5. Instead of a metal pestle any other equipment can be used to extract the proteins.

6. All the extraction steps are carried out at 0 °C or 4 °C.
7. Instead of a fresh BSA standard curve the protein concentration can be estimated using the following formula: protein concentration [mg/mL] = $OD_{595}/(0.0283 \times$ used volume)
8. We never freeze-thaw the samples because we find that enzyme activity often disappears afterwards. To avoid a dephosphorylation of MAPKs due to freezing–thawing, SDS-PAGE or immunoprecipitation should be performed with freshly prepared protein extracts.
9. MAPKs usually have a molecular weight around 38–50 kDa. The gel electrophoresis should be performed long enough to properly separate different MAPK from each other.
10. To reduce the background, we used this secondary antibody in a dilution of up to 1:10,000 with good signal intensities.
11. Alternatively, a secondary antibody coupled to horseradish peroxidase can be used and detected using the ECL detection system.
12. In all steps involving pipetting of beads, cut-off pipette tips should be used.
13. As MBP has a molecular weight of approximately 18 kDa, the upper part of the membrane could be cut off and used for western blot analysis with MAPK-specific antibodies.

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Chapter 4

Detection of Protein Phosphorylation and Charge Isoforms Using Vertical One-Dimensional Isoelectric Focusing Gels

Jeffrey C. Anderson and Scott C. Peck

Abstract

During many biological responses, changes in protein modifications (e.g., phosphorylation) are often more critical than changes in protein abundance in determining the outcome of cellular responses. These important regulatory changes can alter a protein's location, activity, or binding partners. Monitoring modifications such as phosphorylation is often impeded, or even prevented, because of the need for specialized reagents and equipment that are expensive and/or time-consuming to produce. However, many protein modifications alter the isoelectric point (p*I*) of a protein. Therefore, we developed a denaturing, one-dimensional isoelectric focusing (IEF) procedure that separates proteins based on their p*I* to resolve different isoforms, allowing a relatively simple strategy for detecting changes in protein modifications. Although similar results can be achieved by two-dimensional gel electrophoresis, the method described here uses a multi-well SDS-PAGE format that allows many more samples to be assayed within a single gel, thereby greatly decreasing both the time and cost needed to assess modifications of a single protein in response many different treatment conditions. To increase the sensitivity of detection, we also optimized a procedure to transfer proteins from these gels to membranes for subsequent immunodetection. This combination of techniques provides the means of interrogating the number and stoichiometry of isoforms from total protein extracts without a priori knowledge of which modification may occur.

Key words Isoelectric focusing, Protein phosphorylation, Protein isoforms, Immunoblotting, Posttranslational modifications

1 Introduction

Proteins can undergo a wide variety of posttranslational modifications (PTMs) that greatly alter protein location or function. However, detecting these changes often requires either costly investment in specialized reagents or access to expensive technology which may not be easily available to many researchers. The protocol for monitoring PTMs described here is amenable to any biological system and has been successfully used to detect in vivo and in vitro phosphorylation of proteins from both plant and animal cells [1–3]. This protocol has also been used to discriminate between protein isoforms that share similar apparent molecular

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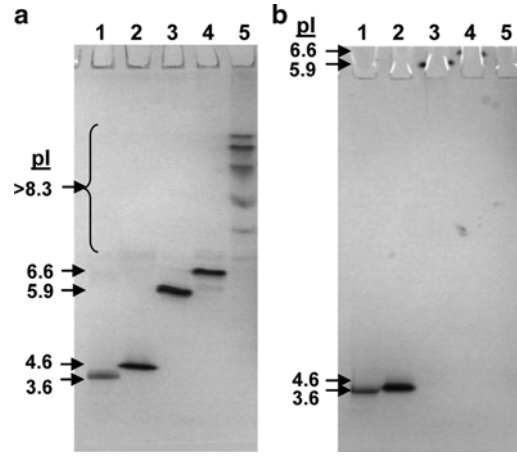

Fig. 1 Isoelectric focusing in a mini-gel format under denaturing conditions. Individual protein p*I* standards were focused in vertical IEF mini-gels under denaturing conditions using either (**a**) 25 mM Tris pH 10.5 or (**b**) 25 mM HEPES pH 5.4 as the cathode buffer and 10 mM phosphoric acid pH 1.9 as the anode buffer. Proteins were detected by Coomassie Blue. *Lane 1*, amyloglucosidase, p*I* = 3.6; *lane 2*, trypsin inhibitor, p*I* = 4.6; *lane 3*, carbonic anhydrase II, p*I* = 5.9; *lane 4*, carbonic anhydrase I, p*I* = 6.6; *lane 5*, glyceraldehyde-3-phosphate dehydrogenase, multiple isoforms p*I* = 8.3 and greater. "Reproduced from Anderson and Peck (2008) with permission from John Wiley & Sons Publications"

masses yet differ sufficiently in amino acid composition to alter their p*I* [4]. Successful detection of protein isoforms requires well-resolved separation of forms in the gel. Therefore, it is very important that the samples be sufficiently free of charged contaminants such as salts and ionic detergents that would otherwise interfere with proper focusing. To address this potential problem we have included a simple protocol that can be used to remove these contaminants from any protein sample prior to IEF. Although this method could, in theory, be applied to any protein of interest, proteins that typically do not resolve well by two-dimensional gel electrophoresis, such as hydrophobic or extremely acidic/basic proteins, may not work well (or at all) with this protocol.

A key consideration in using this protocol is how to obtain the desired separation of protein isoforms. This is a somewhat empirical optimization step with the primary variables being the choice of anode and cathode buffers as well as the ampholyte(s) used for focusing. As shown in Fig. 1 [reproduced from Anderson and Peck (2008) with permission from John Wiley & Sons Publications], separation of a protein mixture is vastly different depending on the combinations of anode and cathode buffers used. We recommend starting with buffer and ampholyte conditions that separate proteins across a broad pH range (Fig. 1a). If sufficient resolution between isoforms is not achieved under these conditions, substituting either of the anode or cathode buffers may further improve

the resolving power of the gels. For example, substituting HEPES for Tris as the cathode buffer narrows the separation range of the gels from approximately pH 3–9 to pH 3–6 (Fig. 1b). In addition to choosing the correct combination of buffers, the type of ampholyte mixtures used is also an important variable to consider when working to resolve isoforms. For example, a phosphorylation-dependent shift in the *Arabidopsis* protein BAG1 was only detected when narrow pH range ampholyte mixtures (pH 3–6 or pH 4–7) were used rather than a broad pH range mixture (pH 3–10) [1].

A limitation of the IEF approach presented here is that the presence of different isoforms will not provide immediate information about which type of modification(s) is present. One must use subsequent investigation to determine which modifications give rise to which forms. For instance, treating protein samples with a protein phosphatase prior to IEF can be used to determine if multiple isoforms are due to the presence of one or more phosphorylated residues [1]. Despite this limitation, the method is a relatively simple way to determine the stoichiometry of the modification (e.g., is 20 % or 90 % of the target protein phosphorylated during a response?), and this type of information is generally much more complicated to obtain using other methods.

2 Materials

2.1 Reagents

1. Acetone.
2. Acrylamide–*N*,*N*′-methylene-bis-acrylamide solution in H_2O, 30 % (29:1 mixture).
3. Ammonium acetate.
4. Ammonium persulfate.
5. Ampholyte mixture, 40 %.
6. Bromophenol blue.
7. CHAPS.
8. Dithiothreitol (DTT).
9. EDTA.
10. Glycerol.
11. HEPES.
12. β-Mercaptoethanol.
13. Methanol.
14. Phosphoric acid.
15. Potassium chloride.
16. Sodium dodecyl sulfate (SDS).
17. TEMED.
18. Thiourea.

19. Trichloroacetic acid (TCA).
20. Tris-buffered phenol.
21. Tris Base.
22. Urea.

2.2 Equipment

1. Standard SDS-PAGE gel casting and running equipment.
2. High voltage power supply (capable of operating at 1,000 V).

2.3 Buffers and Solutions

Highly purified water (e.g., Milli-Q purified) is recommended for preparation all buffers and solutions to avoid trace electrolytes that may affect the current.

2.3.1 Sample Preparation

1. Tris-buffered phenol.
2. Back-extraction buffer: 0.1 M Tris–HCl, pH 8.4, 20 mM KCl, 10 mM EDTA, 0.4 % (v/v) β-mercaptoethanol.
3. 0.1 M ammonium acetate in methanol.
4. 80 % (v/v) acetone.

2.3.2 IEF Gel Preparation and Electrophoresis

1. IEF gel solution: 9 M Urea, 1 % (w/v) CHAPS, 6 % acrylamide–bis-acrylamide (29:1), 0.4 % ampholyte mixture (e.g., 100 μL of 40 % stock per 10 mL).
2. IEF sample buffer: 7 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 0.8 % ampholytes pH 3–10, 50 mM DTT, 4 % (v/v) glycerol, trace bromophenol blue (store in aliquots at −20 °C).
3. Upper chamber buffer: 25 mM Tris base or 25 mM HEPES (*see* **Note 1**).
4. Lower chamber buffer: 10 mM phosphoric acid (i.e., 1 mL of ≥85 % (v/v) phosphoric acid in H_2O solution into 1 L of ultrapure water).

2.3.3 Preparation of IEF Gels for Electrophoretic Transfer to PVDF Membrane

1. 12 % (w/v) trichloroacetic acid in ultrapure water.
2. Resolubilization buffer: 7 M urea, 2 M thiourea, 5 mM DTT in ultrapure water.
3. SDS equilibration buffer: 0.37 mM Tris–HCl pH 8.8, 0.1 % (w/v) SDS (i.e., 1× SDS-PAGE resolving gel buffer).

3 Methods

3.1 Preparation of Protein Samples for Isoelectric Focusing

Charged contaminants (e.g., salts and nucleic acids) must be removed from protein samples prior to isoelectric focusing as they will conduct current resulting in overheating and poor focusing. The following is a general protocol for desalting protein extracts derived from plant tissues. Other commonly used methods of

desalting (e.g., acetone precipitation, dialysis) may work equally well but have not been empirically tested with this protocol.

1. Grind tissue in liquid nitrogen using mortar and pestle and extract proteins into a suitable buffer containing protease and phosphatase inhibitors, if necessary. Clear the cell debris by centrifugation and collect the supernatant in a fresh tube. In some plant tissues, endogenous polyphenolics can modify or damage proteins. In these cases, including 1 % (w/v) or more of insoluble polyvinylpolypyrrolidone (PVPP) and/or soluble polyvinylpyrrolidone (PVP) can be used to protect proteins.
2. Add equal volume of Tris-buffered phenol to protein extract and vortex for 10 s to mix thoroughly. Incubate the samples on ice for 5 min and then centrifuge for 10 min at 10,000 × *g* at 4 °C. Two phases should be evident after centrifugation. The phenol phase at the bottom primarily contains proteins, whereas the aqueous phase on top mostly contains nucleic acids (*see* **Note 2**). Remove and discard the upper aqueous phase, being careful not to disturb the interface between phases. To ensure complete removal of contaminants, add an equal volume of back-extraction buffer to the phenol phase, vortex, and centrifuge for 10 min at 10,000 × *g*. Remove and discard the aqueous phase and repeat this back-extraction step again.
3. Precipitate proteins from phenol phase by adding at least five volumes of 0.1 M ammonium acetate in methanol. Briefly vortex samples and place at −20 °C for at least 30 min to precipitate proteins. After precipitate forms, centrifuge samples for 5 min at 10,000 × *g* to pellet proteins. Wash the pellet twice with 0.1 M ammonium acetate in methanol, followed by two washes in 80 % (v/v) acetone. To efficiently remove contaminating salts, briefly place the sample tubes in a sonicating water bath to break up the pellets into a fine suspension during washes (*see* **Note 3**). Proteins can be stored as a suspension in 80 % (v/v) acetone at −20 °C until ready for use.
4. Prior to running the IEF gels, briefly centrifuge the protein acetone suspensions, remove supernatant, and air dry pellet (*see* **Note 4**). Resuspend the pellet in IEF sample buffer. We typically load 25–50 μg protein per gel lane, and resuspend this amount in IEF buffer to a concentration of ≤2 μg/μL. Allow the protein to dissolve in the IEF sample buffer for several minutes at room temperature, using gentle pipetting to help resuspend (*see* **Note 5**). Before loading onto gel, briefly centrifuge sample to remove any remaining insoluble material.

3.2 Preparation of Isoelectric Focusing Gels

This protocol was designed using the Bio-Rad Protean III mini-gel SDS-PAGE system. Other SDS-PAGE systems and gel formats may work equally well with this protocol but have not been tested.

1. Prepare IEF gel solution and mix with a magnetic stir bar at room temperature until all components are in solution (*see* **Note 5**).
2. After the urea has dissolved completely, add both ammonium persulfate and TEMED to 0.1 % (w/v) and cast a 1.5 mm thick Bio-Rad mini-protean gel using your choice of well comb (*see* **Note 6**). Allow at least 15 min for the gel to polymerize.
3. Remove gel from casting tray and clean away excess urea from the outside of the gel using a wet paper towel. If gel(s) will not be used immediately, wrap them tightly in plastic wrap and store at room temperature (*see* **Note 7**).
4. Place gel(s) in running tank (*see* **Note 8**). For a broad range pH gradient (e.g., pH 3–10), use Tris buffer in the upper chamber and phosphoric acid in the lower chamber. For narrow range (~pH 4–6), use HEPES buffer in the upper chamber and phosphoric acid in the lower chamber. Fill the upper chamber to above sample wells and check for leaks from upper chamber (*see* **Note 9**). Then fill the lower chamber to only ~0.5 cm above the lower edge of the gel glass plates (*see* **Note 10**). Load samples directly into wells.
5. Run the gel under voltage limiting conditions as described below using the Bio-Rad high voltage power supply (or other suitable high voltage power supply). Stir both the upper and lower chambers of the running tank with small magnetic stir bars during the run. Also, place the running tank in a shallow dish filled with room temperature water (~2 in.) to help dissipate heat during the run. Running conditions are as follows: 200 V for 30 min, 500 V for 15 min, 750 V for 15 min, and finally 1,000 V for 75 min.

3.3 Preparing Gels for Immunoblot Analysis

1. Remove gel from glass plates and wash for 5 min in deionized H_2O with several changes (this step and all subsequent steps should be performed with gentle shaking as would be used for incubating an immunoblot). Place gel in ~50 mL of 12 % (w/v) TCA and place at 4 °C overnight (*see* **Note 11**). This step precipitates the proteins in the gel and removes the ampholytes which otherwise interfere with protein transfer.
2. Wash gels 3×10 min at room temperature with deionized H_2O to remove TCA. Incubate gel in resolubilization buffer for 5–10 min, and then wash for 5 min with H_2O, followed by 3×5 min with SDS equilibration buffer. Gel is now ready for blotting to PVDF or nitrocellulose. Use the same transfer conditions that work well for transferring your protein of interest from SDS-PAGE gels.

3.4 Preparing Gels for Coomassie Blue Staining

1. After IEF separation, precipitate proteins in gel using TCA method outlined for immunoblotting in Subheading 3.3 above. Wash the gels 3 × 10 min with water to remove TCA, then stain gel with standard Coomassie Blue stain (0.1 % (w/v) Coomassie Blue R250, 40 % (v/v) methanol, 10 % (v/v) acetic acid) for 30 min followed by destaining for 1–4 h in destain solution (40 % (v/v) methanol, 10 % (v/v) acetic acid) (*see* **Note 12**).

4 Notes

1. Do not adjust the pH of these buffers after dissolving into water. The pH of unadjusted Tris and HEPES buffers should be ~10.5 and ~5.5, respectively.
2. If the extraction buffer is too viscous, phase separation will not occur. In this circumstance, add additional water or back-extraction buffer until phases separate after centrifugation.
3. Sonication is more efficient if wash volumes are kept small. We typically sonicate pellets in 100–200 μL volumes.
4. To determine the amount of protein in an acetone suspension, transfer an aliquot of sample to a separate tube and air dry to remove acetone. Add 10–20 μL of 1 % (w/v) SDS to pellet. Work with pipet tip to resuspend thoroughly and allow proteins to dissolve into solution. Heating to 65 °C can assist resuspension of samples. The concentration of proteins in the resuspended sample can then be determined using the standard BCA method.
5. Never heat urea solutions above room temperature. Heating breaks down urea into isocyanic acid, a compound that can carbamylate amino acids resulting in artifactual charge shifts during isoelectric focusing.
6. IEF gels do not have a stacking gel layer as is typically used for SDS-PAGE. The entire gel should be cast with IEF gel solution in a single pour.
7. We have kept gels for several days without noticing any adverse effects as long as care is taken to avoid letting the gels dehydrate.
8. Either 1 or 2 IEF gels can be run in a single Bio-Rad electrophoresis tank.
9. Unlike SDS-PAGE, the upper and lower chamber buffers have different pH values, and maintaining this difference in pH is critical for proper focusing to occur. Fill the upper chamber first and wait ~5 min to make sure there are no small leaks before adding the buffer to the lower chamber.

10. Add the buffer slowly to prevent bubbles from forming on lower gel edge. We typically do not add the buffers until we are ready to load the samples to minimize diffusion of buffers between gel and tank.
11. In our experience gels can be kept at 4 °C in TCA for at least several days without any problems.
12. If significant background staining is persists (this is particularly problematic if basic ampholytes are used), increase the length of time for the water washes after TCA precipitation and/or increase the destaining time.

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Chapter 5

Affinity-Based SDS PAGE Identification of Phosphorylated *Arabidopsis* MAPKs and Substrates by Acrylamide Pendant Phos-Tag™

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Abstract

Protein phosphorylation is the most abundant and best studied protein posttranslational modification, dedicated to the regulation of protein function and subcellular localization as well as to protein–protein interactions. Identification and quantitation of the dynamic, conditional protein phosphorylation can be achieved by either metabolic labeling of the protein of interest with ^{32}P-labeled ATP followed by autoradiographic analysis, the use of specific monoclonal or polyclonal antibodies against the phosphorylated protein species and finally by phosphoproteome delineation using mass spectrometry.

Hereby we present a fourth alternative which relies on the enforced—affinity-based—electrophoretic separation of phosphorylated from non-phosphorylated protein species by standard SDS-PAGE systems co-polymerized with Phos-Tag™ and Mn^{2+} or Zn^{2+} cations. Phosphate groups of phosphorylated Ser, Thr, and Tyr residues form complexes with Mn^{2+} and Zn^{2+} cations with polyacrylamide immobilized Phos-Tag™. Following appropriate treatment of the gels, separated proteins can be quantitatively transferred to PVDF or nitrocellulose membranes and probed with common—not phosphorylation state specific—antibodies and delineate the occurrence of a certain phosphoprotein species against its non-phosphorylated counterpart.

Key words Mitogen-activated protein kinase, Lambda phosphatase, Phos-Tag™, Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

1 Introduction

Protein phosphorylation is probably the most frequent posttranslational modification of proteins regulating among others, enzyme activities, subcellular localization, and protein–protein interactions [1]. Phosphate groups are primarily added to hydroxyl groups of serine, threonine, and tyrosine residues and more rarely to histidine or aspartate residues [2], while in all cases the transfer and addition of phosphate groups requires ATP as a donor and an enzyme catalyst which is called protein kinase [3]. Today there are many

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protein kinase families identified and their classification takes into account their substrate specificities as well as structural similarities between members of the same family [4].

Mitogen activated protein kinases (MAPKs) are classified within the CMGC (CDK/MAPK/GSK3/CLK) group of protein kinases (along with other important members such as cyclin-dependent kinases and glycogen synthase kinases [5]). They phosphorylate serine or threonine residues on their targets, and such phosphorylation is directed by immediately downstream proline residues [6]. Importantly, the mechanism of MAPK activation relies on the simultaneous phosphorylation of one threonine and one tyrosine residue arranged in the form of a TXY motif in the activation loop of MAPKs by an upstream, dual specificity MAPKK [6, 7]. Since the phosphorylation-dependent MAPK activation and the phosphorylation of the downstream substrates are conditional, time- and dose-dependent, it is important to follow the phosphorylation status of both MAPKs and substrates under the physiological conditions of interest.

Traditionally, phosphorylation-dependent activation of MAPKs can be followed by in-gel kinase assays whereby activated MAPKs in complex protein mixtures are separated by SDS-PAGE in gels copolymerized with a general substrate like myelin basic protein [8], subsequently renatured and documented with the addition of [γ^{32}P]-ATP and autoradiography to reveal the reactive bands. In a similar manner, the phosphorylation of presumed MAPK substrates can be followed by selectively immunoprecipitating the active MAPK, incubating with recombinant substrate in the presence of [γ^{32}P]-ATP and finally visualize the outcome of the reaction after SDS-PAGE and autoradiographic analysis [9]. The above approaches offer specificity and signal amplification but suffer from the risks and the technical demands associated with handling of ^{32}P [10].

Alternatively the phosphorylation of MAPKs can be followed with standard SDS-PAGE and western blotting using antibodies generated against the dually phosphorylated TXY motif within the activation loop of MAPKs. Such antibodies are widely available in the market and they have been in use for plant MAPK in several studies [11–13]. Unfortunately, this is not the case for putative plant MAPK substrates since such antibodies need to be produced by custom order at a considerable cost.

On the other hand, many companies have produced a large number of antibodies against a wide array of plant proteins. In this case phosphorylated proteins maybe discriminated from their non-phosphorylated counterparts by 1D-isoelectric focusing and both forms can be then detected after standard western blotting with a single antibody [14].

In this chapter we present an alternative approach, using phospho-affinity based denaturing SDS-PAGE separation of phosphorylated and non-phosphorylated proteins followed by western

blotting. In this method SDS-PAGE gels are copolymerized with 1,3-bis[bis(2-pyridinylmethyl)amino]-2-propanolato dimetal(II) complex. The acrylamide pendant tag which is trademarked as Phos-Tag™ [15] can then retard the migration of phosphorylated proteins compared to their non-phosphorylated counterparts by forming a complex with phosphate groups in the presence of either Mn^{2+} [16] or Zn^{2+} [17, 18] cations. Such method can confer exceptional resolution regarding the differential phosphorylation which may occur in multiple residues of a single protein [19]. Therefore, phospho- versus non-phospho-proteins will be discriminated as individual bands which can be followed by standard western blot analysis with non-phosphorylation state specific antibodies against the protein of interest. In plant research, Phos-Tag™ received modest attention but among others helped to identify MPK4-mediated phosphorylation of microtubule associated protein 65-1 [20, 21] and recently it helped to uncover the stress-induced phosphorylation of α-tubulin by an atypical kinase activity of the Propyzamide Hypersensitive 1 MAPK phosphatase (*PHS1*; [22, 23]).

Conclusively, we will provide an experience-driven, plant-optimized protocol, accompanied with appropriate controls aiming to facilitate nonradioactive phosphorylation studies in plants circumventing the need of phospho-specific antibodies. An example of MPK3 phosphorylation and activation upon mild oxidative treatment with 800 μM H_2O_2 for 30 min is shown in Fig. 1.

2 Material

2.1 Plant Material and Culture

1. We routinely use *Arabidopsis thaliana* ecotypes Columbia (Col-0), Landsberg erecta (Ler), and Wassilevskaja (Ws) as well as mutants generated on the above backgrounds and related to MAPK signaling. Plant material including a wide array of MAPKKK, MAPKK, and MAPK mutants can be purchased from the SALK Institute.
2. Round (Ø12 mm) or square (100 × 100 × 15 mm) plastic, sterile petri dishes.
3. Sterile forceps, scalpels, razors, and wooden toothpicks.
4. Sterilization solution (100 mL): 10 % (v/v) commercial bleach supplemented with one drop of Tween 20 (*see* **Note 1**).
5. 70 % (v/v) aqueous ethanol.
6. Half-strength Murashige–Skoog medium (1/2 MS; liquid or solid; 1 L): 2.15 g MS devoid of vitamins (Duchefa), 1 % (w/v) sucrose, pH 5.8 (KOH). For solid medium, add 0.4–1.0 % (w/v) Phytagel, autoclave to sterilize and dissolve Phytagel pour to plates prior to cooling and allow to gel under sterile conditions before capping the dishes.

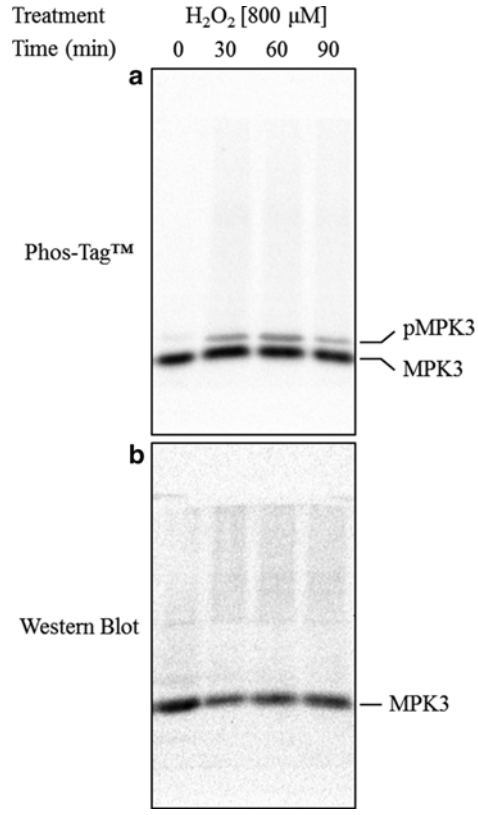

Fig. 1 Phos-Tag™ discrimination between phosphorylated and activated MPK3 following treatment of 14-day-old *Arabidopsis* seedlings with 800 μM H_2O_2 for the designated time points. The first gel (**a**) is a Zn^{2+}-Phos-Tag™ gel and the second one (**b**) is an acrylamide–bis-acrylamide gel excluding Phos-Tag and Zn^{2+}. The two gels were identically run in the same electrophoresis unit. In the Phos-Tag™ gel MPK3 appears as two separated bands (**a**), upper band is phosphorylated MPK3 (pMPK3) and lower band is non-phosphorylated (MPK3). By contrast, total MPK3 appears as a single band in the regular western blot (MPK3; **b**)

2.2 Equipment for Plant Growth, Protein Preparation, SDS-PAGE, and Western Blotting

Seedlings of *Arabidopsis thaliana* will have to grow in environmental chambers with controlled humidity, temperature and photoperiod. Porcelain mortars/pestles precooled with liquid nitrogen (LN_2), preweighted, and appropriately labeled sample tubes of 1.5 and 2 mL, Styrofoam boxes with ice, funnels, sterile medical gauge, 1–5 L Dewar vessels for LN_2 transport and storage, refrigerated benchtop microcentrifuge capable for at least 20,000 × *g* centrifugal force, heating block with adjustable temperature, water bath, shaking platforms, razors, scalpels, and tweezers for sample acquisition and collection, mini format polyacrylamide gel assemblies (glasses, spacers, combs, frames, and stands for casting gels and tank and lid for running gels) for vertical SDS-PAGE (e.g., Mini Protean II system from Bio-Rad), casting frames for wet electrotransfer and low voltage power supply units.

2.3 Chemicals and Consumables for Protein Preparation, Phos-Tag™ PAGE and Western Blotting

All common chemicals should be of specified purity (best pro analysis grade) and can be purchased by any vendor of choice. All solutions should be prepared and handled accordingly (*see* **Note 2**). Water used for preparation of solutions should be of the highest possible purity (deionized and double-distilled with specific resistivity 18.2 MΩ×cm) and solutions should be filtered to remove water insoluble impurities. Accordingly labware used for solution preparation and electrophoresis (volumetric cylinders, beakers, stirring magnets, gel casting glasses, combs, electrophoretic tanks, etc) should be cleaned with laboratory certified detergent (e.g., Alconox®, Sigma) and not with common dishwashing liquid (*see* **Note 3**). Phos-Tag™ as well as precast Phos-Tag™ gels, are manufactured and distributed by Wako Pure Chemical Industries, Ltd. Antibodies against *Arabidopsis* MAPKKKs or MAPKKs are at present very limited (e.g., Santa Cruz Biotechnology offers anti-CTR1 (constitutive triple response 1; an ethylene responsive MAPKKK), and anti-MKK1, anti-MKK2, and anti-MKK3 against the respective *Arabidopsis* MAPKKs). Commercial peptide-specific, affinity purified antibodies against MPK3, MPK4, and MPK6 are currently available from various companies (e.g., Sigma, Abcam, and Agrisera). Likewise, antibodies against MPK substrates including the microtubule associated protein MAP65-1 (a cytoskeletal substrate for MPK4 and MPK6; Bioss), the prototypical MPK4 substrate MKS1 (MAP kinase substrate 1; Statens Serum Institute), and WRKY transcription factors (routinely regulated by MAPKs; Agrisera) of *Arabidopsis thaliana* have recently been commercially released. Peptide, affinity purified antibodies against pTEpY MAPK motifs of mammalian ERKs are manufactured by several companies (e.g., Cell Signaling) and are fully applicable to plant MAPKs bearing the same motif. Horseradish peroxidase (HRP)-conjugated secondary antibodies for enhanced chemiluminescence (ECL) development of western blots are widely available in the market (e.g., Santa Cruz Biotechnology). For chemical inhibition of inducible MAPK signaling, MEK inhibitors including PD98059, or U0126 are routinely used in plant research [20] and are widely available from the market (e.g., Calbiochem). Ready-to-use ECL reagents for development and documentation of western blots are also widely commercially available. Polyvinyldifluoride (PVDF) or nitrocellulose membranes are widely available in the market as well as blotting papers of variable thickness. For documenting and archiving western blots either use X-ray film (e.g., Kodak BioMax™ MR-1, Perkin Elmer), or western blot documentation machine (e.g., ChemiDoc™ MP System, Bio-Rad).

2.4 Solutions

1. Phosphate buffered saline (PBS) pH 7.4: 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl (*see* **Note 4**).
2. Universal extraction buffer: 50 mM K–HEPES (or Tris–Cl) pH 7.4, 150 mM KCl, 5 mM EGTA, 0.1 % (w/v) sodium

dodecyl sulfate (SDS), 0.5 % (w/v) Na-deoxycholate, 100 mM dithiothreitol (DTT), protease inhibitor cocktail (e.g., Complete™ EDTA-Free tablets, Roche), and phosphatase inhibitors (*see* **Note 5**; 10 mM NaF, 10–50 mM β-glycerophosphate, 0.1–1 mM sodium orthovanadate (Na_3VO_4), or commercially available formulations, e.g., PhosStop™, Roche; *see* **Note 6**).

3. Homogenization buffer for phenol extraction: 0.7 M sucrose, 0.5 M Tris–Cl pH 7.4, 50 mM EDTA, 0.1 M KCI, 2 % (v/v) β-mercaptoethanol, protease and phosphatase inhibitors (*see* **Note 7**).
4. Activated Na_3VO_4 aqueous solution [24]: prepare 100 mM Na_3VO_4 in ultrapure water. Titrate to pH 9 with 1 N HCl. The solution will become dark orange. Heat until the solution clears (*see* **Note 8**); allow cooling at room temperature. Repeat the procedure until pH is stable at a value of 9 and restore original volume by adding ultrapure water. Aliquot and store indefinitely at −20 °C.
5. Tris-buffered phenol: Ultrapure phenol equilibrated with 100 mM Tris–Cl pH 7.4 at a volume ratio of 1:10 (pre-equilibrated mixtures are commercially available; *see* **Note 9**). Mix thoroughly and allow phases to separate. Store at 4 °C and acquire phenol from the bottom phase.
6. Methanolic ammonium acetate (NH_4OAc): 100 mM NH_4OAc in absolute methanol.
7. Absolute acetone dried over molecular sieves: Acetone stored over type 3A molecular sieves, and stored under −20 °C.
8. Solubilization buffer: 7 M urea, 2 M thiourea, 2–4 % (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.1 % (w/v) SDS, 20 mM DTT.
9. Protein loading buffer: 65 mM Tris–Cl pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol (*see* **Note 10**), 0.01 % (w/v) bromophenol blue. Should be prepared as a 5× stock solution without β-mercaptoethanol, which should be freshly added to the sample prior to electrophoresis.
10. Protein loading buffer for resuspending acetone precipitates: The same as protein loading buffer but with the addition of 6 M urea (*see* **Note 11**)
11. Stock solutions of MEK inhibitors PD98059 or U0126 should be prepared as 10 mM stocks dissolved in DMSO (*see* **Note 12**).

2.5 Material for Lambda-Phosphatase (λ-PPase) Treatment

1. λ-PPase reaction buffer: 50 mM Tris–Cl, pH 7.5, 0.1 mM Na_2EDTA, 5 mM DTT. Can be prepared as 10× stock solution, aliquoted and stored indefinitely under −20 °C.

Before reaction supplement with 2 mM $MnCl_2$ and 0.01 % (v/v) Triton X-100.

2. λ-PPase storage buffer.
3. phosphatase inhibitor cocktail: 10 mM NaF, 10–50 mM β-glycerophosphate, 0.1–1 mM activated Na_3VO_4 (*see* **Note 13**).

2.6 Material for Acrylamide Pendant SDS-PAGE and Western Blotting

1. Phos-Tag™ stock solution: to the vial which contains 10 mg Phos-Tag™ add 100 μL absolute methanol and vortex until Phos-Tag™ comes in solution. Subsequently add 3.2 mL ultrapure water to prepare a 5 mM stock solution.
2. $MnCl_2$ stock solution: 10 mM in ultrapure water.
3. $ZnNO_3$ stock solution: 10 mM in ultrapure water.
4. 1.5 M Tris–Cl pH 8.8.
5. 0.5 M Tris–Cl pH 6.8.
6. 1.25 Bis-Tris–Cl pH 6.8.
7. 30 % w/v acrylamide–bis-acrylamide 37.5:1 (e.g., Sigma).
8. 10 % (w/v) SDS in ultrapure water.
9. 10 % (w/v) ammonium persulfate (APS) in ultrapure water.
10. Tetramethylethylenediamine (TEMED).
11. Separation gel mixture for Mn^{2+}-Phos-Tag™ gels (10 mL, T8%): 4.47 mL ultrapure water, 2.67 mL 30 % acrylamide–bis-acrylamide 37.5:1, 2.5 mL 1.5 M Tris–Cl pH 8.8, 100 μL 10 % (w/v) SDS, 100 μL stock Phos-Tag™ (final concentration 50 μM) and 100 μL stock $MnCl_2$ solution (*see* **Note 14**), 25 μL 10 % (w/v) APS, 5 μL TEMED. Allow at least 2 h for complete polymerization.
12. Separation gel mixture for Zn^{2+}-Phos-Tag™ gels (10 mL, T7%): 4.58 mL ultrapure water, 2.86 mL 1.25 M Bis-Tris–Cl pH 6.8, 2.33 mL 30 % acrylamide–bis-acrylamide 37.5:1 (*see* **Note 15**), 100 μL stock Phos-Tag™ (final concentration 50 μM) and 100 μL stock $ZnNO_3$ solution (*see* **Note 16**), 25 μL 10 % (w/v) APS, 5 μL TEMED. Allow at least 2 h for complete polymerization.
13. Stacking gel mixture for Mn^{2+} Phos-Tag™ gels (5 mL, T4%): 3 mL ultrapure water, 1.25 mL 0.5 M Tris–Cl pH 6.8, 0.67 mL 30 % acrylamide–bis-acrylamide 37.5:1, 50 μL 10 % (w/v) SDS, 50 μL 10 % (w/v) APS, 5 μL TEMED.
14. Stacking gel mixture for Zn^{2+} Phos-Tag™ gels (5 mL, T4%): 2.27 mL ultrapure water, 1.43 mL 1.25 M Bis-Tris–Cl pH 6.8, 0.67 mL 30 % acrylamide–bis-acrylamide 37.5:1, 25 μL 10 % (w/v) APS, 5 μL TEMED.
15. Gel running buffer I for Mn^{2+}-Phos-Tag™ gels: 25 mM Tris base, 192 mM glycine, 0.1 % (w/v) SDS (can be prepared as 10× stock; *see* **Note 17**).

16. Gel running buffer II for Zn^{2+}-Phos-Tag™ gels: 50 mM MOPS, 50 mM Tris base, 0.1 % SDS, pH 7.8, 5 mM sodium bisulfite (*see* **Note 18**).
17. Gel transfer buffer for Mn^{2+} and Zn^{2+}-Phos-Tag™ gels: 25 mM Tris base, 192 mM glycine, add 5–10 % (v/v) Methanol to the diluted 1× buffer immediately before transfer (*see* **Note 19**).
18. Ponceau staining solution: 0.1 % (w/v) Ponceau S in 5 % (v/v) aqueous acetic acid.
19. Tris-buffered saline supplemented with Tween-20 (TBST): 20 mM Tris–CI pH 7.5, 150 mM NaCl, 0.1–0.5 % (v/v) Tween 20 (*see* **Note 20**).
20. Blocking buffer I: 2.5 % (w/v) bovine serum albumin (BSA) and 2.5 % (w/v) nonfat dry milk in TBST (*see* **Note 21**).
21. Blocking buffer II: 1 % (w/v) Hammarsten grade casein in TBST (e.g., Affymetrix; *see* **Note 22**).
22. Blocking buffer III: 3–5 % (w/v) BSA w/v in TBST (*see* **Note 23**).
23. Stripping buffer I: 0.5 N NaOH in ultrapure water (*see* **Note 24**).
24. Stripping buffer II: 15 g glycine, 1 g SDS, 10 mL Tween 20, adjust pH to 2.2, adjust volume to 1 l with ultrapure water (*see* **Note 25**).
25. Stripping buffer III: 20 mL SDS 10 % (w/v), 12.5 mL Tris–HCl pH 6.8 0.5 M, 67.5 mL ultrapure water, add 0.8 mL ß-mercaptoethanol under the fume hood (*see* **Note 26**).

3 Methods

3.1 Arabidopsis thaliana Plant Cultures and Treatments

1. Surface-sterilize seeds with 10 % (v/v) solution of household bleach supplemented with a drop of Tween 20 for 10–20 min with occasional vortexing (*see* **Note 27**).
2. After transferring in a sterile laminar flow box, rapidly wash seeds with 70 % (v/v) ethanol in distilled, autoclaved water, followed by three to four changes with sterile, distilled water and then disperse on sterile filter-paper-lined petri dishes and allow to air-dry.
3. Plate seeds in a single row (*see* **Note 28**) on solid medium of medium hardness (1/2 MS medium pH 5.8 with 10 mM K-MES, without vitamins and with the addition of sucrose to 1–5 % (w/v) (*see* **Note 29**), solidified with 0.4–1.0 % (w/v) Phytagel (*see* **Note 30**) in round or square sterile petri dishes, seal with gas permeable film, and stratify at 4 °C for 1–4 days before transferring to growth chamber in order to synchronize germination and subsequent growth of seedlings.
4. Following stratification, transfer to environmental growth chamber with controllable temperature at 21 °C, photoperiod

(16 h/8 h light/dark cycle), light intensity (120–150 μmol/m^2 s), and humidity (50 %). Place petri dishes in vertical position. When petri dishes are inclined, many roots tend to embed into the medium which will eventually contaminate the protein extract.

5. Allow growth for at least 14 days to ensure adequate seedling growth and sufficient root and shoot material for protein isolation.
6. Prior to treatments place petri dishes on platform shaker and allow standing in horizontal position for at least 2 h (*see* **Note 31**). Subsequently pour 10 mL of the treatment solution at the opposite end of the petri dish and slowly shake on a rotating table for the desired time. For each treatment allow one petri dish to ensure adequate material.
7. For MEK inhibitor studies, inhibitors should be applied alone at the appropriate dilution (*see* **Note 32**) for sufficient time before the application of any stress and also during the application of the desired stress treatment (*see* **Note 33**).

3.2 Protein Extraction

1. Following treatments and with the aid of a sharp razor blade, rapidly separate roots from aerial parts. Pick up roots as fast as possible with the aid of forceps taking care to avoid contamination with solid medium.
2. Briefly wash with ice-cold PBS, blot excess of liquid by tapping roots on filter paper and flash freeze in liquid nitrogen. Aerial parts are not adhering on the medium, so they can be washed off the plate with a squirt bottle with ice-cold PBS and collected in a sterile medical gauge lined funnel. Following draining and adequate blotting of excess liquid, freeze accordingly aerial parts in LN_2.
3. Grind frozen roots and aerial parts to fine powder with the aid of LN_2 prechilled mortar/pestle and collect powders into preweighted 1.5 or 2.0 mL sample tubes stored under LN_2. Material powdered in LN_2 can be used immediately for protein extraction or stored in deep freezer (−80 °C) for later processing.
4. Following collection, LN_2 powders from roots or aerial parts should be rapidly weighted and immediately mixed with 2 volumes of universal extraction buffer (Subheading 2.4). After brief vortexing, homogenates should rest on ice with occasional mixing for 15–30 min.
5. Alternatively LN_2 powders can be homogenized and extracted with homogenization buffer (*see* Subheading 2.4, **item 3**) if phenol extraction will follow. In this case homogenization/extraction is the same as described in the step above.

6. The homogenates are then clarified by brief centrifugation (13,000 × *g*, 10 min, 2–4 °C).
7. Provided that the cleared homogenate has sufficient protein content (as determined by Bradford assay; *see* **Note 34**), it can be mixed proportionally with 5× protein loading buffer, heat-denatured (90 °C, 3–5 min) and used directly for Phos-Tag™ SDS PAGE.
8. If the protein concentration of the resulting supernatant is low (as determined by Bradford assay; *see* **Note 34**), then it is distributed in equal volumes between fresh 2 mL microfuge tubes (ca. 250–300 μL of extract per tube) and mixed with at least 4–6 volumes of ice-cold, molecular-sieve anhydrous acetone and transferred to −20 °C freezer overnight to precipitate proteins.
9. At the next day, coagulated proteins are pelleted again by brief centrifugation (13,000 × *g*, 10 min, 2–4 °C).
10. Following centrifugation, the supernatant is discarded and the pellet is allowed to air dry at room temperature until all acetone has visibly evaporated (*see* **Note 35**).
11. Resuspend in solubilization buffer (use 1/10th of the volume of the original cleared supernate), measure protein content, and mix proportionally with protein loading buffer supplemented with 6 M urea.

3.3 Phenol Protein Extraction

This is meant for cleaner protein samples than those obtained as described in Subheading 3.2.

1. Protein supernatants acquired as described in Subheading 3.2, **step 5** may be alternatively extracted by phenol phase extraction by mixing with an equal volume of buffered phenol.
2. Let mixture stand on ice for 30 min with vortexing at frequent intervals.
3. Separate phases by centrifugation (10,000 × *g*, 10 min, 4 °C).
4. Remove phenol phase to fresh microfuge tube and back-extract with equal volume of homogenization buffer.
5. Back-extract aqueous phase with Tris-buffered phenol.
6. Repeat phase separation, collect and combine phenol phases, and distribute them into clean 2 mL microfuge tubes (ca. 250–300 μL per tube).
7. Add 5 volumes of 100 mM methanolic ammonium acetate and allow proteins to precipitate overnight at −20 °C.
8. Recover precipitate at 10,000 × *g*, 10 min, 4 °C.
9. Resuspend pellet in 100 mM methanolic ammonium acetate, vortex to disperse, and repeat **step 8** in Subheading 3.3.

10. Resuspend pellet in 80 % (v/v) acetone in water, disperse by vortexing, and repeat **step 8** in Subheading 3.3 (repeat twice).
11. Allow final pellet to air-dry (*see* **Note 35**).
12. Resuspend in solubilization buffer (use 1/10th of the volume of the original cleared supernate), measure protein content, and mix proportionally with protein loading buffer supplemented with 6 M urea.

3.4 Treatment of Protein Extracts with Lambda Phosphatase

1. Thoroughly resuspend protein pellet in a minimal volume of λPPase reaction buffer by thoroughly pipetting on ice. Any residual clumps of denatured protein should be removed by a quick spin-down (13,000 × *g*, 1–2 min, 4 °C).
2. A small amount of cleared supernate should be kept for protein content determination by any colorimetric assay (Bradford, bicinchoninic acid, etc., available from many vendors).
3. Dilute 50–100 μg of total protein with λPPase reaction buffer and add 200–400 units of λPPase (*see* **Note 36**). Allow reaction at 30 °C for 20–30 min. In parallel prepare an identical sample supplemented with phosphatase inhibitors and one sample loaded with heat-denatured λPPase as negative controls.
4. After designated time, stop reaction by mixing proportionally with 5× loading sample buffer and heating.
5. Alternatively, precipitate with 80 % (v/v) ice-cold acetone and resuspend protein pellet with 1× urea sample buffer without heating.

3.5 Conditions for Electrophoretic Separation of Phospho- and Non-phosphorylated Proteins by Acrylamide Pendant Phos-Tag™ SDS PAGE: Mn^{2+} Protocol

1. Fill upper and lower chamber with running buffer I.
2. Set power supply unit to 5–15 mA/gel constant current.
3. Allow electrophoresis to run until 25 kDa prestained protein marker just exits the gel assembly.

3.6 Conditions for Electrophoretic Separation of Phospho- and Non-phosphorylated Proteins by Acrylamide Pendant Phos-Tag™ SDS PAGE: Zn^{2+} Protocol

1. Fill upper and lower chamber with running buffer II.
2. Set power supply unit to 5–15 mA/gel constant current.
3. Allow electrophoresis to run until 25 kDa prestained protein marker just exits the gel assembly.

3.7 Post-processing of Phos-Tag™ Gels and Electrophoretic Transfer to PVDF Membranes

1. After disassembling glass plates, immerse Phos-Tag™ gels in transfer buffer containing 10 mM EDTA (*see* **Note 37**) and place on a rocking table for 15–30 min.
2. Wash gels and equilibrate gels with transfer buffer supplemented with 1 mM EDTA.
3. Wet sponges and thick blotting paper sheets in transfer buffer supplemented with 1 mM EDTA.
4. Pretreat PVDF membranes with absolute methanol for 30s and subsequently immerse them in transfer buffer not allowing them to dry out.
5. Assemble sponge, blotting paper, Phos-Tag™ gel, and PVDF membrane sandwiches accordingly for wet transfer.
6. Transfer at a constant voltage of 100 V for 1½ h or at 24 V overnight at 4 °C.
7. After transfer is complete, remove membranes and allow them to air-dry (*see* **Note 38**). After drying is complete, reactivate them in absolute methanol and wet them in TBST.
8. Confirm transfer efficiency by Ponceau S staining of the membrane.
9. Block membranes with blocking agent as described in Subheading 2.4 for 1½ h at RT to prevent unspecific antibody binding (*see* **Note 39**).
10. Incubate with primary antibody diluted appropriately in manufacturer defined blocking buffer for 1½ h (room temperature) to overnight (refrigerator).
11. Wash thoroughly in TBST (6 × 10 min).
12. Incubate with appropriately diluted horseradish peroxidase-conjugated secondary antibody.
13. Repeat **step 9**.
14. Incubate membranes in enhanced chemiluminescent reagent according to manufacturer's instructions (*see* **Note 40**).
15. Document membranes either on X-ray film or in gel/blot documentation machine according to manufacturer's instructions. For comparisons *see* [25].
16. Should reprobing of the membranes with another antibody has to be done, strip antibodies off the membrane by 30 min incubation in stripping buffer. For reprobing of stripped membranes, repeat **steps 9–15**.

4 Notes

1. If concentrated Tween is added directly to the diluted bleach, the solution may cloud. For this reason, Tween 20 is first diluted in 1 mL of distilled water and then added slowly to the bleach solution with a micropipette with continuous stirring.
2. It is important to work with all safety precautions associated with good laboratory practice. Working area and hardware must be clean, and work should be conducted with appropriate clothing and latex or nitrile hand gloves. All toxic solutions should be handled under a fume hood and discarded according to institutional safety regulations.
3. Even trace phosphate contaminations in chemicals, water, or detergent used for cleaning glassware will impede Phos-Tag™ based separation of phosphorylated proteins.
4. PBS can be prepared as a 10× stock solution, supplemented with 0.01 % (w/v) thimerosal and stored indefinitely at room temperature.
5. Phosphatase inhibitors are essential to preserve phosphorylation status in the primary extract. However, they may interfere with λPPase treatment. In this case, it is essential to remove such contaminants by selectively precipitating proteins.
6. According to material safety data sheet PhosStop™ is a mixture of okadaic acid (a marine natural product that specifically and potently inhibits serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A)) and cantharidine (potent and specific inhibitor of protein phosphatases 1 (PP1) and 2A (PP2A)). The product is therefore highly toxic and should be handled with appropriate safety precautions.
7. The buoyant density of the homogenization buffer will allow it to separate as a lower phase during phenol extraction and allow the acquisition of the phenol phase without contamination from the aqueous one.
8. This will take time.
9. Phenol is toxic. Handle under fume hood.
10. β-Mercaptoethanol is toxic, carcinogenic, and foul smelling. Always work under a dedicated fume hood and handle with gloves.
11. Avoid heating to prevent urea decomposition and carbamoylation of proteins
12. Dissolve PD98059 or U0126 in the minimum possible amount of DMSO, as this will ensure that DMSO in the treatment solution will be the least possible.

13. When using Na_3VO_4 as phosphatase inhibitor exclude reducing agents like DTT or β-mercaptoethanol from the same solution as they will promote oligomerization of vanadates.
14. Do not use other Mn^{2+} salts.
15. Acrylamide is neurotoxic. Dispensing and handling acrylamide solutions or acrylamide and bis-acrylamide powders before gel polymerization should be carried out under fume hood with gloves, mask, and protective clothing.
16. Do not use other Zn^{2+} salts.
17. pH does not need adjustment.
18. Sodium bisulfite is mutagenic. Always handle with gloves under fume hood.
19. Methanol is toxic. Handle under fume hood.
20. Increasing of Tween-20 concentration during blocking of the membrane may decrease nonspecific antibody reactivities.
21. Allow sufficient time for dissolution as milk tends to form clumps difficult to dissolve. Always filter before use. Avoid using this blocking solution when probing membrane with phospho-specific antibodies as variably phosphorylated casein in milk formulations may lead to heavy background staining of PVDF or nitrocellulose membranes.
22. Casein will require mild heating to dissolve. Place solution in a water bath at 50 °C. Also avoid this blocking buffer when probing with phospho-specific antibodies.
23. Solubility of BSA increases at low temperatures. Therefore precool the desirable amount of TBST on ice prior to accelerate dissolution of BSA.
24. This solution is intended only for PVDF membranes.
25. This buffer applies to both nitrocellulose and PVDF membranes. Note that due to the presence of SDS proteins bound on the membranes may be removed.
26. Due to the high content of mercaptoethanol and the stripping procedure at elevated temperatures (ca. 50 °C), prepare this buffer and handle it only under fume hood. Membranes stripped in such way should be kept in a tightly closed and sealed container.
27. Other sterilization solutions may include 3 % (v/v) H_2O_2 (diluted from perhydrol) in 70 % (v/v) ethanol. Chlorine gas produced by mixing concentrated HCl with sodium hypochlorite is an alternative sterilization procedure for dry seeds but it must be practiced in a closed place and sterilization will require longer time (e.g., 12 h for 50 μL of packed, dry seeds).

28. Overcrowding seedlings in a single petri dish may cause aberrant growth and emergence of stress related phenotypes due to ethylene production.
29. Lack of sucrose in the growth medium may allow the better identification of some MAPK mutants while higher concentration of sucrose in the culture medium might rescue some of these mutants.
30. Hard media, i.e., those containing Phytagel concentrations between 0.8 and 1.5 % (w/v) can help to reveal phenotypes related to root cell elongation defects.
31. Rotating the plants from vertical to horizontal position may rapidly induce MAPK activation independently of the desired treatment. Thus, a considerable time of acclimation at the horizontal position (e.g., 2 h) will be necessary before starting the experiment.
32. MEK inhibitors should be used at concentrations near their published IC50 values (5–10 μM for PD98059 and 10 μM for U0126) to ensure specificity of the treatment.
33. If the involvement of MAPKs is intended to be addressed after some physiological treatment, then MEK inhibitors should be applied for sufficient time alone, prior to the onset of the treatment to ensure adequate intracellular diffusion of the inhibitors.
34. Even trace amounts of phosphatase inhibitors in the protein extract will interfere with λPPase assay. These can be quantitatively removed by sequential precipitation of proteins in 100 mM methanolic ammonium acetate and anhydrous acetone.
35. Prior to drying the pellet is milky white. After drying it will be translucent with a pinkish discoloration. Do not allow to overdry and be patient with resolubilization.
36. The original protocol [26] calls for a 10 min wash with 1 mM EDTA in transfer buffer. However, we and others [22, 23] found that this is not sufficient to allow the quantitative transfer of proteins from the Phos-Tag™ gel to the blotting membrane. Thus, 10 mM EDTA is used for gel washing while the transfer buffer is supplemented with 1 mM EDTA.
37. Drying of PVDF membranes prior to further processing ensures that most of the proteins bound will remain on the membrane after subsequent processing. This is particularly important when a membrane will have to be stripped and reprobed, and will allow multiple rounds of stripping and reprobing when original material is sparse.
38. If background staining is still a problem, block overnight at 4 °C.

39. A custom ECL can be made with p-coumaric acid as enhancer of luminol chemiluminescence [27].
40. Always drain excess of ECL reagent prior to development. If reaction is too strong, try to briefly wash with TBST before development.

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Part II

Genetic Manipulation of MAPK Function

Chapter 6

Identification of Constitutively Active AtMPK6 Mutants Using a Functional Screen in *Saccharomyces cerevisiae*

Elodie Hudik, Souha Berriri, Heribert Hirt, and Jean Colcombet

Abstract

MAPK (Mitogen-Activated Protein Kinases) mutants which are active independently of phosphorylation by upstream MAPK Kinases (MAPKKs) help to clarify signal transduction processes through MAPK modules and provide a useful tool to understand MAPK roles in the cell. The identification of such mutations is tricky. In this chapter, we provide a detailed protocol for their screening, taking advantage of a functional expression assay in yeast.

Key words MAP Kinase, Functional genetic screen, Constitutive activity, *Saccharomyces cerevisiae*, In vivo cloning, Random mutagenesis

1 Introduction

Constitutively active (CA) protein kinases considerably extend the repertoire of tools for the functional analysis of signal transduction pathways [1]. Until recently, no strategy to render MAPKs (Mitogen-Activated Protein Kinases) was available for plants. In a recent work, we took advantage of a functional expression screen in yeast to identify point mutations triggering MAP2K-independent constitutive activity to a model MAPK of *Arabidopsis*, MPK6 [2]. In this chapter, we provide a detailed protocol for the screening of CA MAPKs in yeast.

The *Saccharomyces cerevisiae* genome codes for four functional MAPK modules [3]. The high osmolarity glycerol (hog) pathway, defined by the MAPKK pbs2 and the MAPK hog1, is activated in response to high osmolarity and allows the yeast to survive with large concentrations of salt or solutes in the environment (Fig. 1). Single and double mutants of the pathway are hypersensitive to salt and will die on high salt concentrations. In a previous work, we showed that a number of MAP2K/MAPK modules, including *Arabidopsis* AtMKK2/AtMPK6, are able to functionally complement the defect of growth of *pbs2Δhog1Δ* double mutants [4].

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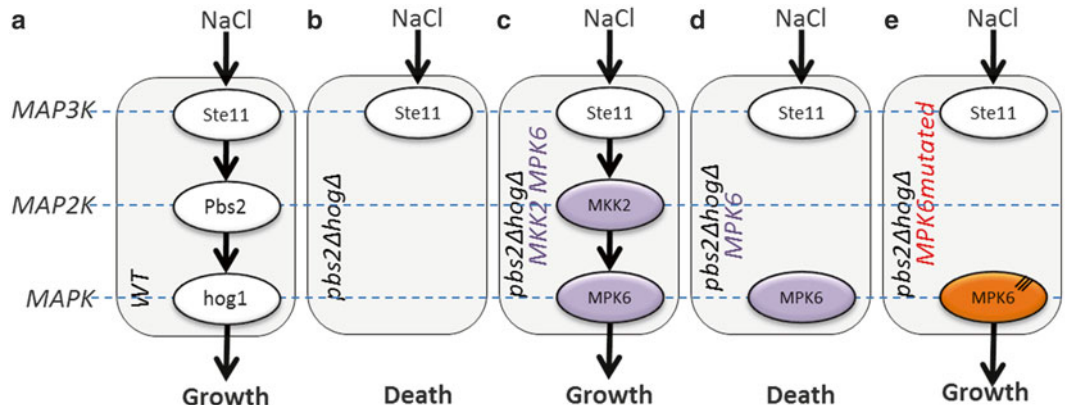

Fig. 1 Overview of the screening strategy using a mutant yeast strain defective in hog1 pathways. In *Saccharomyces cerevisiae*, NaCl activates the high-osmolarity glycerol 1 (Hog1) MAPK module, allowing the cells to grow on media supplemented with salt (**a**). *pbs2*Δ*hog*Δ double-mutant is unable to cope with high salt concentrations (**b**), but this growth defect is complemented by *Arabidopsis thaliana MPK6* and *MKK2* genes (**c**). The fact that *MPK6* alone does not complement the defect suggests that MKK2 is necessary for MPK6 activation (**d**). The functional screen consists to identify MPK6 mutations triggering the yeast growth on salt media without its upstream MAP2K MKK2 (**e**)

Interestingly, MKK2 activation of MPK6 is necessary for growth recovery, as MPK6 alone is unable to restore growth on hyperosmotic conditions. This defines a suitable screening condition to identify mutations which allow MAP2K-independent MAPK activity as mutations in MPK6 triggering improved catalytic activity should complement the growth defect phenotype of the yeast double mutant on salt (Fig. 1).

hog1-related mutants are able to grow on normal synthetic media, but not under hyperosmotic conditions, allowing for an easy selection system in the laboratory. Additionally, the CA MAPK screen using such strains is a positive screen: the interesting clones will be the ones able to grow on a selective hyperosmotic medium. The protocol we describe here is largely adapted of the work of Englberg and coworkers [5]. The major modification we performed consists in generating mutations using a PCR-based random mutagenesis strategy coupled to an in vitro cloning step instead of using an *E. coli* strain inducing mutations to amplify the plasmids. Figure 2 shows an overview of the molecular cloning steps allowing the creation of a library of randomly mutated MPK6 open reading frames (ORFs) ready to be expressed in the reporter yeast strain (e.g., Fig. 2). With this technique (we used GeneMorph® II Random Mutagenesis Kit from Stratagene), the number of mutations is accurately selected ranging from 0–4.5 to 9–16 mutations per kb depending on the PCR conditions. The Mutazyme II polymerase activity of the kit is a mixture of several Taq DNA Polymerase enzymes performing different kinds of errors during

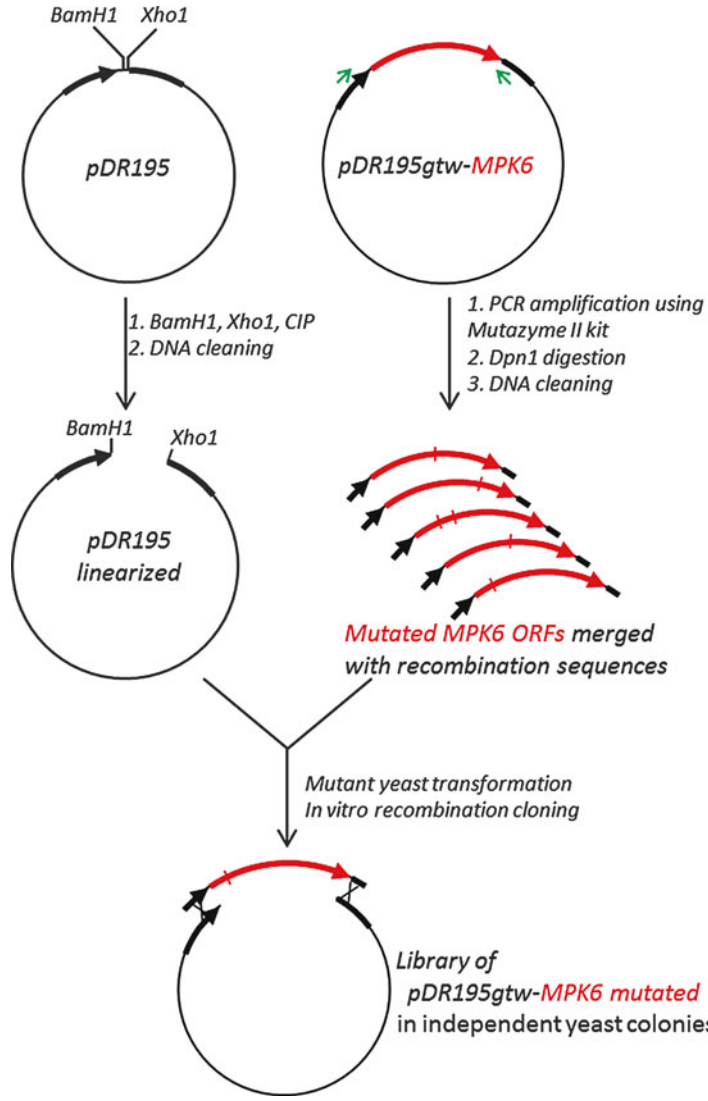

Fig. 2 Reconstruction in yeast of a plasmid library allowing for the expression of randomly mutated MPK6

the DNA synthesis in a way to have similar probabilities to obtain all types of mutations. Please note however that each randomly created mutation has about a 5 % chance to induce a STOP codon. Therefore, there is a risk to lose a large number of clones which are truncated by using a high mutation rate protocol on long sequences.

2 Materials

A list of materials is provided as example, but similar materials from other brands may be purchased.

2.1 Strains and DNA

1. *Saccharomyces cerevisiae pbs2Δhog1Δ* double mutant in the genetic background W303 [4, 6].
2. DH5alpha thermo-competent *Escherichia coli* cells prepared as previously described [7].
3. pDR195 and pDR195gtw-MPK6 (6, 1). pDR195-based vectors contain the URA3 autotrophy marker gene. Other expression vectors and MAPK model gene may be used to perform a screen as long as they allow the complementation of the strain defect as described in Fig. 1. This will allow for the identification of new mutations.
4. Primers pPMA1prom (TTT CTC TTT CTT TCC TAT AAC ACC AAT AGT G) and pADH1term (GTG TCA ACA ACG TAT CTA CCA ACG ATT TGA CC) for MPK6 amplification from pDR195gtw-MPK6.
5. Primers pMPK6_F (GTA TTT TCT TTA CCA GAT CCT CCG TG) and pMPK6_R (CTG GTG CAC GGT ACC ATC TCG TGA C) for the sequencing of the MPK6 coding sequence.

2.2 Enzymes and Kits

1. Endonucleases BamH1 and Xho1, calf intestinal alkaline phosphatase, and NEBuffer3 were all from NEB.
2. Wizard® SV Gel and PCR Clean-Up System (Promega).
3. Wizard® SV plus Minipreps DNA Purification System (Promega).
4. GeneMorph® II Random Mutagenesis Kit (Stratagene).

2.3 Medium and Buffer

1. YPD medium: (1 % (w/v) Yeast extract, 2 % (w/v) peptone, 2 % (w/v) d-glucose, ±2 % (w/v) Agar for plates, autoclaved).
2. SC-U (Synthetic medium minus Uracil) medium: (0.17 % (w/v) Yeast Nitrogen Base w/o amino acids and ammonium sulfate, 0.5 % (w/v) NH_4SO_4, 2 % (w/v) d-Glucose, Drop-out mix minus Uracil (US Biological), pH 5.6 adjusted with NaOH, ± 2 % (w/v) Agar for plates, autoclaved).
3. LB (Lysogeny Broth) medium and LB agar.
4. TE Buffer: 10 mM Tris–HCl pH 7.5, 1 mM EDTA–NaOH pH 8.0.
5. TEL0.5 Buffer: (0.5 mM EDTA–NaOH pH 8.0, 5 mM Tris–HCl pH 7.5, 100 mM lithium acetate pH 7.5, filtered).
6. TEL5 Buffer: 5 mM EDTA–NaOH pH 8.0, 50 mM Tris–HCl pH 7.5, 500 mM lithium acetate pH 7.5, filtered.
7. PEG solution: 50 % (w/v) PEG-3350 in water, filtered.
8. PegTEL Buffer: 1 volume of TEL5 Buffer + 4 volumes PEG solution.
9. Yeastmaker Carrier DNA (Clonetech).

10. Yeast Extraction Buffer: 100 mM NaCl, 10 mM Tris–HCl pH 8, 1 mM EDTA, 0.1 % (w/v) SDS.

2.4 Specific Materials

1. Nanodrop 2000c (Thermo Scientific).
2. Replicator (Replica-Plating Tool, Scienceware) with adapted autoclaved cotton velveteen squares.

2.5 Consumables

1. Glass beads, acid washed (e.g., Sigma).
2. Plating beads (autoclaved).
3. Ø 14.5 cm round petri dish.
4. Ø 8 cm round petri dish.
5. 96-wells plates with 2 mL square wells for microbiological culture.
6. Gas-Permeable Adhesive Seals (e.g., Thermo Scientific).

3 Methods

The pipeline of the whole process is presented in Fig. 3.

3.1 Generation of DNA Mixture for pbs2Δ hog1Δ Yeast Transformation

1. Prepare mutagenized PCR product using GeneMorph® II Random Mutagenesis Kit (Stratagene), pPMA1prom and pADH1term as primers and pDR195gtw-MPK6 as matrix. pPMA1prom and pADH1term couple of primers amplifies a

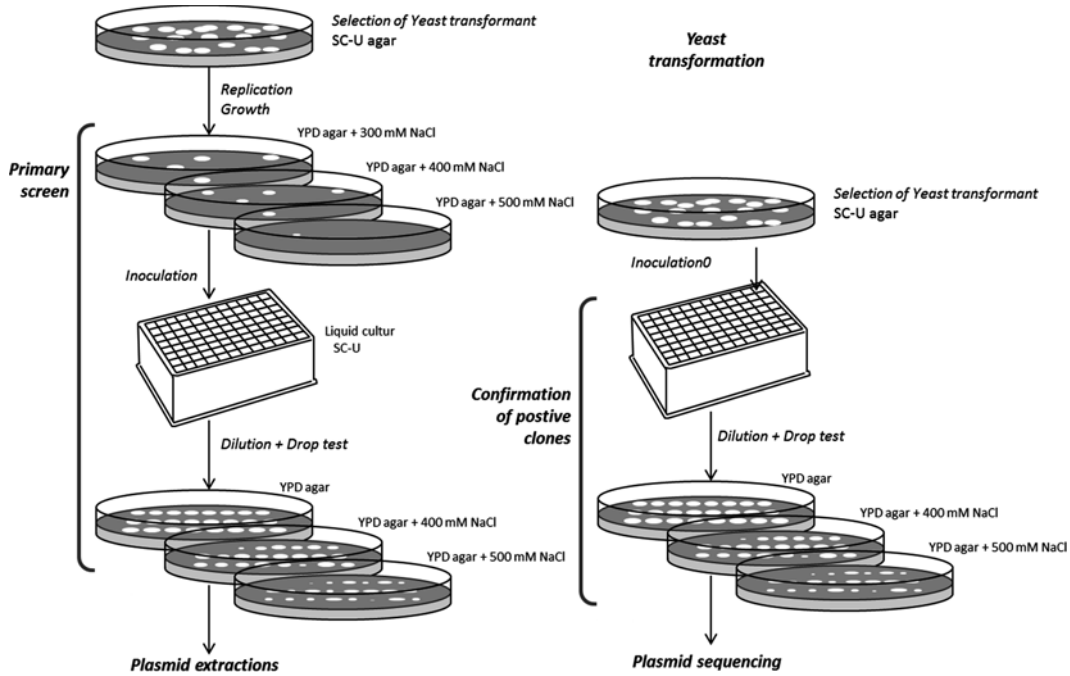

Fig. 3 Overview of the protocol

1,621-bp DNA fragment from pDR195gtw-MPK6. We generally perform three 50 µL PCR reactions in parallel in order to rich different mutation rates as stated by the manufacturer (Low: 0–4.5, Medium: 4.5–9, and high: 9–16 mutations/kilobases).

2. After the amplification reaction, digest the matrix by adding 2 µL Dpn1. Mix well by pipetting, spin down, and incubate at 37 °C for 2 h.
3. Clean the PCR DNA using a micro-column. We commonly use Wizard® SV Gel and PCR Clean-Up System (Promega).
4. Measure the DNA concentration using Nanodrop.
5. Meanwhile, linearize 5 µg of pDR195 with BamH1 and Xho1 in the presence of CIP by mixing:

DNA	5 µg
NEB buffer 3 (10×)	5 µL
BamH1	2 µL
Xho1	2 µL
CIP	0.5 µL
H_2O	up to 50 µL

6. Spin down the reaction mix at maximum speed for 10 s and incubate 2–4 h at 37 °C.
7. Clean the DNA using a micro-column. We commonly use Wizard® SV Gel and PCR Clean-Up System (Promega).
8. Measure the DNA concentration using a Nanodrop or another adapted device.
9. Prepare in 1.5 mL tubes, DNA mixtures with the linearized vector and the PCR product with a 1:1 ratio in molarity. Prepare controls as followed. Keep on ice for transformation.

3.2 Transformation of pbs2Δ hog1Δ Yeast

1. Inoculate an YPD agar plate with the glycerol stock of *pbs2Δhog1Δ* yeast using a sterile inoculator loop. Grow the yeast 2–3 days at 30 °C.
2. Inoculate 5 mL YPD in 50 mL culture tube with a single colony and grow the culture at 30 °C overnight (ON) at 180 RPM.
3. Dilute the 5 mL ON culture in 50 mL YPD in 200 mL Erlen Flask and grow for an additional 5–6 h at 30 °C with 180 RPM.
4. Spin down the cells at 2500 RPM (~1,300×*g*) at 20 °C for 5 min.
5. Resuspend by pipetting the pellet in few milliliters of TE buffer. Complete to 50 mL with TE buffer.

Table 1
Overview of the DNA mixes to be prepared for the yeast transformation

DNA mix	Number of tubes to be prepared	Meaning	Expected number of expected colonies
Water::water	1	Test of the contamination of the solutions used in the yeast transformation protocol	None
Linearized vectors::water	1	Check the linearization and the ability of the yeast to repair DNA	Few (ideally none)
Water::Mutated PCR fragment with a low mutation level	1	Check the Dpn1 degradation of the matrix pDR195-MPK6 used for the mutagenesis	Few (ideally none)
Water::Mutated PCR fragment with a medium mutation level	1	Check the Dpn1 degradation of the matrix pDR195-MPK6 used for the mutagenesis	Few (ideally none)
Water::Mutated PCR fragment with a high mutation level	1	Check the Dpn1 degradation of the matrix pDR195-MPK6 used for the mutagenesis	Few (ideally none)
Linearized vectors + Mutated PCR fragment at low level	5[a]	Putative interesting clones	Many
Linearized vectors + Mutated PCR fragment at medium level	5[a]	Putative interesting clones	Many
Linearized vectors + Mutated PCR fragment at high level	5[a]	Putative interesting clones	Many
pDR195 + MPK6 (1 µg)	1	Quantification of the yeast transformation efficiency	Many

[a]The total number of transformation tubes is about 20 corresponding to the use of 2 mL of yeast competent cells (*see* below)

6. Spin down the cells at 2500 RPM (~1,300×*g*) at 20 °C for 5 min.
7. Resuspend the pellet in 2 mL TEL0.5 buffer.
8. Keep the pellet for 10 min at room temperature (and longer on ice).
9. Meanwhile mix each transformation DNA mixture (*see* Table 1) with 10 µL denaturated carrier DNA. Process controls at the same time (*see* Table 1). Keep them on ice up to next step.
10. Add 100 µL of competent yeast. Mix well by pipetting.
11. Add 700 µL of PegTEL Buffer. Mix well by vortexing.
12. Incubate at 30 °C for 30 min with slow shaking.
13. Add 88 µL DMSO and vortex.

14. Heat-shock the cells in a water-bath at 42 °C for 7 min.
15. Spin down the cells 1 min at 5,000 RPM (~2,700×*g*) in a microtube centrifuge.
16. Remove the supernatant and resuspend the cells in 1 mL TE Buffer.
17. Spin down the cells 1 min at 5,000 RPM (~2,700×*g*) in a microtube centrifuge.
18. Remove supernatant using a 1 mL pipette and resuspend the cells in 200 µL sterile TE Buffer.
19. Pour the appropriate number (20) of SC-U agar plates (usually before the experiment or during the 30 min incubation at 37 °C). Let them solidify and dry without lid up to the point the agar surface presents wavelets.
20. Place 5–30 plating glass beads on each LB agar plates.
21. Pipette the transformed yeast cells (~200 µL) on the medium surface.
22. Spread the yeast solution at the agar surface using horizontal shaking long enough for the liquid to be sucked by the agar medium.
23. Invert the plates to collect the glass beads in the cover. Throw them in a collector to be later on cleaned and reused.
24. Let the yeast colonies grow during 2–3 days at 30 °C.
25. Roughly evaluate the number of transformant colonies (*see* **Note 1**).

3.3 Identification of Yeast Colonies Able to Grow on Salt

1. Prepare YPD agar selective plates containing salt by mixing 25 mL of melted YPD agar medium with an appropriate volume of sterile 5 M NaCl. We usually prepare for each plate of primary transformant colonies two series of five plates with various concentrations of NaCl (350—400—450—500—550 mM). Label them with the Salt concentration, the order they will have in the replication process and a reference to the first SC-U agar plate containing the primary transformant colonies.
2. Sterilize the replicator using 70 % (v/v) ethanol. Velvet squares should have been autoclaved.
3. Place cotton velveteen square on the replicator and clamp it using the adapted locking ring.
4. Orientate the SC-U agar plate containing the colonies, the 10 salt YPD plates, and the replicator by drawing a dot on the bottom of the plates and on the ring of the replicator.
5. Apply medium surface of the SC-U agar plate containing the primary colonies on the replicator velvet, marrying the two orientation dots up. Press gently for yeast cells to be transferred onto the velvet. Remove the plate.

6. Apply one by one the 10 YPD agar plates containing Salt, marrying the orientation dots up. Each time, check carefully that the velvet tissue is in contact with the agar medium by gentle tapping fingers.
7. Incubate the plates long enough to see colonies appearing (*see* **Note 2**).
8. Strip candidate colonies on a SC-U plate to back them up long enough to run the experiment to the end.

3.4 First Confirmation of Yeast Candidates

1. Fill a 96-well plate with 500 µL SC-U liquid medium.
2. Inoculate using sterile toothpicks SC-U medium with individual candidate yeast colonies. Include also the strain transformed with pDR195gtw-MPK6 as negative control.
3. Seal the plate with gas-permeable adhesive seal.
4. Grow the yeast overnight at 30 °C under constant shaking.
5. The morning after, pour large petri dishes with 75 mL YPD supplemented with a range of salt concentrations as defined above by mixing melted YPD agar with an appropriate volume of sterile 5 M NaCl. Let solidify and dry up.
6. Fill a 96-well PCR plate (dilution plate) with 200 µM of TE.
7. As yeast cells tend to sediment, resuspend the overnight yeast culture by pipetting. Faster work can be performed using a 300 µL multichannel pipette.
8. Dilute 10 µL of each yeast culture in 200 µL TE. Mix well.
9. Use a multichannel pipette to drop 5 µL of each diluted yeast candidate clone on medium.
10. Let the yeast drop dry.
11. Incubate the plates at 30 °C for 2–4 days.

3.5 Recovery of Plasmids from Candidate Yeast Colonies

1. Inoculate with the confirmed yeast strains recovered from the selective salt YPD agar, 5 mL SC-U liquid culture in 50 mL culture tubes. Incubate overnight at 30 °C with shaking.
2. Fill a 2 mL Round bottom microfuge tube and spin down the cells from 2 mL of culture (5,000 RPM (~2,700 × *g*) for 1 min).
3. Decant the supernatant by inverting the tubes.
4. Add about 300 µL of acid-wash glass beads.
5. Add 300 µL of Yeast Extraction Buffer.
6. Vortex 10 times during 30 s and keep the tubes on ice during 30 s in between.
7. Add 500 µL Phenol. Mix well.
8. Centrifuge at maximum speed for 5 min.
9. Transfer the supernatant (about 400 µL) in a new clean 2 mL tube. Add 1/10 volume Acetate Na 3 M and 3 volumes of

absolute cold ethanol. Mix well and keep in freezer for at least 30 min.

10. Centrifuge at 14,000 RPM (~20,000 × *g*) for 10 min.
11. Drain out the supernatant and wash the pellet with ice-cold ethanol 70 % (v/v) for 1 min.
12. Centrifuge at 14,000 RPM (~20,000 × *g*) for 2 min.
13. Rapidly remove the supernatant with a 1 mL pipette and briefly spin down to drain the remaining liquid and remove it by pipetting.
14. Let the pellet dry at room temperature.
15. Dissolve the pellet in 30 μL TE. Warm to 40 °C for 5 min to help dilution if necessary.
16. Transform 5 μL of the yeast extract in DH5 alpha thermocompetent *E. coli* cells. Plate the bacteria on LB agar plate supplemented with 100 ng/mL Carbenicillin (*see* **Note 3**).
17. Incubate at 37 °C overnight.
18. Select a single colony for each candidate to start 5 mL LB liquid culture in 50 mL tubes. Grow them overnight at 37 °C under constant shaking.
19. Extract the plasmid. We commonly use Wizard® SV plus Minipreps DNA Purification System (Promega).

3.6 Confirmation that the Mutations of the Candidate Plasmids Confer Salt Tolerance to pbs2Δhog1Δ Yeast

1. Using the yeast transformation protocol described above, transform 5 μL DNA of each candidate plasmid in the yeast mutant *pbs2Δhog1Δ*. Add pDR195 and pDR195gtw-MPK6 plasmids as negative controls. Do not forget a technical negative control as well (transformation with water) for which not a single yeast colony should be recovered. Plate the transformed yeast on SC-U in 8 cm plates (*see* **Note 4**). Grow the colonies at 30 °C for 2–3 days.
2. For each transformation, select 3 single colonies and inoculate cultures in 500 μL liquid SC-U in 96-well culture square plates. Keep a careful map of the plates. Seal the plates with a gas-permeable seal. Incubate overnight with shaking at 30 °C.
3. Prepare large YPD agar plates containing increasing concentrations of salt as described above.
4. Resuspend the cells by pipetting and dilute 5 μL of the culture in 200 μL TE in 96-well plates as described above.
5. Drop 5 μL of the dilutions on the YPD agar plates as described above.
6. Let the droplets dry and incubate the plates at 30 °C. Check daily the growth of the clones.
7. About 60 % of the candidate yeast clones initially identified in the primary screen were confirmed at this step. The DNA was sequenced using MPK6_F and MPK6_R primers.

4 Notes

1. This number should be significantly higher on plates for yeast which had the opportunity to recombine a functional vector using the linearized plasmid and the PCR product (*see* Table 1). Yeast transformant number largely varies from one experiment to another. The ideal density of colonies on plates is obviously as much as possible with the limit to have separated colonies.

   The number of colonies to be replicated in order to saturate the screen may be easily calculated from the length of the mutated ORF and the expected mutation efficiency selected in the mutagenesis protocol.

2. In our experience, spontaneous reversion of yeast growth on salt medium often occurs but independently of mutations in MPK6. Therefore, it is important to daily follow the plates and retain candidate colonies only if they may be found at the coinciding position on several plates of the replication series. Depending on the NaCl concentration as well as the amount of yeast transferred on the plate medium, interesting colonies may appear after 3–4 days.

3. Make sure that the competency of the bacteria is high enough as often this step is limiting. Alternatively, commercial competent cells may be used.

4. To avoid preparing many plates and yeast dilutions, we usually resuspend the transformed yeast in 20 μL TE, spot them as a drop on the SC-U medium surface and use inoculator loops to spread the yeast in rough gradient of density to have separated colonies.

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Chapter 7

Virus-Induced Gene Silencing in Plant MAPK Research

Christian Hettenhausen, Ian T. Baldwin, and Jianqiang Wu

Abstract

Virus-induced gene silencing (VIGS) technology has become more and more widely used in various plant species for rapid screening of gene functions. VIGS does not require time-consuming tissue culture steps that are needed for stable transformation in most plant species and it can be used for studying gene function even in plants that are very difficult to stably transform. Furthermore, VIGS technology provides high gene silencing efficiency (up to 95 %) and specificity. Here, we describe a VIGS protocol that can be used for studying the functions of MAPKs and other genes in a wild tobacco species, *Nicotiana attenuata*. This method is also suitable for other *Nicotiana* species and tomato with minor modifications.

Key words Mitogen-activated protein kinase, *Nicotiana attenuata*, Virus-induced gene silencing, Transient transformation

1 Introduction

Gene silencing is probably the most important means of studying gene function. Mostly, this is done by transforming plants with vectors containing a fragment of the target gene in an antisense or inverted repeat fashion. Agrobacterium- or biolistics-mediated plant stable transformation is available for a small number of plant species. However, except *Arabidopsis* (*Arabidopsis thaliana*), whose transformation can be easily done by floral dipping [1], transformation generally requires lengthy tissue culture procedures. This has greatly limited functional analyses of genes in these plants, especially in the age of functional genomics.

VIGS is a technique that utilizes recombinant viruses to specifically silence genes of interest in plants. In plants, posttranscriptional gene silencing (PTGS) is an innate resistance to limit viral proliferation. The underlying mechanism of this form of resistance is very similar to the RNA interference (RNAi). To initiate VIGS, a viral vector carrying a fragment of the target gene is introduced into plant cells by *Agrobacterium*- or biolistics-based transient transformation, and consequently, plants start to accumulate

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recombinant viruses, which in turn induce silencing of the plant target genes. Compared with gene silencing mediated by stable transformation, VIGS procedures have several advantages: (1) Viral vectors are simple to construct; (2) VIGS does not require tissue culture and thus can be used to study functions of many genes in short time; (3) VIGS can be used in many plant species, such as tomato, tobacco, *Arabidopsis*, and even in plants that are hard to stably transform, such as dicots, grapevine and soybean, and monocots, barley and maize [2]. Given the relatively high throughput property of VIGS technology, it is an excellent tool to study functional genomics. Plant mitogen-activated protein kinases (MAPKs) belong to a large gene family and have important signaling functions [3] and VIGS has been successfully used to obtain plants silenced in target MAPK genes in short time [4–8].

A tobacco rattle virus (TRV)-based VIGS system has often been used in a few solanaceous plants, namely, tobacco (*Nicotiana tabacum*), *N. attenuata*, *N. benthamiana*, and tomato (*Solanum lycopersicum*). Compared with other viruses, such as potato virus X (PVX), TRV more effectively infects solanaceous plants and can uniformly spread to most parts of a plant including newly emerging leaves, and this results better silencing efficiency and can be used for studying many plant organs, including leaves, flowers, and roots [9]. TRV has a positive-strand bipartite viral genome. RNA1 was cloned into pBINTRA6, and RNA2 was cloned into a construct named pTV00 [9]. A fragment of the gene of interest can be cloned into pTV00 to silence this gene in the host plants. Here, we describe a detailed protocol, in which the TRV-based VIGS system [9, 10] is used to silence target MAPK genes for studying their functions in *N. attenuata* [4]. This method is also suitable for studying other genes and in other *Nicotiana* species and tomato with minor modifications [6, 11].

2 Materials

Prepare all solutions using pure or ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Waste should be disposed following regulations, including the genetically modified bacteria and plants.

2.1 Preparation of Agrobacterium Strains

2.1.1 Preparation of A. tumefaciens GV3101 Electro-competent Cells

1. Sterile YEP medium (10 g/L yeast extract, 10 g/L peptone, 5 g/L sodium chloride; adjusted to pH 7.0) (0.5 L are enough to prepare about 60 aliquots of competent cells).
2. Antibiotics (25 mg/mL rifampicin and 5 mg/mL tetracycline stock solutions, store at −20 °C).
3. Sterile H_2O (1 L). Sterile 10 % (v/v) glycerol (0.5 L).
4. Sterile conical flasks/Erlenmeyer flasks (1.0 L).

5. Sterile 1.5 mL and 50 mL centrifuge tubes.
6. Spectrophotometer for determination of bacterial density (OD_{600}).
7. Refrigerated centrifuge.
8. Shaker for cultivation of bacterial cultures.
9. Freezer (−80 °C).

2.1.2 Preparation of Gene-Specific VIGS Constructs

1. Gene-specific PCR primers.
2. Restriction enzymes and buffers.
3. DNA ligase kit; PCR polymerase kit.
4. *E. coli* competent cells.
5. Sterile liquid LB medium and LB agar with antibiotics (50 mg/L kanamycin).
6. Gel and plasmid DNA extraction kits (e.g., MN NucleoSpinExtract II; MN NucleoSpin Plasmid; http://www.mn-net.com).
7. Agarose gel electrophoresis equipment (chamber, power supply, gel trays, etc.).
8. PCR machine, centrifuge, and electroporation instrument.
9. Shaker for cultivation of bacterial cultures.

2.1.3 Transformation of *A. tumefaciens* GV3101

1. Sterile electroporation cuvette, 1 mm gap (use UV or 70 % (v/v) ethanol to sterilize); electroporation instrument.
2. Sterile S.O.C. media (2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose).
3. Sterile LB agar and liquid LB medium with antibiotics.
4. Shaker for cultivation of bacterial cultures at 28 °C.
5. Sterile 50 % (v/v) glycerol.

2.2 Materials for Plant Growth

1. *N. attenuata* Torr. ex S. Watson seeds.
2. Seed sterilization solution, freshly prepared: 2 % (w/v) dichloroisocyanuric acid sodium salt with 0.005 % (v/v) Tween-20 in H_2O (1 mL is enough for 50–100 seeds).
3. Stock solution of 0.1 M GA_3 (gibberellic acid) in ethanol. Keep at 4 °C.
4. 50× diluted liquid smoke (House of Herbs, Passaic, NJ), in H_2O, autoclaved.
5. Sterile H_2O (100 mL).
6. Petri dishes with 0.6 % (w/v) plant agar- or Phytagel-supported Gamborg's B5 cultivation media (1× strength).

7. Small Teku plastic pots and 1 L pots; soil.
8. Growth chamber for plates with seedlings (16 h light/8 h dark, 26 °C, 150–200 μmol/s m PAR); air-conditioned (22–24 °C) growth chamber for plant cultivation.

2.3 Materials for Bacteria Cultivation and Plant Inoculation

1. *A. tumefaciens* GV3101 strains carrying constructs: pBINTRA6, pTV00 (empty vector control), pTV-(MAPK Gene-of-Interest; hereafter GOI), and pTV-PDS (fragment of phytoene desaturase gene).
2. *N. attenuata* plants in young rosette stage, growing in a climate chamber at a constant temperature program of 22–24 °C, 16 h day/8 h night, ca. 65 % relative humidity.
3. YEP medium: 10 g/L yeast extract, 10 g/L peptone (from soy), and 5 g/L sodium chloride, autoclaved; LB medium can also be used as a substitute for YEP, but *A. tumefaciens* grows more slowly in LB.
4. 1,000× kanamycin stock solution in H_2O (50 mg/mL), filter-sterilized.
5. Erlenmeyer flasks for growing *Agrobacterium* cultures, autoclaved.
6. Shaker for cultivation of bacterial cultures in flasks at 28 °C.
7. Spectrophotometer for determination of bacterial density.
8. Centrifuge.
9. Resuspension buffer: 5 mM $MgCl_2$/5 mM 2-N-morpholinoethanesulfonic acid (MES) (adjust pH of MES to 6.0 with KOH).
10. 1 mL syringes without needle.

3 Methods

3.1 Construction of VIGS Vectors

3.1.1 Preparation of A. tumefaciens GV3101 Electro-competent Cells

1. Inoculate 20 mL YEP-medium containing 25 mg/L rifampicin and 5 mg/L tetracycline with *A. tumefaciens* GV3101 and incubate in a shaker overnight at 200 rpm and 28 °C.
2. Inoculate 0.5 L YEP medium (no antibiotics) in a sterile 1 L conical flask with 10 mL of the overnight pre-culture. Let the bacteria grow at 200 rpm and 28 °C for about 8 h until they reach an OD_{600} of 0.5–0.7 (logarithmic growth phase).
3. Fill the bacterial cultures into sterile 50 mL centrifuge tubes (maximum 40 mL volume) and spin them in a fixed angle rotor at 3,800 × *g* for 10 min at 4 °C.
4. Pour off the supernatants, thereby preserving the pellets. Refill the tubes with the same volume of the remaining bacterial culture. Repeat the centrifugation step to pellet all bacteria.

Keep cell suspensions or pellets on ice between each centrifugation step!

5. Carefully remove all supernatants. If necessary, briefly spin the tubes again after emptying and remove the last drops with a pipette tip. It is important to remove as much salt as possible to obtain cells with lowest possible conductivity for highest possible competence.
6. Resuspend the cells in 10 mL precooled sterile H_2O (resuspend the pellet gently without vortexing). Combine the bacterial suspensions from the individual tubes.
7. Spin for 10 min and remove the supernatant as before. Continue with two more cycles of washing and until all bacteria are pooled into one 50 mL centrifuge tube.
8. Resuspend the cells in 40 mL 10 % (v/v) glycerol. Centrifuge and remove supernatant as before. Repeat at least three times.
9. Resuspend the final pellet in 1.5 mL 10 % (v/v) glycerol and aliquot 40 μL per 1.5 mL microcentrifuge tube.
10. Freeze the aliquots in liquid nitrogen and store at −80 °C.

3.1.2 Cloning of Gene Fragments into the VIGS Vector pTV00 to Form pTV-GOI

A fragment of the GOI to be silenced must be cloned in the polylinker of pTV00 (*see* detailed description at http://www.plantsci.cam.ac.uk/research/baulcombe/methods.htmL). Best results may be obtained if the fragment is inserted in an antisense orientation after choosing appropriate unique cloning sites. Because of enzyme buffer compatibility (assuming that you are using enzymes from New England Biolabs), BamHI and SalI sites are preferred for cloning GOI into pTV00, but other combinations of restriction sites can also be used if BamHI or SalI sites cannot be avoided on the GOI fragment (unique sites in the polylinker are SpeI, BamHI, SmaI, XmaI, HindIII, BspDI, ClaI, AccI, ApaI, and KpnI consecutively).

1. Find a region of sufficient size (150–400 bp; *see* **Note 1**) in the GOI cDNA sequence that does not contain the chosen restriction sites. If only one member of a gene family should be silenced, the fragment having the least identity with other family members should be chosen (a 23 or more than 23 nt continuous sequence perfect match may induce silencing of the other homologues [12]; even constructs designed from the UTR regions are possible). For silencing of homologous genes, choose the region with the highest identity. In our experience, it is also possible to silence two independent genes by tandem insertion of two 150–200 bp sequences specific for each GOI.
2. The primers should carry a 5′ GC rich sequence (AGCT) followed by the restriction site of one of the cloning enzymes and sufficient nucleotides for annealing to the GOI (about 21–25 nucleotides).

3. PCR-amplify the desired GOI fragment with the primers using cDNA prepared from plant tissues expressing the GOI. Preferably, use a proofreading polymerase and follow the manufacturer's instructions for optimal performance. Pipette a 50 μL reaction and divide it into 10 μL aliquots. Run each aliquot at a different annealing temperature in the range of 55–65 °C.
4. Run your PCR products along with a molecular mass standard on a 1 % (w/v) agarose gel. Excise PCR fragments of the expected size and extract the DNA with an appropriate kit (e.g., MN NucleoSpin Extract II, http://www.mn-net.com). About 200 ng of PCR fragment will be needed for further cloning. If the amount of the PCR fragment is too low, repeat the PCR at the optimal annealing temperature. If the PCR result is unsatisfying at all annealing temperatures, design a new primer pair.
5. Digest, in separate reactions, 200 ng of pTV00 vector and 200 ng of the PCR fragment with the restriction enzymes according to the manufacturer's instructions.
6. Run both digestions using a 1 % (w/v) agarose gel; excise the 5.5 kb pTV00 band and the digested PCR fragment. Extract DNA.
7. Set up a 20 μL ligation reaction with approximately 50 ng of digested pTV00 and 100 ng digested PCR fragment. Ligate overnight at 16 °C.
8. Transform an electro- or chemical-competent *E. coli* strain carrying lacIq (e.g., TOP10, http://www.invitrogen.com) with the ligation mixture (1–2 μL). Add warm (37 °C) S.O.C medium to cells and incubate cells in a 37 °C shaker at 200 rpm for 30–60 min.
9. Plate 50–200 μL of the transformed cells on LB plates containing 50 mg/L kanamycin and incubate the bacteria at 37 °C overnight.
10. Pick a few single colonies (e.g., 4) and inoculate each of them into 15 mL culture tubes containing 5 mL of LB liquid medium (50 mg/L kanamycin). Shake overnight at 200 rpm at 37 °C.
11. Isolate plasmid DNA using a standard kit (e.g., MN NucleoSpin Plasmid, Macherey-Nagel).
12. Digest 1 μL of each plasmid with appropriate restriction enzymes and run the digestion products on a 1 % (w/v) agarose gel. Plasmids that contain both the correct pTV00 and GOI fragment are used to confirm the correct sequence of the insert by sequencing with the primers: TRV FOR 5′-GCTGCTAGTTCATCTGCAC-3′; TRV REV 5′-GCAC GGATCTACTTAAAGAAC-3′.

13. One plasmid with the correct sequence is used to transform *A. tumefaciens* electro-competent cells as described in the following section.

3.1.3 Transformation of A. tumefaciens GV3101 by Electroporation

1. Pre-warm 1 mL of S.O.C. per construct to 28 °C.
2. Slowly thaw on ice one aliquot (40 μL) of competent *A. tumefaciens* GV3101 cells.
3. After thawing, mix the cells with 50 ng of plasmid DNA from a correct pTV-GOI clone.
4. Fill the mixture into an ice-cold electroporation cuvette with a gap of 1 mm.
5. Perform electroporation using an electroporator (e.g., Bio-Rad MicroPulser, http://www.bio-rad.com) following the instrument instruction.
6. Add 1 mL of pre-warmed S.O.C. immediately after pulse.
7. Mix the cells with the S.O.C., pipette the suspension into a 1.5 mL microcentrifuge tube, and incubate for 30 min at 28 °C at 200 rpm.
8. Plate 50 μL on a LB agar plates containing 50 mg/L kanamycin; meanwhile plate 10 μL to another plate in order to avoid that too many colonies may appear.
9. Incubate the plates for 48 h at 28 °C.
10. Isolate a single colony. Use this colony to inoculate 3 mL of liquid LB medium containing 50 mg/L kanamycin. Grow the bacteria in a shaker at 200 rpm and 28 °C for about 20 h until they reach an OD_{600} of about 1.
11. Prepare a glycerol stock by mixing 0.5 mL of this culture with 0.22 mL of 50 % (v/v) glycerol, freeze in liquid nitrogen and store at −80 °C. The culture or the glycerol stock can be used as starting culture for the VIGS procedure.

3.2 Plant Inoculation

3.2.1 Plant Growth

1. Prepare fresh sterilization solution containing 2 % (w/v) of dichloroisocyanuric acid sodium salt and 0.005 % (v/v) Tween-20 in H_2O.
2. Incubate 50–100 *N. attenuata* seeds (*see* **Notes 2** and **3**) in 1 mL of sterilization solution for 5 min using 1.5 mL centrifugation tubes; shake occasionally. The following steps should be carried out under a sterile hood.
3. Decant solution and wash seeds in 1 mL sterile H_2O. Repeat decanting and washing steps at least three times.
4. Add 1 mL of diluted smoke solution and 10 μL of 0.1 M GA_3 stock and incubate seeds for 1 h (*see* **Note 4**). Shaking occasionally or inclining the tubes to provide a larger surface area of seed exposure during incubation yields more even germination and greater germination efficiency.

5. Decant smoke/GA_3 solution, wash seeds three times with sterile H_2O, and distribute 30–40 seeds/plate with 0.6 % (w/v) agar or Phytagel-supported Gamborg's B5 cultivation media.
6. Allow plates to dry briefly until any water transferred with seeds has evaporated, then close with Parafilm (Pechiney Plastic Packaging Company, Chicago, IL) and transfer to a growth chamber (16 h light/8 h dark, 26 °C, 150–200 μmol/s 1 PAR).
7. After 10 days carefully remove seedlings from agar and transfer to low-nutrient soil in small Teku plastic pots.
8. After 10 days in Teku pots, transfer seedlings to 1 L pots in soil. Place pots in groups of 10–11 into flat plastic trays with a ca. 5.5 cm rim; this is convenient for the VIGS inoculation. Transfer to a climate chamber with a constant temperature of 22 °C and 16 h day/8 h night light regime, ca. 65 % relative humidity, and medium light intensity (200–250 μmol/s m PAR).

3.2.2 Inoculation of N. attenuata Seedlings by Syringe Infiltration

1. Plants should be inoculated at 4–6 days post-potting, namely, 24–26 days post-germination. It is very important that plants are in the right stage because with older plants, VIGS has been found to be less efficient (*see* **Note 5**).
2. The whole experiment is carried out in a climate chamber with a constant temperature of 22 °C (*see* **Note 6**) and 16 h day/8 h night light regime, ca. 65 % relative humidity, and medium light intensity (200–250 μmol/s m).
3. In parallel with plant growth, streak *A. tumefaciens* cultures pTV00 (empty vector control; *see* **Note 7**), pTV-GOI, pTV-PDS (as the positive control), and pBINTRA6 onto YEP or LB agar plates with 50 mg/L kanamycin. Allow to grow for 2 days at 28 °C. These plates can be stored at 4 °C for approximately 1 month.
4. Prepare sterile Erlenmeyer flasks and enough YEP medium. For every 1 mL of inoculation solution, you will need 2.5 mL of liquid YEP culture with pTV-GOI plus 2.5 mL pBINTRA6 liquid YEP culture; calculate 1 mL inoculation solution per plant. Do not fill flasks to more than 50 % of their maximum volume to allow sufficient gas exchange while shaking cultures.
5. On day 3 after potting, check the size of the plants. If the plants are too small, postpone the VIGS experiment for 1–2 days; ideally, the leaf size should be 2–3 cm in length during infiltration (*see* **Note 5**). If the size is sufficient, start small overnight pre-cultures of *A. tumefaciens* GV3101 carrying viral vectors in 15 mL culture tubes with YEP + kanamycin

(50 mg/L) at 28 °C and 200 rpm. The pre-culture can be used with 1/30–1/10 dilutions for making the larger cultures.

6. Water plants in the morning of the day that you want to VIGS, or the day before. Open stomata are important for the efficient bacteria inoculation into the leaves.
7. On day 4 after potting (or later depending on plant growth), add pre-cultures (final dilution 1/30–1/10) to Erlenmeyer flasks containing suitable amount of YEP medium (with 50 mg/L kanamycin) and incubate at 28 °C and 200 rpm. These need to grow at least 5–6 h to reach the correct OD_{600} (0.4–0.6). Alternatively, you can inoculate an 1:1,000 aliquot of your pre-culture into the prepared Erlenmeyer flasks and grow the cultures overnight (about 16 h).
8. When the culture reaches an OD_{600} of 0.4–0.6, spin down the bacteria at $4{,}000 \times g$ for 10 min. You can centrifuge 40 mL at a time in 50 mL Falcon tubes.
9. Discard the supernatants and resuspend the pellets by shaking (do not vortex) in the inoculation solution, which contains 5 mM $MgCl_2$ and 5 mM MES. The volume of the inoculation solution should be 1/5 the volume of your culture, so that the final OD_{600} of the inoculation solution is 2.0–3.0.
10. For every construct, make a final inoculation solution by mixing equal volumes of resuspended *Agrobacterium* carrying pTV-GOI construct and pBINTRA6. To avoid contamination, add suspensions of *Agrobacterium* containing pTV-GOI construct into aliquots of *Agrobacterium* with pBINTRA6.
11. Perform inoculations using a 1 mL syringe without a needle: pressure-inject *Agrobacterium* suspended in inoculation solution into 3 leaves per plant, with one to four inoculations on the underside of each leaf. You should try to saturate at least 75 % of each inoculated leaf (the leaf turns dark green). It is easiest to inoculate plants during the light period when the stomata are open.
12. Cover the whole tray of plants with a plastic bag or with an upside-down tray (make sure the upside-down tray is tightly closed against the tray holding the plants to provide high humidity for establishment of *A. tumefaciens* transformation), and leave the lights off for 2 days.
13. Remove the plastic bags or trays and switch on the lights.
14. The viral spread is always accompanied by a characteristic leaf phenotype: after about 10–12 days, newly developing leaves are wrinkled, and leaves which mature post-inoculation often appear lighter than the oldest rosette leaves which were not VIGSed. You should discard plants that do not develop wrinkled leaves by day 16 post-inoculation as they will not show gene silencing.

VIGSed plants should also be smaller than non-VIGSed plants grown under the same conditions. The wrinkled-leaf phenotype will ameliorate as plants start to elongate.

15. Bleaching of the leaves on the PDS positive control plants should appear at approximately 10–14 days post-inoculation. Experiments should begin once clear and even bleaching is visible in pTV-PDS positive control plants; tissues which were already established at the time of inoculation will not bleach, and the corresponding tissues should be avoided in experiments with GOI-silenced and EV control plants.

4 Notes

1. GOI fragments at a size of about 300 bp provide effective gene silencing; fragments shorter than 150 bp may result in a weak silencing effect, while fragments longer than 300 bp increase the probability of unspecific silencing of genes with short sequence homologies.
2. *N. attenuata* seeds can be obtained upon request from Prof. Ian T. Baldwin (baldwin@ice.mpg.de), Department of Molecular Ecology, Max Planck Institute for Chemical Ecology in Jena, Germany.
3. RNAi stably silenced lines of one gene also can be used as starting material for a successful VIGS of another independent target GOI.
4. *N. attenuata* seeds require smoke cues to break dormancy and germinate. Treatment with smoke solution is not required for other tobacco species like *N. benthamiana*; however, it is still recommended to use the GA_3 solution for increased and synchronized germination in other tobacco species.
5. Proper plant age is critical for the establishment of VIGS. Older plants are usually more resistant to *A. tumefaciens* infection, while younger plantlets may not survive damage to the leaves caused during infiltration. Plants that we normally use for infiltration have about six to nine leaves with leaf blades 2–3 cm long.
6. Low temperature is important for proper VIGS establishment, but the temperature should permit normal development of plants and larvae. We find the best compromise is to establish VIGS at 22 °C.
7. pTV00 should be used as a negative control to exclude nonspecific phenotypic effects of VIGS as virus infection is known to change hormone and metabolic homeostasis in plant cells. In most cases virus-infected plants will contain higher levels of the pathogenesis-associated hormone salicylic acid.

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Chapter 8

RNA Interference of Plant MAPK Cascades for Functional Studies

Juan Xu and Shuqun Zhang

Abstract

Arabidopsis genome contains 20 genes encoding mitogen-activated protein kinases (MAPKs, or MPKs), and ten genes encoding MAPK kinases (MAPKKs, or MKKs), the upstream kinases that activate MAPKs in the signaling cascades. They play critical roles in many different biological processes ranging from growth/development to response to environmental stimuli and pathogen invasion. T-DNA knockout lines are not currently available for all these genes. There is also functional redundancy at both MAPK and MAPKK levels. In addition, embryo lethality is associated with some double mutant combinations, which makes it difficult to investigate their specific functions in plants. In such situation, the use of RNA interference technology by which mRNA of interested gene is targeted by double-stranded RNA (dsRNA) for degradation and gene silencing provides a powerful tool for loss-of-function analyses. In this chapter, we describe the hairpin-RNA interference (hpRNAi) method we employed to silence MPK3/MPK6 and their upstream MKK4/MKK5 in the model plant *Arabidopsis*, with particular emphasis on the generation of hpRNAi constructs for single gene RNAi, tandem RNAi of two MAPKK genes, and tissue-specific RNAi.

Key words MAPK cascade, RNA interference, Hairpin RNAi construct, Tandem RNAi, Tissue/cell-specific RNAi

1 Introduction

Mitogen-activated protein kinases (MAPKs or MPKs) cascades are highly conserved signaling modules. They play critical roles in regulating plant growth/development and responses to environmental stimuli including pathogen invasion. MAPKs are at the bottom tier of the three-kinase module in MAPK cascades, and their activities are regulated by MAPK kinase (MAPKKs, or MKKs). The activities of MAPKKs are, in turn, regulated by MAPKK kinases (MAPKKKs) [1]. There are multiple members at each level of the MAPK cascades, and functional redundancy and crosstalk also exist. Loss-of-function genetic systems are essential for us to understand their distinct functions in specific signaling pathways.

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However, T-DNA knockout lines are currently unavailable for some members of the MAPK and MAPKK families. To explore the functions of these MAPK and MAPKK genes, people have used RNA interference strategy to silence specific members in the MAPK cascades [2–6].

RNA interference is an endogenous sequence-specific degradation of target mRNA, triggered by the double-stranded RNA (dsRNA) [7]. It has been widely used in various organisms to generate loss-of-function data and to facilitate functional studies of genes of interest [8]. The intron-containing hpRNA (ihpRNA) transgenic plants generally have 90 % silencing efficiency of the target genes, which is comparable with null loss-of-function mutants [9, 10]. The variation in the degree of gene silencing can be exploited to study gene-dosage effects or to generate a non-null mutant to circumvent the embryo lethality of the null mutant. In addition, the use of tissue/cell-specific promoter has allowed the study of gene function in specific tissues/cells [2].

To obtain stable RNAi lines of target MAPK or MAPKK genes, one needs to insert appropriate ihpRNAi constructs into the plant genome. The ihpRNA constructs require two inverted repeats of the target gene fragment with an intron spacer region that can stabilize the hpRNA structure and increase the efficiency of gene silencing [9, 10]. The inverted fragment of the target gene is generally 400–800 bp long and can be derived from different regions of the target mRNA including the coding region, 5′-untranslated region (5′-UTRs), and 3′-UTRs. The pHANNIBAL and pKANNIBAL vectors are well suited for the generation of ihpRNAi constructs with a 30–50 bp intron spacer sequences between the two inverted sequences that corresponding to a target gene [9]. Researchers can choose appropriate restriction sites according to the sequence of their specific target genes.

This RNA interference strategy has been successfully used to discover the crucial functions of MKK4/MKK5-MPK3/MPK6 cascades in plant growth and development including stomatal differentiation [3], floral organ abscission [4], localized cell division, and inflorescence architecture [5]. Besides silencing a single MAPK gene, we also expanded this method to tandem RNAi to target two functional redundant MAPKK genes, and RNAi constructs driven by tissue/cell-specific promoter to target MAPK gene in specific tissues. Similar strategy was also used successfully by other research groups to study MAPK functions in plants [11]. Here we provide detailed materials and methods for the generation of ihpRNAi constructs to silence the expression of MAPK or MAPKK genes in *Arabidopsis*.

2 Materials

2.1 Generation of ihpRNAi Constructs to Silencing MAPK and MAPKK Genes

1. Design of the constructs: sequences of MAPK/MAPKK genes of interest for hairpin RNA design (FASTA format), gene-specific primers, cloning vector pBluescript, pHANNIBAL/pKANNIBAL vector [9], a modified pBI121 binary vector with Xho I and Spe I added to the multi-cloning sites (*see* **Note 1**).
2. Enzymes: high-fidelity Pfu Ultra (Stratagene) or Phusion polymerase (Finnzymes), fast digest restriction enzymes (Fermentas), T4 DNA ligase (NEB), Taq DNA polymerase.
3. Reagent and kit: dNTPs, LB medium, ampicillin, kanamycin, IPTG (isopropylthiogalactoside), X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactoside) (*see* **Note 2**). DNA Gel Recovery kit (Zymo Research), and mini plasmid isolation kit (Invitrogen).
4. Transformation: XL-1 Blue competent cells, and SOC medium (2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.05 % (w/v) NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, pH 7.0).
5. Equipment: NanoDrop (Thermo), PCR machine, electrophoresis apparatus, water bath, centrifuges, incubator, shaker with controllable temperature, autoclave, and pH meter.

2.2 Preparation of Agrobacterium and Arabidopsis Plants for Transformation

1. Electro-competent cells of *Agrobacterium* GV3101 strain.
2. LB medium, ½ Murashige and Skoog (MS) medium containing 1 % (w/v) sucrose and 0.5 % (w/v) Phytoagar, kanamycin, gentamycin.
3. *Arabidopsis* Col-0 seeds, soil, pots, and domes.
4. A growth chamber with controllable temperature and light cycle.
5. Transformation flower dipping medium: ½ MS salt, 5 % (w/v) sucrose, 0.05 % (w/v) MES, 0.045 μM benzylaminopurine (BAP), 0.02 % (v/v) Silwet L-77, adjust the pH to 5.8 with 1 M KOH.
6. BIO-RAD Gene Pulser Xcell Electroporation System, centrifuge, incubator, shaker with controllable temperature, autoclave, and pH meter.

2.3 Analysis of Transgenic Plants by Scoring Phenotype and Real-Time qPCR

1. Transgenic plant screening: ½ MS medium containing 1 % (w/v) sucrose and 0.5 % (w/v) phytoagar with appropriate antibiotics, soil, pots, plastic domes, and growth chamber.
2. RNA isolation: TRIzol reagent (Invitrogen), chloroform, isopropanol, ethanol, diethylpyrocarbonate (DEPC)-treated H_2O, TURBO DNA-free kit (Ambion), cDNA reverse transcription kit (Fermentas).

3. Real-time qPCR: SYBR Green PCR Master mix (Takara), forward and reverse primer designed for real-time PCR of target genes and internal controls, e.g., EF-1α.
4. 96-well plate and optically clear PCR film or strips for real-time PCR, real-time PCR cycler (Eppendorf, Mastercycler ep realplex).

3 Method

3.1 Generation of RNAi Construct to Silence a Single MAPK Gene

In *Arabidopsis*, MPK3 and MPK6 are two key players with functional redundancy in regulating plant growth/development and defense responses [12]. Although the T-DNA knock out lines are available for both MPK3 and MPK6, embryo lethality of the mpk3 mpk6 double mutant make it impossible to clarify their roles in other biological processes. To circumvent the embryo lethality of the null double mutant, we generated MPK3 ihpRNAi constructs to silence MPK3 in the mpk6 T-DNA mutant background [3] as shown in Fig. 1a. The procedure is detailed below:

1. Amplify a 693-bp region of the MPK3 cDNA (*see* **Note 3**) from total cDNAs of *Arabidopsis* (Col-0) using two pairs of

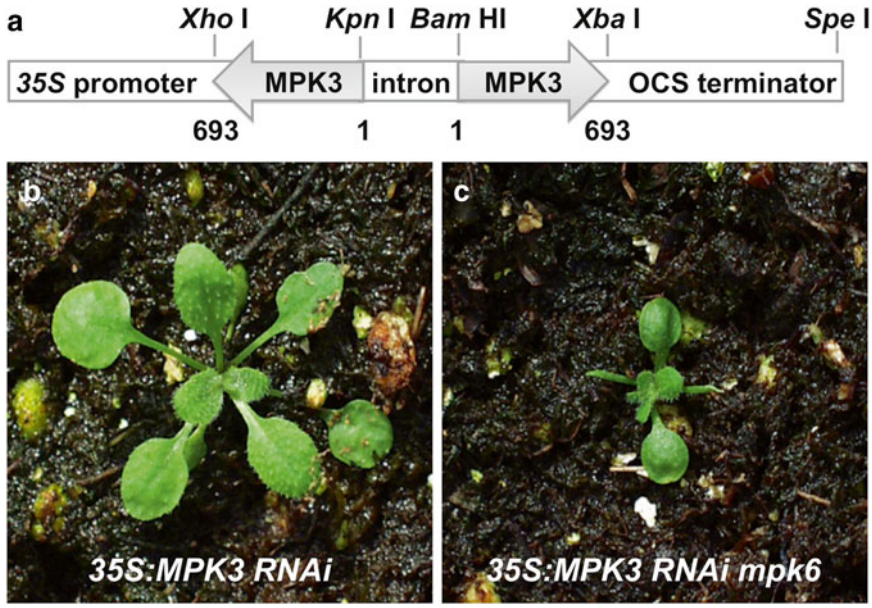

Fig. 1 Map of MPK3 RNAi construct and phenotype of the MPK3RNAi seedlings. (**a**) Diagram showing the restriction enzyme sites used to generate the MPK3 RNAi construct (modified from Supplemental Figure S7 in [3] with permission from American Society of Plant Biologists). The numbers underneath the diagram indicate the base pair numbers in the open reading frame of MPK3. (**b**) MPK3RNAi seedling in Col-0 background shows no obvious phenotype. (**c**) A MPK3 RNAi seedling in mpk6 background showed arrested growth and development, and later restored growth/development due to the silencing of the MPK3 RNAi transgene

primers with different restriction sites at each end for directional cloning of the same MPK3 region in inverted orientations into the pHANNIBAL vector (*see* **Note 4**). The added restriction sites are shown in lower case letters.

For the sense orientation: 5′-ggatccATGAACACCGGCGGT-3′ (Bam HI) and 5′-tctagaCTCCATAAAGATACAACC-3′ (Xba I).

For the antisense orientation: 5′ ggtaccATGAACACCGGC-GGT-3′ (Kpn I) and 5′-ctcgagCTCCATAAAGATA-CAACC-3′ (Xho I).

Set up PCR reactions using Phusion polymerase according to the manufacturer's instructions.

2. Analyze the PCR products by electrophoresis in a 1 % (w/v) agarose gel in 1× TBE buffer with 0.5 μg/mL of ethidium bromide. Check the sizes of the amplified PCR products against a DNA molecular weight standard (*see* **Note 5**).
3. Recover the PCR products using the Gel DNA Recovery kit according to instruction manual.
4. Digest the pBluescript vector with Sma I restriction enzyme and recover the digested pBluescript vector using the DNA Gel Recovery kit (*see* **Note 6**).
5. Clone each of the purified PCR products (*from* **step 3**) into the Sma I-digested pBluescript vector (*from* **step 4**). Set up the ligation reaction according to the instruction manual of T4-DNA ligase (*see* **Note 7**).
6. Transform the ligation products to XL-1 Blue competent cells by heat shock method (*see* **Note 8**). Plate all cells on the LB plates containing 100 μg/mL ampicillin, 1 mM IPTG, and 40 μg/mL X-gal. Incubate the plates in a 37 °C incubator overnight.
7. Pick five to ten white colonies and perform colony PCR using T3 and T7 primer pair with Taq polymerase. Inoculate one positive clone of each construct to 4 mL LB medium containing 100 μg/mL ampicillin and incubate the cultures at 37 °C in a shaker (180–220 rpm) overnight (12–16 h).
8. Purify the plasmids from the bacterial cultures using mini plasmid preparations kit. Digest 2 μg plasmid with the restriction enzymes that cut the restrict sites incorporated into the primers, and in this case Bam HI/Xba I and Kpn I/Xho I pairs, respectively. Check the digested products by electrophoresis as described in **step 2**. If the sizes of the inserts are correct, their sequences are further validated by Sanger sequencing using T3 or T7 primer.
9. Recover the inserts from sequence-validated plasmids and clone them into the pHANNIBAL vector. First, clone the fragment

in sense orientation into pHANNIBAL vector using Bam HI and Xba I sites overhangs. Validate by colony PCR and restriction digestion (*see* **Note 9**). Second, clone the other fragment in an antisense orientation into pHANNIBAL vector using Kpn I and Xho I. Validate the insert by restriction mapping (*see* **Note 10**).

10. Digest the whole cassette including the inverted repeats with the Pdk intron and the OCS terminator from the pHANNIBAL vector with Xho I and Spe I as indicated in Fig. 1a. Recover the cassette using the DNA Gel Recovery kit.
11. Digest the modified pBI121 binary vector with Xho I and Spe I and recover the vector backbone using the DNA Gel Recovery kit.
12. Clone the fragment from **step 9** into the pBI121 binary vector from **step 10** (*see* **Note 11**). Validate the insert by restriction mapping. In the final construct shown in Fig. 1a, the 35S promoter is from pBI121 vector, and the OCS terminator is from pHANNIBAL vector.

3.2 Tandem RNAi of Target MAPKK Genes

In *Arabidopsis*, MKK4 and MKK5 share 78 % protein sequence similarity and may play redundant functions. T-DNA knockout line is not available for either gene. To provide loss-of-function data to demonstrate that MKK4 and MKK5 are upstream MAPKKs of MPK3/MPK6 in the same cascade, we also constructed MKK4 and MKK5 ihpRNAi constructs (Fig. 2a). We found that RNAi gene silencing of each MAPKK gene showed no obvious phenotypes.

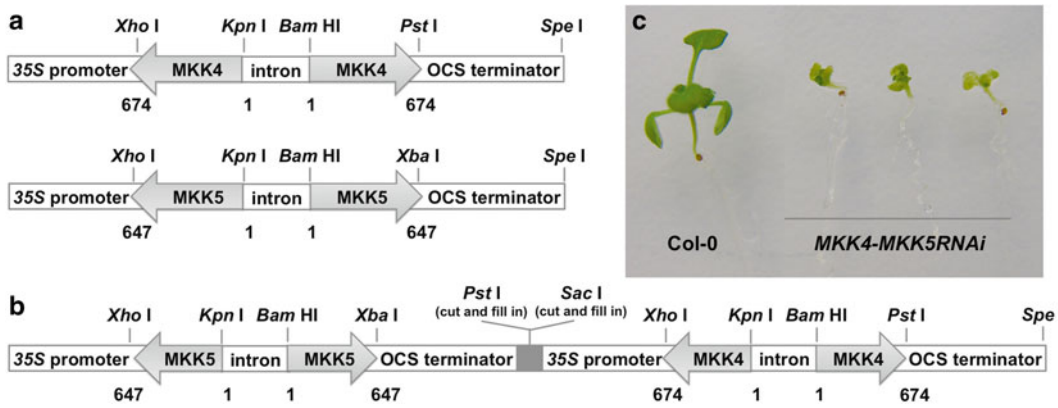

Fig. 2 Maps of ihpRNAi constructs for silencing MKK4, MKK5, and MKK4/MKK5, and the phenotype of tandem MKK4-MKK5 RNAi seedlings. (**a**, **b**) Diagrams showing the restriction enzyme sites used to generate the MKK4 RNAi, MKK5 RNAi and tandem MKK4-MKK5 RNAi constructs (modified from Supplemental Figure S7 in [3] with permission from American Society of Plant Biologists). The numbers underneath the diagram indicate the base pair numbers in the open reading frames of MKK4 and MKK5 genes. (**c**) MKK4-MKK5 RNAi seedlings with severe growth and development defects. The epidermal layers of such seedlings only have guard cells [3]

The use of a tandem MKK4-MKK5 RNAi provided key information about their functions in specific signaling pathways [3–5]. The procedure for the generation of the tandem RNAi construct used to silence both MKK4 and MKK5 is as follows (*see* **Note 12**):

1. Generate the single ihpRNAi constructs of MKK4 and MKK5 in the same way as described above for MPK3 RNAi construct. Figure 2a shows the maps of MKK4 and MKK5 single RNAi constructs. The single ihpRNAi constructs were then used to generate the tandem MKK4-MKK5 RNAi construct. RNAi lines with single MAPKK gene silencing are good controls of the tandem RNAi lines.
2. Digest the MKK5 RNAi construct in pHANNIBAL vector with Pst I, fill the ends using Klenow, and then digested with Spe I to generate a linearized plasmid with a blunt end close to the MKK5 RNAi cassette and Spe I on the other end for insertion of MKK4 RNAi cassette.
3. Digest the MKK4 RNAi construct in pHANNIBAL vector with Sac I, a site situated before the 35S promoter, and then fill the ends with Klenow to generate a blunt end. After this, digest the plasmid with Spe I to drop out the MKK4 RNAi cassette.
4. Gel-purify the linearized MKK5 RNAi construct in pHANNIBAL backbone (from **step 2**) and MKK4 RNAi cassette (from **step 3**), and ligate them together to generate the tandem MKK4-MKK5 RNAi construct in pHANNIBAL vector.
5. Digest the MKK4-MKK5 RNAi construct in pHANNIBAL vector with Xho I (partial digestion) and Spe I restriction enzymes. Recover the MKK5-MKK4 RNAi cassette for cloning into the modified pBI121 vector digested with the same enzymes. Figure 2b is a map of the final construct. The first 35S promoter is from the pBI121 vector (*see* **Note 13**).

3.3 Tissue-Specific RNAi of MAPKs

Some MAPKs play important roles in different biological processes at different developmental stages throughout the life cycle of a plant. The suppression of MAPKs at an early stage will prevent the examination of other functions of the MAPKs at later stages. It is also possible that the RNAi lines will give pleiotropic phenotypes, which will complicate the interpretation of the loss-of-function data. In such cases, a tissue-specific promoter-driven RNAi construct will allow the suppression of MAPKs in specific tissues/cells at specific developmental stages, which can avoid the complications associated with constitutive suppression using the 35S promoter-driven RNAi construct. For example, to study the function of MPK3 in stomatal opening and closure, Gudesblat and colleagues have used guard cell-specific KST1 promoter-driven C-terminal coding region of MPK3 to silence MPK3 expression in guard cells [2].

To understand the function of MPK3/MPK6 in pollen development, we have used an RNAi construct to suppress the expression of MPK3 specifically in pollen in the mpk6 mutant background, which allowed us to obtain loss-of-function data in the developing pollen grains (Guan et al., unpublished data). In brief, we modify the MPK3 RNAi construct by replacing the cauliflower mosaic virus 35S promoter with the LAT52 promoter [13], which confers pollen-specific expression of MPK3 RNAi, and specifically suppress the MPK3 function in pollen. The detailed procedure is as follows:

1. Digest pBI121 vector with Hind III and Xho I to remove the CaMV 35S promoter region. Recover the digested pBI121 vector backbone using the DNA Gel Recovery kit.
2. Amplify the LAT52 promoter with the forward and reverse primers, to which Hind III and Xho I restriction enzyme sites were added, respectively.
3. Clone the LAT52 promoter to pBluescript vector as indicated in Subheading 3.1, **steps 3–6**.
4. Digest the LAT52 promoter region from sequence validated plasmid with Hind III and Xho I enzymes. Clone the recovered LAT52 promoter fragment to the binary pBI121 vector backbone to obtain the modified binary vector with the pollen-specific promoter LAT52.
5. Clone the MPK3 RNAi hairpin insert described in Subheading 3.1, **step 9** into the modified pBI121 vector digested with Xho I and Spe I as described in Subheading 3.1, **steps 10–12**.

3.4 Agrobacterium-Mediated Transformation of ihpRNAi Constructs

All the binary vectors described above were transformed into *Agrobacterium* strain GV3101 by electroporation. *Arabidopsis* transformation was performed by the floral dip procedure [14].

1. Surface sterilize the *Arabidopsis thaliana* seeds and imbibe at 4 °C for 3–5 days (*see* **Note 14**).
2. Plate the seeds on ½ MS plate containing 1 % (w/v) sucrose and 0.5 % (w/v) phytoagar (pH 5.8). Incubate the plates in a tissue culture chamber at 22 °C with 14 h light/10 h dark cycle (70 μE/m^2 s) for 7 days.
3. Transplant seedlings into pots. Put the pots in a tray and cover the tray with a clear plastic dome for 3–5 days in a growth chamber at 22 °C with a 14 h light/10 h dark cycle. It is important to start the plants early. It takes more than 5 weeks for the *Arabidopsis* plants to flower, which is the stage for transformation by flower-dipping method.
4. Transform the ihpRNAi binary constructs into *Agrobacterium* strain GV3101 by electroporation. Plate the transformed cells on LB plates containing 50 mg/L kanamycin and 25 mg/L

gentamycin (*see* **Note 15**). Incubate the plates at 28 °C for 2 days until colonies appear.

5. Inoculate the positive colony to 5 mL LB medium with antibiotics, grow at 28 °C for 1 day, and then inoculate 2–200 mL LB medium with antibiotics, grow at 28 °C overnight until the OD_{600} is about 2.0.
6. Spin down the agrobacteria at 3,000 × *g* at 28 °C for 10 min and resuspend the cells in infiltration medium (*see* Subheading 2.2, **item 5**) to OD_{600} = 0.8–1.0.
7. Dip the bolts and part of the rosettes of *Arabidopsis* plants in a beaker with agrobacterial cells in infiltration medium for 5 min (*see* **Note 16**). Set the plants on their sides in a tray and cover with plastic dome to maintain humidity for 24 h. Next day, uncover the tray and set the plants upright (*see* **Note 17**).
8. Harvest the seeds 5–7 weeks after transformation for transgenic plants selection.

3.5 Analysis of Transgenic Plants by Scoring Phenotype and Real-Time PCR

1. Surface sterilize the T1 seeds collected from the T0 transformed *Arabidopsis* plants (*see* **Note 18**) and imbibe at 4 °C for 3–5 days.
2. Plate the seeds on ½ MS plates containing 1 % (w/v) sucrose, 0.5 % (w/v) phytoagar, 25 μg/mL kanamycin and 50 μg/mL timentin (pH 5.8) (*see* **Note 19**).
3. Phenotypes of gene silencing may start to appear as early as 4–5 days after seed germination. Keep observing the identified phenotypes, which may prevent the transgenic seedlings from growing/developing normally (Fig. 2c) (*see* **Note 20**).
4. Transplant the T1 transgenic seedlings to soil 7 days later when the transgenic plants are easily identifiable (*see* **Note 21**). The growth conditions are as mentioned earlier (Subheading 3.4 **step 3**).
5. Isolate total RNA from seedlings (**step 3**) or leaf tissues of 4-week-old T1 transgenic plants (**step 4**) using TRIzol Reagent as the manufacturer's instructions (*see* **Note 22**).
6. Treat the RNA samples using TURBO DNA-free Kit according to the instruction manual to remove the contaminating genomic DNA.
7. Quantify the RNA by measuring the absorbance of the samples using a NanoDrop.
8. Synthesize cDNAs from 2 μg of total RNA using oligo-dT primer and reverse transcriptase per manufacturer's instructions.
9. Design specific forward and reverse primers from the cDNA of the target gene using Primer3 online (http://frodo.wi.mit.edu/). EF1α or UBQ5 (ubiquitin) transcript can be used as an internal

control for all the samples tested using the specific primers (*see* **Note 23**).

10. Set up the real-time PCR reactions with a total volume of 20 μL containing 10 μL 2×SYBR Green PCR Master mix, 300 nmol forward and reverse gene-specific primer in 5 μL, and 5 μL of diluted cDNA (*see* **Note 24**).
11. Analyze the data by the double ΔCT method and calculate the gene silencing efficiency.
12. Harvest the T2 seeds from T1 transgenic plants.
13. Identify those with a single-copy T-DNA insertion based on the 3:1 segregation of T2 progeny on antibiotic or herbicide selection plates. Further identify the homozygote transgenic plants in the T3 progeny based on segregation of antibiotic resistance.

4 Notes

1. Other binary vectors can also be used. The modified pBI121 vector was generated by the addition of Xho I and Spe I sites into the multi-cloning site of the original pBI121 vector. This way, the same insert DNA can be easily cloned into the inducible pTA7002 vector as well [15].
2. X-gal is light sensitive and should be stored in the dark at −20 °C after being dissolved in dimethylformamide (DMF).
3. An additional RNAi construct that targets the 312–1,178 bp coding region of MPK3 gene also worked very efficiently.
4. The primers should be designed to include appropriate restriction sites at the 5′ end of the forward and reverse primers based on the sequence of the target gene itself and the pHANNIBAL vector.
5. Shorten the UV exposure time as much as possible to avoid DNA damage.
6. It is also possible to add protective bases to the restriction sites in the 5′ end of the primers and digest the purified PCR products directly. However, we found that it is more predictable to clone the PCR product and then digest the target fragment from the plasmid.
7. To avoid self-ligation of pBluescript, we usually add a little bit Sma I (usually 0.2–0.5 unit) in the ligation reaction. In case that the DNA fragment has internal Sma I site, Hinc II site in the pBluescript vector can be used as the cloning site.
8. Other *E. coli* strains such as DH5α, and JM109 can also be used in this step.
9. The sequencing validation is not essential in this step since there is no DNA amplification by PCR.

10. PCR rarely works when one tries to amplify a piece of DNA with inverted repeats in a hairpin structure. It is also difficult to sequence it using PCR-based sequencing technique. As a result, we choose to clone each fragment after PCR amplification and perform sequencing to verify them first. It is also possible to add additional nucleotides to the primers and digest the PCR products for direct cloning into pHANNIBAL vector. However, the sequencing verification of the inverted repeats in this vector can be difficult.
11. We also mobilize the cassette into pTA7002 binary vector in order to control the expression of RNAi in a DEX-dependent manner. One can also use other inducible binary vectors according to the specific experimental design.
12. Nowadays, new technology like CRISPR/Cas interference by which people can silence multiple gene expression by encoding multiple target guide sequences into a single CRISPR array provides a new powerful reverse genetic tool [16, 17]. Researchers can employ the new technology to save themselves from complicated and time-consuming constructs generation.
13. It is also possible to generate double RNAi plants after obtaining good single MKK4 and MKK5 RNAi lines by crossing them together. However, if the suppression is almost complete in both lines, the double RNAi will be lethal, similar to the MKK4-MKK5RNAi T1 seedlings with severe gene suppression (Fig. 2c).
14. Surface sterilization of seeds was performed by placing seeds (in microcentrifuge tubes with lids open) in a sealed desiccator with a beaker containing 120 mL H_2O, 80 mL bleach and 3 mL concentrated hydrochloric acid for 4 h. Concentrated hydrochloric acid was added into the beaker right before the desiccator was sealed. Generally, the surface sterilize time is dependent on the amount of the seeds in the tubes. Sterilize 100–300 seeds (~25–100 μL in volume) usually take 10–14 h.
15. Because of the high-efficiency of electroporation transformation, do not plate all the cells on the selection plate. Generally, 50 μL of cells are enough. It is better to spread the cells at different densities in different sections on the plate to ensure the formation of isolated colonies.
16. Water the plants well 1 day before transformation.
17. Keep plants transformed with different constructs separate to avoid contamination.
18. The time for surface sterilization of transgenic seeds using chlorine gas could be a little longer to prevent possible *Agrobacterium* carryover. We found that 14 h sterilization works well for 50 μL transgenic seeds.

19. Add antibiotics when the autoclaved medium is not hot to avoid losing efficacy of the antibiotics. Timentin inhibits the growth of *Agrobacterium*, which is only essential for the selection of T1 transgenic plants. Choose appropriate antibiotic for your specific binary vector. Here, kanamycin is added for the pB1121 vector.
20. Sometimes, the insertion of transgene may cause T-DNA insertional mutation of other genes in the genome. It is important to record the frequency of a phenotype in the T1 generation to exclude this possibility. In addition, RNAi transgene can be silenced as well. As shown in Fig. 1c, MPK3 RNAi in the mpk6 mutant background resulted in growth arrest in seedlings. The first pair of true leaves had no leaf blade. No additional leaf emerged for a long time until the transgene was silenced, and then normal leaves emerged. The newly emerged leaves no longer had gene silencing and grew and developed as wild-type plants.
21. The transgenic plants can be distinguished on the kanamycin selection plates at 4–5 day after germination. The positive seedlings are green and have long roots (if RNAi construct does not cause growth/developmental defect), while the non-transgenic seedlings are yellow, smaller, without emerging true leaves, and with short roots.
22. Do not forget to have a wide-type plants or vector-transformed plants as controls for gene expression analysis in this experiment.
23. We found that the primers targeting the 3′ region of the target cDNA sequence usually give better results in qPCR quantitation.
24. We usually dilute the cDNA from RT reaction (**step 7**) by 40-fold, and use 5 μL in a total of 20 μL real-time reaction to reduce the pipetting variation.

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Part III

Localization Studies of MAPKs and Their Interactions

Chapter 9

Immunofluorescent Localization of MAPKs and Colocalization with Microtubules in *Arabidopsis* Seedling Whole-Mount Probes

Olga Šamajová, George Komis, and Jozef Šamaj

Abstract

In all eukaryotes, signaling by mitogen-activated protein kinase (MAPK) pathways plays a crucial role in signal transduction during regulation of cell growth, differentiation, proliferation as well as death and stress responses. In this chapter we describe a reliable method to immunolocalize MAPKs in roots of *Arabidopsis thaliana* by using whole-mount seedling probes. This method relies on quick and efficient chemical fixation, partial cell wall digestion, plasma membrane permeabilization, subsequent antibody incubation, and visualization by high-end confocal laser scanning microscopy (CLSM) performed on whole *Arabidopsis* seedlings. Protocols are provided for immunofluorescent localization of MPK3, MPK4, and MPK6, representing three major developmentally and stress-regulated MAPKs of *Arabidopsis*. In addition, protocols for colocalization of these MAPKs with microtubules are also provided.

Key words *Arabidopsis thaliana*, Mitogen activated protein kinase, Root, Whole mount, Immunolocalization

1 Introduction

Plant mitogen-activated protein kinase (MAPK) signaling is organized into interconnected pathways which ultimately control developmental processes and conditional responses of plants to their surrounding environment [1]. MAPK signaling undergoes spatiotemporal regulation in which cellular and subcellular MAPK localizations play important roles [2, 3]. From extensive studies in the animal field, it became apparent that although MAPKs are rapidly transported into the nucleus following activation [4], a significant fraction of their active form persists in the cytoplasm, and it is tethered to different structures including the cytoskeleton [5] and various endomembrane elements [6]. In the resting cell, inactive MAPKs are associated with different intracellular compartments [6], suggesting that MAPK cascades have definite

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spatiotemporal distribution, allowing them to function differentially upon activation. However, very little information is known about the localization and organization of individual MAPK modules on subcellular level in plant cells [2, 3].

Although immunofluorescence localization was readily implemented in cultured animal cells through the seminal work of Elias Lazarides and Klaus Weber [7], its application to plants required to overcome an important physical barrier represented by the cell wall. This was achieved by enzymatic digestion of cell walls in order to facilitate the intracellular diffusion of antibodies against tubulin, the building block of the microtubule cytoskeleton in all eukaryotes [8]. In that original work which employed onion roots, cells were after fixation macerated with cell wall-digesting enzymes and mechanically separated to a so-called root squash preparation allowing the wide-field immunofluorescent elucidation of the major plant microtubule arrays. Root squashes and similar separated cell preparations of other plant tissues [9] provide excellent resolution of intracellular structures at a single cell level. However, tissue integrity is disrupted and developmentally important information is lost. Therefore, the development of whole-mount methods is imperative to elucidate intracellular protein localization in the tissue and organ context. Tissue integrity preservation becomes an even more important issue in the case of MAPKs as they are involved in the development of diverse organs [10, 11] as well as in the stomatal lineage formation in cotyledons and leaves [12].

The protocols described in this chapter provide the basis for studying subcellular localization of three developmentally and stress-regulated MAPKs represented by MPK3, MPK4 and MPK6 [3] in root cells by using whole-mount probes prepared from young seedlings. These protocols can be combined with immunolocalization of microtubules (Fig. 1) in order to reveal spatiotemporal relationships between above mentioned MAPKs and cytoskeleton in intact *Arabidopsis* roots [10, 11]. Moreover, by using combination of general and phospho-specific antibodies (e.g., against the pTEpY motif of mammalian ERKs and plant MAPKs; [13]) this method has also the power to discriminate between activated versus non-activated pool of respective MAPKs. Thus, whole-mount protocols for MAPK immunofluorescent localizations, should take into account the phosphorylation dependent nature of their activity status. Finally, described whole-mount immunolocalization method, along with complementary approaches such as GFP-tagging of MAPKs can provide an interesting source of information about localization of MAPKs at subcellular level and thus contribute to better understanding of MAPK signaling.

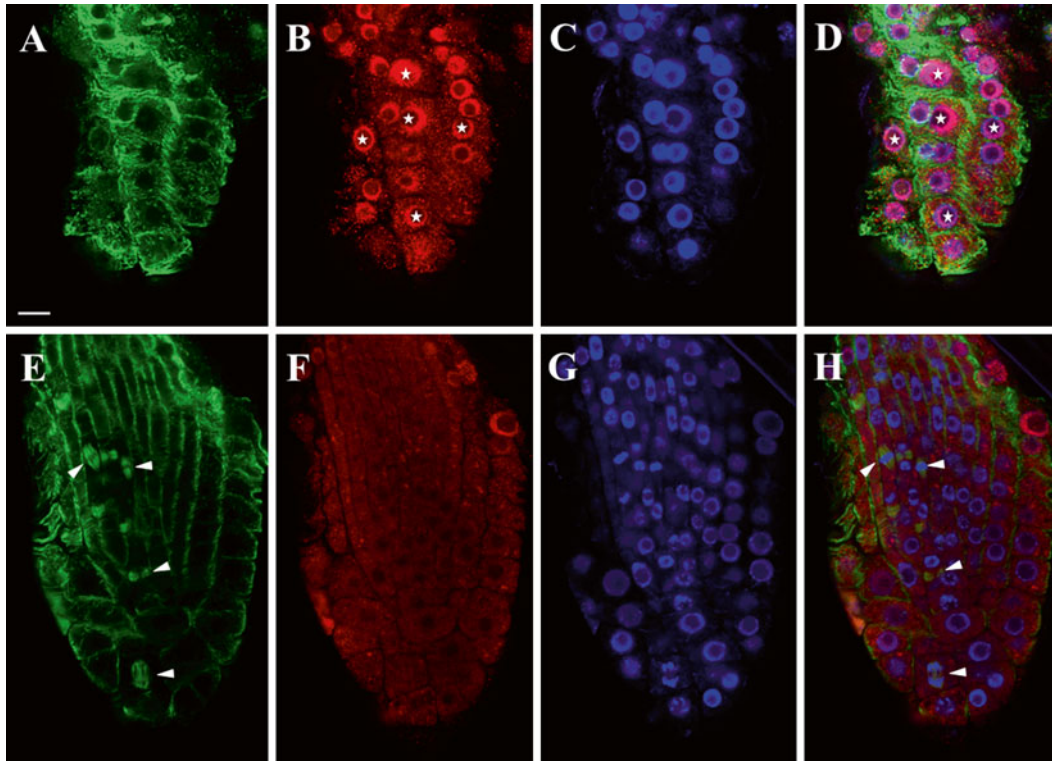

Fig. 1 Example of triple localization of microtubules (**A**, **E**), MPK6 (**B**, **F**), nuclei (**C**, **G**) and the resulting merged images (**D**, **H**) in single epidermal (**A–D**) and central (**E–H**) confocal sections in co-immunolabeled roots of *Arabidopsis thaliana*. Note the prominent nuclear localization of MPK6 in some epidermal cells (*asterisks*). Several mitotic and cytokinetic microtubular arrays are denoted by *arrowheads*. Scale bar is 10 μm for all figures

2 Materials

2.1 Plant Material

Seeds of *Arabidopsis thaliana* grown on half-strength Murashige–Skoog (1/2 MS, Duchefa) medium (2.2 g/L) supplemented with 10 g/L of sucrose and 6 g/L of Phytagel (pH 5.8).

2.2 Reagents for Immunolocalization

All chemicals should be of analytical grade. Solutions should be prepared in ultrapure water. Handling of toxic chemicals should comply with institutional safety regulations.

1. Stock formaldehyde solution: Prepare fresh 8 % (w/v) paraformaldehyde stock solution by dissolving 8 g paraformaldehyde in 90 mL microtubule stabilizing buffer (MTSB, see composition below) (using warm water bath) and 10 N NaOH to clear solution (usually 5–10 drops) (maximum 60 °C), remove from bath and carefully adjust pH to 7.2–7.4 with NaOH, bring to final volume 100 mL and keep in 4 °C not more as 1 week (*see* **Note 1**).

2. Stock glutaraldehyde solution: Always use frozen (−20 °C) aliquots of 25 % (v/v) glutaraldehyde stock solution (e.g., Sigma; *see* **Note 2**).
3. Fixation solution: Mixture of 1.5 % (w/v) paraformaldehyde and 0.5 % (v/v) glutaraldehyde in MTSB. Glutaraldehyde should be added last.
4. MTSB consists from 50 mM PIPES (*see* **Note 3**), 5 mM $MgSO_4 \times 7H_2O$, and 5 mM EGTA, pH 6.9 (*see* **Note 4**).
5. Phosphate buffered saline (PBS): 0.14 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4 \times 2H_2O$ and 1.5 mM KH_2PO_4, pH 7.3 (*see* **Note 5**).
6. Reduction solution: 0.05 % (w/v) sodium borohydride ($NaBH_4$) in PBS (must be always freshly prepared).
7. Cell wall digestion cocktail: Mixture of 1 % (w/v) meicelase (Desert Biologicals), 1 % (w/v) macerozyme R10 and 1 % (w/v) cellulase R10 (Yakult Honsha Ltd.) in PBS. Alternatively, use 2 % (w/v) driselase (Sigma), 2 % (w/v) cellulase R10, and 1 % (w/v) pectolyase Y23 (e.g., Duchefa) in PBS. Spin down in centrifuge before use (12,000 × *g*, 2 min).
8. Permeabilization solution: 10 % (v/v) dimethyl sulfoxide (DMSO), 2 % (v/v) Nonidet P-40 in PBS (*see* **Note 6**).
9. Blocking solution: 3 % (w/v) bovine serum albumin (BSA) in PBS.
10. Primary antibodies: Antibodies against the three major *Arabidopsis thaliana* MAPKs (MPK3, MPK4 and MPK6) are commercially available by different vendors (e.g., Sigma). Likewise, commercially available antibodies also exist for *Arabidopsis* MAPKKs (e.g., Santa Cruz Biotechnology, Inc.). Phosphorylated and activated forms of *Arabidopsis* MAPKs can be detected with phospho-specific antibodies against the pTEpY motif of mammalian ERKs (e.g., Cell Signaling Technology). For microtubule localization, we routinely work with rat monoclonal antibodies [14] raised against yeast α-tubulin (YOL1/34; e.g., Serotec) which give nice results with plant material [10].
11. Secondary antibodies: We routinely use anti-rabbit, anti-mouse, and anti-rat IgGs coupled to a wide range of Alexa Fluor dyes (e.g., Invitrogen; *see* **Note 7**). These provide high quantum yield, very good photo stability, being also rather insensitive to pH, yielding samples with high signal-to-noise ratios which can be archived for long term.
12. Solutions with antibodies: Primary antibodies are diluted 1:350 (anti-AtMPK3 and anti-AtMPK4), 1:750 (anti-AtMPK6), and 1:300 (anti-tubulin) in PBS containing 2 % (w/v) BSA. Secondary antibodies are diluted 1:500 in PBS

containing 2 % (w/v) BSA. Specific dilutions should follow manufacturer's instructions and be optimized on a personal experience basis.

13. 4,6-Diamidino-2-phenylidone (DAPI) staining solution: Stock solution of 0.1 mg/mL (w/v) DAPI (e.g., Sigma) in DMSO, aliquoted and stored at –20 °C in the dark. Prepare working solution by diluting 1:1,000 with PBS.
14. Mounting medium with anti-fading agent: 0.1 % (w/v) paraphenylene diamine (*see* **Note 8**) in 90 % (v/v) glycerol buffered with PBS or 1 M Tris–HCl at pH 8.0 (*see* **Note 9**). Store in dark at –20 °C. Alternatively, use commercial antifade medium (e.g., VECTASHIELD, Vector Laboratories).

2.3 Equipment and Consumables

Standard plastic labware, Parafilm, fine forceps, cell culture plates (24 wells) or small fixation vessels (1–3 mL), small plastic baskets (diameter 14 mm, available from Intavis) with mesh grids (100 μm), microscopic SuperFrost slides, coverslips, sterile laminar flow hood, refrigerator, culture chamber, fume hood, desiccator coupled to vacuum pump, centrifuge, incubator operating at 37 °C, magnetic stirrer with heating, pH meter, CLSM platform.

3 Methods

3.1 Growth of Arabidopsis Seedling Plants

1. Seeds of *Arabidopsis* are surface-sterilized with 70 % (v/v) ethanol for 5 min followed with 98 % (v/v) ethanol for 2–3 min and finally washed in sterile H_2O for 10 min.
2. Plate seeds on sterile Petri dishes with ½ MS medium, closed with Parafilm and stratified at 4 °C for 2 days.
3. Transfer Petri dishes to environmental chamber (21 °C; 16 h light, 8 h dark) for 3–5 days.

3.2 Processing for Immunofluorescent Localization

1. Select 3–5 days old seedling plants of *Arabidopsis thaliana*.
2. Quickly but very carefully transfer selected seedlings by using fine forceps or tweezers (EM grade) to fixation solution in microtiter plates or in fixation vessels (*see* **Notes 10** and **11**).
3. Fix probes under gentle vacuum at room temperature (RT) for 1 h (*see* **Note 12**).
4. Remove fixative and wash probes 2 × 10 min with MTSB and 2 × 10 min with PBS.
5. Reduce residual aldehyde groups with $NaBH_4$ in PBS for 3 × 5 min.
6. Wash probes 3 × 5 min with PBS. Fixation and reduction steps are done in the fume hood.
7. Digest cell walls with enzyme cocktail at 37 °C for 30 min.

8. Wash probes 4 × 5 min with PBS.
9. Permeabilize cell membranes with permeabilization solution at RT for 1 h.
10. Wash probes 4 × 10 min with PBS.
11. Block samples with blocking solution for 1 h at RT, to prevent unspecific binding.
12. Remove blocking solution and without washing add primary antibody appropriately diluted in PBS containing 2 % (w/v) BSA. Allow incubation at 4 °C overnight.
13. Remove primary antibody solution (*see* **Note 13**) and wash at least 6 × 10 min with blocking solution (*see* **Note 14**).
14. After final washing step, add the appropriately diluted Alexa Fluor-conjugated secondary antibody and incubate: 1½ h at 37 °C and 1½ h at 25 °C.
15. Wash probes 4 × 10 min with PBS (*see* **Note 15**).
16. Incubate probes with DAPI solution for staining of nuclei and chromosomes at RT for 10 min.
17. Rinse probes with PBS for 10 min.
18. Carefully mount each seedling in a drop of mounting medium and cover with coverslip. Remove excess of mounting medium with filter paper. Seal with transparent nail polish. Allow polish to fully harden before storing samples in boxes for microscopic slides at −20 °C in the dark to preserve fluorescent signal.
19. Perform adequate controls (*see* **Note 16**).

3.3 Confocal Laser Scanning Microscopy (CLSM)

1. Turn on microscope, lasers and associated computer and open acquisition program according to the operational manual from the manufacturer.
2. Select accordingly the channels that will be acquired (in most cases blue for DAPI, green for Alexa Fluor 488 and red for Alexa Fluor 546).
3. Arrange acquisition protocol: we normally acquire individual channels sequentially and not simultaneously (*see* **Note 17**) and we set photomultiplier tube voltage values during continuous imaging at the range indicator mode (*see* **Note 18**).
4. Set *Z*-axis according to Nyquist criteria (*see* **Note 19**).
5. For quantitative co-localization studies acquire all channels with the same pinhole size ([15]; *see* **Note 20**).
6. Save acquired images and export them as uncompressed, 16-bit TIFFs for further image processing and analysis.

3.4 Optional Procedure

Liquid handling pipetting robot (e.g., InSitu Pro, Intavis, Germany) may be recommended for easier and large scale handling of probes for whole-mount immunolocalization procedure.

4 Notes

1. Formaldehyde is highly toxic and carcinogenic. Always handle under fume hood with appropriate protective clothing and gloves. Dispose it according to institutional safety regulations for toxic waste.
2. Glutaraldehyde is toxic and should be handled and disposed as formaldehyde.
3. Put solid KOH to dissolve PIPES first.
4. Prepare 0.5 M EGTA stock solution. Free acid form of EGTA is not water soluble and it requires titration with solid KOH until the solution clears and pH is 8.0.
5. Prepare 10× PBS stock solution, supplemented with 0.01 % (w/v) sodium azide (NaN_3) to prevent contaminations and stored at RT. Alternatively it may be autoclaved, stored at RT but always handled under sterile laminar flow box, as it is prone to contaminations.
6. DMSO is a developmental neurotoxin and should be handled with appropriate safety precautions.
7. Rat and mouse immunoglobulins can be combined for simultaneous double immunolocalizations, provided that the respective secondary fluorophore conjugated antibodies are pre-adsorbed. If not, such colocalizations using immunoglobulins raised in closely related species are better done sequentially to avoid cross-reactivities.
8. Para-phenylene diamine is highly toxic, and should be handled and disposed accordingly following institutional safety regulations.
9. Weight 50 mg para-phenylene diamine, add 100–150 μL DMSO, swirl the glass vessel fast and vigorously until powder is dissolved, add 5 mL of 1 M Tris–HCl buffer (pH 8.4–8.8) or phosphate buffer (pH 8.2–8.6), by using glass rod mix gently but quickly with 45 mL of 100 % glycerol (DMSO is oxidant so it must be rapidly diluted with Tri–HCl and glycerol to prevent oxidation of para-phenylene diamine). Aliquot and store solution at −20 °C or −80 °C in dark, maximum 3–4 months (color should not turn to pink or brown during storage). Alternatively, para-phenylene diamine may be dissolved without DMSO, by bubbling argon gas in the Tris–HCl solution before adding glycerol.
10. Change solutions very carefully using 1 mL micropipette, avoid direct contact with seedlings, especially with their root tips. We recommend using small plastic baskets (diameter 14 mm) with nylon mesh grids (100 μm), which are placed to

24 wells cell culture plates for better preservation of probes and convenient handling during all steps of this protocol.

11. Transfer carefully containers in new wells of microtiter plate filled with fresh solutions during all steps in this protocol by using forceps (this pipetting-free procedure is minimizing sample damage).
12. Place the plates in vacuum desiccator and use membrane pump; incubate samples at RT for 60 min.
13. Diluted primary antibodies can be saved for two to three subsequent immunolocalizations. After removing the diluted primary antibody solution, collect it in a microfuge tube, spin it down to remove cell debris and store solution at 4 °C.
14. Washing away the primary antibody is critical for reduction of background fluorescence. The regime given is the minimum time that in our hands gave clean results. However, this step can be prolonged.
15. Diluted fluorophore-derivatized secondary antibodies are not recommended for repeated use. Discard after each labeling.
16. A control sample omitting of primary antibody can determine amount of background signal caused by the secondary antibody. No labeling in this case indicates that there is no nonspecific binding of the secondary antibody. Another control labeling is with pre-immune serum, which determines specificity of primary antibody.
17. With sequential acquisition, emission bleed-through that may confound colocalization studies of two fluorophores with partially overlapping emission spectra is avoided.
18. The range indicator mode allows us to avoid saturation during acquisition which is very important during quantitative image analysis.
19. This requires sampling at the half of Rayleigh criterion [15]. In Zen 2012 software (Zeiss) the optimal Z-thickness is automatically determined and can be selected by the user.
20. Pinhole size is normally set to 1 Airy unit for optimal resolution. However, the size of the Airy unit depends on the emission wavelength. For colocalization studies it is important to ensure the same Z-section thickness, since this will determine the amount of photons collected in each respective channel.

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Chapter 10

Immunofluorescent Localization of MAPKs in Steedman's Wax Sections

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Abstract

Signals of different nature are transduced in cells through signal transduction pathways, where mitogen-activated protein kinases (MAPKs) play an important role as signaling molecules. Views into intracellular localization of MAPKs are critical for the understanding of their spatial and temporal functions, like activation-based relocation, compartmentation, or interactions with local substrates. Localization of MAPKs in cells is thus very useful cell biological approach, extending complex mode of cell signaling characterization in plants. Here, we present a method for subcellular immunofluorescence localization of MAPKs using protein- or phospho-specific antibodies, performed on sectioned fixed plant samples. It is based on embedding of samples in the Steedman's wax, a low-melting point polyester wax embedding medium, which maintains high antigenicity of studied proteins. In addition, exposure of dewaxed sections to antibodies allows for their efficient penetration. Altogether, it makes this simple method a good tool in the efficient subcellular localization of diverse proteins, including plant MAPKs.

Key words *Arabidopsis thaliana* L, Immunofluorescence microscopy, MAPK-specific antibody, *Medicago sativa* L, Oxidative stress, Salt stress, Sections, Steedman's wax

1 Introduction

Responses of plants to various stimuli are mediated by the recognition of related signals at the plasma membrane and their intracellular transduction via signal transduction cascades. Mitogen activated protein kinases (MAPKs) are signaling molecules involved in the transduction of signals by reversible phosphorylation. MAPK signaling pathways are organized into modules. Activation of MAPK modules and regulation of different cellular processes by MAPK signaling occurs in distinct subcellular compartments [1]. Thus, understanding of the spatial and temporal organization of MAPK signaling modules and their molecular interactions requires their proper localization within the cells.

Even though that the most powerful way of subcellular localization is in vivo expression of MAPKs tagged with fluorescent

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proteins, several microscopy techniques localizing MAPKs in situ brought comprehensive data about subcellular distribution of MAPKs during plant development and under stress responses. Whole-mount immunolocalization technique, employing precise tissue- and cell-specific localization in roots with very good subcellular resolution, was used for characterization of developmental roles of *Arabidopsis* MAPKs AtMPK4 and AtMPK6, as related to cytoskeleton, endomembranes, and cell division [2, 3].

Success in the immunofluorescence analyses of MAPKs on sectioned plant samples benefited from thorough testing of suitable embedding medium, which can effectively protect protein antigenicity during processing. Steedman's wax was introduced to histochemistry in 1957 [4]. This ribbon-forming sectioning medium has several advantages making it for a perfect tool in the indirect immunofluorescence detection of proteins. This low melting-point polyester wax has a melting point of 35–37 °C and is soluble in ethanol, which allows for maintenance of high antigenicity of diverse proteins. Because Steedman's wax is soluble in ethanol, the use of hazardous organic solvents, often used with other embedding media, can be completely avoided, thus minimizing health risks and waste disposal costs. Steedman's wax has been introduced to plant science some 25 years ago and proved suitable for the immunofluorescence detection of microtubules [5, 6], actin filaments [7–11], plasma membrane H^+-ATPases [12], PSTAIR proteins [13], cyclins [14, 15], MAP kinases [16–19], arabinogalactan proteins [20, 21], pectins and xyloglucans [8, 22], expansins [23], calreticulin and HDEL proteins [24], profilin [25], myosin VIII [26–28], ARP3 protein [29], PIN proteins [8], and auxin [30].

Due to excellent penetration ability of antibodies into sectioned samples and due to maintenance of high antigenicity of proteins, Steedman's wax proved to be particularly useful for visualization of MAPKs. Immunofluorescent localization of MAPKs in sectioned root samples revealed localization of MAPKs in the nucleus of resting cells, being exported out and relocated to cytoplasmic structures upon activation. This was shown to stress-induced MAPK (SIMK) in root cells of *Medicago sativa* L. during cell division and salt stress [16] or during root hair development [17, 18]. Immunofluorescence microscopy in elongating root cells of *M. sativa* L. using the Steedman's wax embedding allowed also for correlative localization of SIMK protein with its activity. Utilization of a phospho-specific antibody against the dually phosphorylated pTEpY motif of SIMK revealed that recruitment of SIMK at tips of growing root hairs is specific for its activated form [17].

The full protocol for sample preparation, Steedman's wax embedding, and making of sections for immunofluorescent detection of MAPKs is thus provided in detail. Included are also practical advices for treatments of experimental plants for specific activation of MAPKs, like salt stress and oxidative stress.

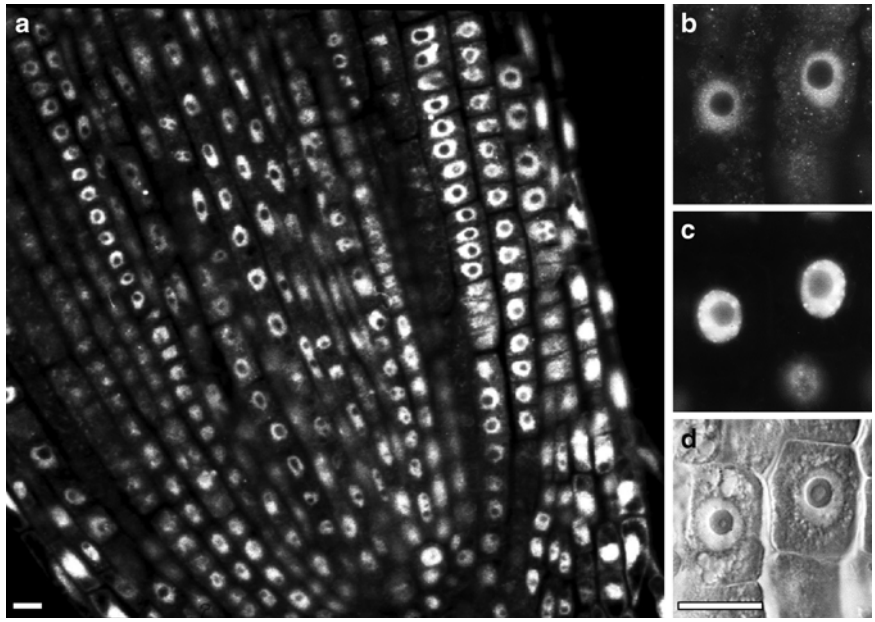

Fig. 1 Immunolocalization of SIMK in root cells of *Medicago sativa* L. Excised roots were fixed in 3.7 % (w/v) buffered formaldehyde, dehydrated and embedded in Steedman's wax. After sectioning and dewaxing, SIMK was localized by indirect immunofluorescence with affinity purified SIMK antibody (M23). (**a**) Overview of SIMK localization in different tissues of root apical meristem. (**b**) SIMK localization in individual root cells. (**c**) DAPI staining, (**d**) DIC image. Bar = 10 μm

The whole protocol is described for preparation of samples from roots of *Medicago sativa* L., with noting of all modifications that are related to preparation of *Arabidopsis thaliana* L. root samples. The anticipated result is exemplified to Fig. 1.

2 Materials

All chemicals should be of defined purity (pro analysi grade). Solutions should be prepared in ultrapure water (MilliQ or equivalent). The laboratory needs to be equipped with standard equipment.

2.1 Plant Material, Culture Media, and Solutions for Abiotic Stress Treatments

1. Seeds of *Medicago sativa* L., cv. Europe.
2. Seeds of *Arabidopsis thaliana* L., ecotype Columbia (Col-0).
3. Commercially formulated half-strength Murashige and Skoog (1/2 MS; Duchefa) medium is prepared without vitamins, supplemented with 1 % (w/v) sucrose, pH 5.7 (*see* **Note 1**). The medium can be solidified by the addition of 0.6 % (w/v) Phytagel (Sigma-Aldrich).
4. Fahraeus liquid culture medium: 1.36 mM KH_2PO_4, 1.12 mM Na_2PO_4, 1.36 mM $CaCl_2$, 0.97 mM $MgSO_4$, 20 μM Fe-citrate, pH 6.5 [31].

5. Liquid medium for salt stress induction: liquid ½ MS supplemented with 50–250 mM NaCl.
6. Liquid medium for oxidative stress induction: liquid ½ MS supplemented with 1–15 mM H_2O_2.

2.2 Plant Fixation and Embedding

1. Stabilizing buffer: 50 mM K-PIPES pH 6.9, 5 mM $MgSO_4 \times 7H_2O$, 5 mM EGTA (*see* **Notes 2** and **3**).
2. Phosphate buffered saline (PBS): 0.14 M NaCl, 2.7 mM KCl, 6.5 mM $Na_2HPO_4 \times 2H_2O$, 1.5 mM KH_2PO_4. Make it with H_2O, pH set to 7.3 (*see* **Notes 2** and **3**).
3. Formaldehyde stock solution (10 % w/v): weigh out 16 g of paraformaldehyde powder, transfer in a conical flask and add 150 mL of ultrapure water. Place on heated magnetic stirrer; adjust temperature to 60–65 °C and wait until temperature is reached (*see* **Note 4**). Add dropwise and slowly 10 M KOH until the solution clears and allow to cool down at room temperature. For long-term storage aliquot and store under −20 °C or −80 °C.
4. Glutaraldehyde stock solution: 25 % (v/v) glutaraldehyde in ultrapure water (e.g., Electron Microscopy Sciences; *see* **Notes 4** and **5**).
5. Working fixative solution: 3.7 % (w/v) formaldehyde (*see* **Notes 6** and **7**) and 0.5 % (v/v) glutaraldehyde in stabilizing buffer.
6. Embedding medium: Steedman's wax, prepared by mixing polyethylene glycol (PEG) 400 distearate with 1-hexadecanol in 9:1 (w/w). Alternatively, the complete medium is commercially available as Polyester wax. The medium is used at 37 °C (*see* **Note 8**).
7. Bovine serum albumin (BSA) stock solution: 1 % (w/v) BSA in ultrapure water or stabilization buffer. Store it in 1 mL aliquots at −20 °C.

2.3 Sectioning of Plant Samples and Processing of Sections

1. Wooden stubs for fixing of wax-embedded blocks.
2. Rotary microtome operating with metal knives.
3. Razor blades and brushes for manipulation with wax sections.
4. Microscopy glass slides for collection and adhesion of wax sections.
5. Mayer's glycerol–albumen [32] for adhesion of sections: mix 50 mL egg white, 50 mL glycerol, 1 g sodium salicylate and add 0.1 % (w/v) merthiolate or a crystal of thymol as antimicrobial agents. Although it is commercially available (e.g., EMD Millipore), it can be prepared in-house. For other adhesion options *see* **Note 9**.

2.4 Immunofluorescence Microscopy

1. For the present study an affinity purified, peptide antibody against the 7 carboxyl terminal residues of SIMK (FNPEYQQ; designated M23) coupled to a purified derivative of tuberculin [33, 34] was generated in rabbit and used thereon. The phosphorylated form of SIMK was based on antibody generated against the pTEpY motif of SIMK along with surrounding residues (CTDFMpTEpYVVTRWC; designated N103) again coupled to a purified derivative of tuberculin [17]. Alternatively, commercially available anti-pTEpY antibodies can be used. Similar, non-cross-reactive sera were generated for other *Medicago* sp. MAPKs [33, 34]. Antibodies for major *Arabidopsis thaliana* MPKs (AtMPK3, AtMPK4, and AtMPK6) are commercially available (e.g., Sigma; *see* **Note 10**).
2. For direct immunofluorescence detection primary antibodies can be conjugated to fluorophores Alexa Fluor 488 (green fluorescence) and Alexa Fluor 568 (red fluorescence) using protein labelling kits (Invitrogen) according to manufacturer instructions.
3. For indirect immunofluorescence detection commercially available fluorescein isothiocyanate (FITC)-, Alexa Fluor 488-, tetramethyl rhodamine isothiocyanate (TRITC)-, or Alexa Fluor 568-conjugated anti-rabbit IgGs may be used (e.g., Invitrogen).
4. DAPI (4,6-diamidino-2-phenylidone). Stock solution of 0.1 mg/mL in DMSO, stored in −20 °C. Prepare working solution by diluting 1:1,000 with PBS.
5. Toluidine Blue O, 0.01 % (w/v) solution in PBS (*see* **Note 11**).
6. Mounting medium with anti-fading agent: 0.1 % (w/v) paraphenylene diamine (*see* **Note 12**) in 90 % (v/v) glycerol buffered with 100 mM phosphate or 100 mM Tris–Cl buffer at pH 8.0. Store in dark, at −20 °C or −80 °C (*see* **Note 13**).

3 Methods

All experimental procedures should be carried with compliance to safety rules.

3.1 Plant Material and Treatments

1. Seeds of *Medicago sativa* L. cv. Europe are surface sterilized with 1 % (w/v) sodium hypochlorite and 70 % (v/v) ethanol for 3 min, thoroughly washed with sterile water, imbibed in sterile water at 4 °C for 4–6 h, and placed on moist filter paper in petri dishes for germination (*see* **Note 14**). Germinate in culture chambers in darkness at 25 °C.
2. Seeds of *Arabidopsis thaliana* L. are surface sterilized as above and plated on solid ½ MS medium (*see* **Note 15**). Germinate

in environmental chamber at 22 °C, 16/8 h light/dark regime and 50 % humidity.

3. For stress treatments of either *Medicago* or *Arabidopsis* seedlings, apply directly 10 mL of ½ MS supplemented with the desirable NaCl or H_2O_2 concentration and for the desirable time (*see* **Note 16**).

3.2 Sampling and Preparation of Plant Material for Sectioning

1. Select 3-day old seedlings of *Medicago sativa* L. cv. Europe with straight 40–50 mm long primary roots.
2. Remove *Medicago* seedlings growing out of the mesh; place them on Parafilm-supported filter paper wetted by the same solution (culture medium with 250 mM NaCl) and excise root tips 8–10 mm long by sharp scalpel or razor blade. *Medicago* and *Arabidopsis* plants treated on the surface of moist filter paper in petri dishes should be sampled directly on place, still in the presence of NaCl-containing solution. Put excised roots immediately to fixative solution made in stabilizing buffer and fix at room temperature for 1 h (*see* **Note 17**).
3. Wash samples in stabilizing buffer for 3 × 10 min. Discard solutions after washing in the formaldehyde waste in the hood.
4. Wash the samples in PBS for 15 min.
5. Dehydrate the samples in a graded ethanol series diluted in PBS in the following steps: 30, 50, 70, 90 % (v/v) ethanol in PBS and 97 % ethanol, each step at room temperature for 30 min (*see* **Note 18**).
6. Move the samples in 97 % ethanol to the incubator with temperature set to 37 °C and let it warm up for at least 30 min (*see* **Note 19**).
7. Pass the samples through increased Steedman's wax—absolute ethanol dilutions (2:1, 1:1 and 1:2) for careful and proper infiltration with embedding medium. Start with a mixture 2:1, followed by 1:1 and by 1:2 (ethanol–wax, v/v), each step should take 1–2 h (*see* **Note 20**).
8. Transfer the samples to pure Steedman's wax and repeat at least twice, first for 2 h and second for overnight (*see* **Note 21**).
9. Pour fresh pure Steedman's wax into silicon or rubber embedding molds, filling completely the block spaces (*see* **Note 22**).
10. Move the samples very carefully from vials to the molds with help of a preparation needle and add fresh pure Steedman's wax to fill completely the block spaces in the molds (*see* **Note 23**).
11. Take the molds with the samples out of the incubator and adjust the final positioning of the samples in blocks on flat and stable surface (laboratory bench), while the Steedman's wax inside is still liquid and transparent (*see* **Note 24**).

12. Mark correctly each block and let it solidify at room temperature in dry place. Blocks after solidification must be stored in stable environment either at room temperature or well-sealed at 4 °C.

3.3 Sectioning of Steedman's Wax-Embedded Plant Material

1. Place blocks of samples in Steedman's wax on filter paper and trim them carefully with sharp razor blade to remove excessive wax around the embedded tissue (*see* **Note 25**). Note that two opposite edge lines of the block must be aligned in parallel. They will be further aligned with the knife in the microtome, which is indispensable for formation of straight ribbon of sections during sectioning.
2. Stick wax blocks to wooden stubs using pure solidified Steedman's wax. Heat preparation needle in a flame and melt down the Steedman's wax on surface of the wooden stub completely. It should cover the whole surface of the stub. When wax starts to solidify, place the block with sample on it and let it solidify completely (*see* **Note 26**).
3. After full solidification, if necessary, other pieces of solid Steedman's wax may be added to the base of the blocks and carefully melted down by hot preparation needle for better block fixation. Let it solidify completely.
4. Make final trimming if necessary.
5. Clamp the wooden stub into the specimen holder of a microtome. It is important to align the top face of the wax block and its parallel edge lines with the knife.
6. Upon starting of cutting first sections appear at the knife edge, forming subsequently a ribbon of sections. Release the front sections of the ribbon from the knife and hold the whole ribbon out during cutting by paint brush to prevent sticking of the ribbon to the base of metal knife.
7. Detach the ribbon from the knife edge and transfer it carefully on a clean surface that is not sticky to wax (*see* **Note 27**).
8. Prepare microscopy slides coated with Mayer's glycerol–albumen (*see* **Note 28**).
9. Divide ribbon into parts with a razor blade and place them sequentially on a glass slide using the brushes (*see* **Note 29**).
10. Put few drops of water at the side of the ribbons and let them expand. Put enough water to spread sections, but prevent its excess (*see* **Note 30**). Once all sections are expanded enough, remove water by filter paper on one side of the slide and let it dry completely. Slides protected to external moisture could be stored in the fridge at 4 °C or in freezer at –20 °C.

3.4 Processing of Sectioned Plant Samples

1. Sectioned samples must be dewaxed and rehydrated first to enable the penetration of the antibodies for immunodetection. Dewax the sections in ethanol by passing slides through 97 % ethanol for 3×10 min (*see* **Note 31**), continuing by graded ethanol series of 90 % and 70 % ethanol diluted with PBS, 10 min each. The final step is PBS for 10 min.
2. Transfer slides with sections to stabilizing buffer for 30 min.
3. Dip slides into –20 °C precooled methanol for 10 min.
4. Transfer slides to stabilizing buffer for 30 min at room temperature. The samples are ready for immunodetection.

3.5 Immunofluorescence Microscopy

1. Pre-incubate rehydrated sections with 1 % (w/v) BSA in PBS for 10 min to prevent unspecific antibody binding.
2. Remove BSA and incubate sections with primary affinity-purified MAPK-specific antibody diluted with PBS (in the case of SIMK, the dilution was 1:1,000) at room temperature in the darkness for 60 min (*see* **Note 32**).
3. Rinse out the excess of antibody with stabilizing buffer and leave slides in it for additional 10 min.
4. Incubate sections with secondary antibody diluted with PBS (in the case of FITC- or Alexa 488-conjugated anti-rabbit immunoglobulin G raised in goat the dilution was 1:100) at room temperature in darkness for 60 min.
5. Rinse out the excess of antibody with PBS and leave slides in PBS for additional 10 min (*see* **Note 33**).
6. Apply 4,6-diamidino-2-phenylidone (DAPI) for staining of nuclear DNA, diluted 1:1,000 with PBS for 10 min.
7. Wash DAPI out by PBS and put slides into 0.01 % (w/v) Toluidine Blue O diluted in PBS for 10 min.
8. Rinse slides with PBS for 10 min.
9. Mount slides in anti-fade mounting medium containing p-phenylenediamine (*see* **Note 34**). Seal with nail polish and store at –20 °C.
10. Perform adequate controls (*see* **Note 35**).
11. The samples are ready for immunofluorescence observation in epifluorescence of a confocal laser scanning microscope with appropriate selection of fluorescence excitation and emission wavelengths.

4 Notes

1. For buffering ½ MS, MES can be added at 10 mM and titrated to desired pH with KOH. Otherwise, ½ MS can be buffered with KOH without the addition of MES.

2. EGTA can be prepared as 0.5 M stock solution. Free acid form of EGTA is not water soluble, therefore for preparation of the stock solution, KOH pellets need to be added until the solution clears and pH is 8.0.
3. MSB and PBS can be prepared as 10× solutions supplemented with 0.01 % (w/v) sodium azide (NaN_3) or 0.01 % (w/v) merthiolate for preventing microbial contaminations. Alternatively, solutions can be autoclaved.
4. Formaldehyde and glutaraldehyde are highly toxic and carcinogenic; always handle under dedicated fume hood, wearing protective clothing and gloves. Solutions containing formaldehyde and glutaraldehyde should be discarded according to institutional regulations for organic toxic waste.
5. Glutaraldehyde stock solutions should be of Electron Microscopy grade. It is best to buy in small aliquots (ampules). Keep one stored at 4 °C for routine use and the rest stored aliquoted under –20 °C.
6. After thawing the stock solution a slight precipitate may form. Clear the solution by brief warming in waterbath at ca. 50 °C.
7. For most plant tissues, and particularly roots, formaldehyde fixation preserves tissue integrity and antigenicity satisfactorily. Modifications in concentration and combination of different aldehydes in the fixation solution may be, however, necessary to get appropriate preservation of root samples from different plant species. Alternatively, 3.7 % formaldehyde, can be prepared by diluting 37 % formaldehyde solution (formalin). However, formalin solutions are stabilized against oxidation and polymerization of formaldehyde by the addition of generous amounts of methanol (10–12 % v/v) which may interfere with the antigenicity of some structures.
8. To prepare low melting point Steedman's wax, individual ingredients must be melted down at higher temperature first. Put PEG 400 distearate to Erlenmeyer's beaker and melt it down at 65 °C. Add 1-hexadecanol and let it melt down as well. The solution must be thoroughly stirred for 3–4 h at 65 °C. Pour well mixed (clear) wax into aluminum containers and let it cool down at room temperature. Medium should be stored at room temperature in dry and dark place. In our hands, laboratory prepared Steedman's wax had better properties during the sectioning and further processing of sections.
9. Sections can be adhered on commercially available cationized Superfrost Plus® slides (Fischer Scientific), or on slides covered with poly-L-lysine (0.1 % (w/v)).
10. Always ensure specificity of primary antibody by western blot on crude protein extracts prepared from the plant under study.

11. Toluidine Blue O staining quenches autofluorescence and results in increased signal-to-noise ratios.
12. Para-phenylene diamine is extremely toxic. Handle under dedicated fume hood with appropriate protective clothing and gloves. Discard para-phenylene diamine solutions according to institutional regulations for organic toxic waste.
13. Para-phenylene diamine is very prone to oxidation. Mixing should be optimally carried out in an oxygen-free atmosphere, ideally by bubbling argon gas in the solution. Once the mounting medium assumes a dark discoloration it is no longer effective in preventing photobleaching and should be discarded.
14. It is advisable to scarify *Medicago* seeds by incubation in concentrated H_2SO_4 for 5 min. This breaks dormancy, improves germination and synchrony of growth.
15. Prior to transferring to the environmental chamber, place plates with *Arabidopsis* seeds at 4 °C for 2–3 days for stratification.
16. We used two different modes of salt stress application to *Medicago* seedlings. First, solution of 250 mM NaCl in the culture medium was pre-bubbled by air for at least 2 h to prevent oxygen deprivation for plants during longer treatments. Solution was placed to square black plastic jar, overlaid by plastic mesh with holes 2 × 2 mm. Individual seedlings were inserted into the holes allowing submergence of the root to the medium, but leaving the green part above in the air. We inserted seedlings to the mesh holes in control medium first and only then we passed the mesh with all seedlings to salt-containing medium. Second mode of salt stress application was based on pouring of 250 mM NaCl (prepared in culture medium) to plant roots growing on the surface of moist filter paper in petri dishes. Together with *Medicago*, this mode was applied also to *Arabidopsis* seedlings.
17. Allowing fixation of the samples in vacuum immediately after sampling usually enhances penetration of the fixative.
18. Make sure that thin samples are not damaged or lost by pipetting during all washing and dehydration steps.
19. Seedlings should be stained with 0.1 % (w/v) Toluidine Blue (it is applied in the 97 % ethanol step before moving the samples to 37 °C) in order to allow for their visualization during the embedding process and all manipulation steps with wax blocks.
20. First contact of dehydrated samples with Steedman's wax is critical, in particular for large vacuolated cells in the elongation zone of roots. It is thus recommended to prepare a mixture 2:1

(ethanol–wax, v/v) and pour it directly to vial with the samples in 97 % (v/v) ethanol. The samples in ethanol will be pushed up by ethanol–wax mixture, which allows for subsequent slow sedimentation of the samples through ethanol–wax gradient afterwards, which ensures slow and gentler infiltration with wax. In this case, an extra step with pure ethanol–wax mixture at 2:1 should follow.

21. Although all steps before application of pure wax were performed inside of the incubator in sealed vials, it is recommended to perform last steps of infiltration (in particular during the first incubation in pure Steedman's wax) in opened vials. It will make sure that last traces of ethanol will be evaporated.
22. Embedding molds must be pre-warmed first. Put them to the incubator at least 30 min before use.
23. Transferring the samples to the molds with opened door of the incubator leads to temporal dropping of temperature inside. Make sure that after complete transfer of the samples the temperature rise up back to 37 °C before further step.
24. The samples should be positioned with the help of preparation needle along longer axis of the block and close to the lower edge. This facilitates sample recognition in the block after solidification. It is also helping a lot during trimming and subsequent alignment in the microtome.
25. You have to manipulate with wax blocks using a forceps only. Since the melting point of the Steedman's wax is 35–37 °C, blocks melts down quickly by hand touching and could be destroyed.
26. If you do it in too hot wax on the surface of wooden stub, the block could melt down which may damage the sample.
27. Optimal thickness of sections from plant root samples embedded in Steedman's wax, suitable for further immunofluorescence localizations is 5–10 μm.
28. Microscopy slides should be cleaned by detergent first, washed by tap water, rinsed by 97 % (v/v) ethanol and distilled H_2O. Mayer's glycerol–albumen must be applied in small drop only, because in excess it may lead to unspecific binding of antibodies, which rises up the background fluorescence signal. The film of albumen should be thin and homogenous. It is the best to spread the drop on the surface of the glass by alcohol-cleaned finger until it worms up. Let it cool down before putting the sections.
29. We regularly prepare longitudinal sections of roots. In both *Medicago* and *Arabidopsis*, the whole root is cut out within one ribbon of sections. By dividing of ribbon into several parts, all sections in an ordered manner could be placed on one or two

slides. The whole root can be thus thoroughly studied from serial sections.

30. The whole contact surface of sections with the glass must be removed from the slide by water, otherwise compressed sections after sectioning stay permanently attached as such to the slide without any possibility to rescue. Excess of water behind the sections, however, leads to their overextension and disruption.
31. Use three different jars with 97 % (v/v) ethanol for initial steps of dewaxing, since it will contain high concentration of diluted wax components after use. This prevents cross-contamination of other dewaxing solutions. 97 % (v/v) ethanol II and III could be used in two to three dewaxing cycles, but discard always 97 % ethanol I after use.
32. Antibody is applied on the surface of sections in the volume of 200 μL, spread evenly with the help of pipetting tip and kept in wet chambers to prevent evaporation.
33. It is possible to immunolocalize other antigens in the same samples, like other MAPKs or cytoskeletal proteins. It is necessary, however, to use primary antibodies against these antigens raised in different animal than rabbit to prevent cross-reaction.
34. Add few drops of the anti-fade mounting medium on the sections and let it spread a little first. Then cover the slide carefully with a coverslip of an appropriate size. Make sure that no air bubbles remain under the coverslip. Blot excess fluid at the edges of the coverslip with filter paper. Make sure that you seal it with a nail polish only when the edges are clean and excess of mounting medium has been removed.
35. Immunodepletion control with primary antibody pre-incubated with related synthetic peptide used for antibody preparation should block immunofluorescent labelling. No immunolabelling should be seen also when pre-immune serum is used. It avoids any doubts about nonspecific binding of the primary antibody. Another control labelling is with omitting of primary antibody. No labelling in this case indicates that there is no nonspecific binding of the secondary antibody.

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Chapter 11

Fluorescent Protein Tagging of *Arabidopsis* MAPKs for In Vivo Localization Studies

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Abstract

Mitogen-activated protein kinases (MAPK) are key regulatory elements in many processes. They are highly conserved throughout eukaryotes. In plants, MAPKs are involved in biotic and abiotic stress responses; they regulate cell division, cell growth, and also programmed cell death. In vivo visualization of MAPKs is crucial for understanding of their spatiotemporal organization. Cloning of MAPK–fluorescent protein fusions might present difficulties related to the preservation of protein–protein interactions essential for MAPK localization, interactions with upstream and downstream regulators, and finally substrate targeting. In this chapter we describe cloning of MAPKs in the flexible MultiSite Gateway® cloning system followed by easy and quick testing of binary vectors by transient assays in *Arabidopsis thaliana* and *Nicotiana benthamiana*.

Key words Mitogen activated protein kinase, Multisite Gateway® cloning, FAST transient transformation, Green fluorescent protein, GFP-Trap® immunoprecipitation, Native promoter, Floral dipping

1 Introduction

Mitogen-activated protein kinase (MAPK) signaling is a sophisticated machinery of extracellular signal perception and transduction ultimately targeting and regulating various cytoplasmic and nuclear substrates to modify their function via reversible phosphorylation [1]. The three-tiered MAPK module comprises of a linear and sequential phosphorylation cascade. Upon signal perception, the module is initiated by a serine/threonine mitogen-activated protein kinase kinase kinase (MAPKKK) which upon activation phosphorylates and activates the corresponding MAPKK, a dual specificity Ser/Thr and Tyr kinase. The MAPKK then phosphorylates simultaneously a threonine and a tyrosine residue in a TXY motif within the activation loop of the descending MAPK. The then dually phosphorylated and activated MAPK specifically targets and

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phosphorylates proline directed Ser or Thr residues (SP motif) in its substrates, affecting their localization and function. The *Arabidopsis* genome encodes for more than 60 MAPKKKs, 10 MAPKK and 20 MAPK genes [2]. Moreover proteomic and interactomic studies are in course of revealing increasing numbers of MAPK substrates [3]. The above numbers indicate extensive crosstalk and functional redundancy in MAPK signaling [4, 5]. The above as well as the placement of MAPK signaling in the crossroad between hormonal and secondary signaling pathways, merely substantiate the increasing interest for elucidating conditionally or developmentally inflicted MAPK pathways in research model and crop plants [5, 6]. Probably the complexity underlying plant MAPK signaling may be spatiotemporally resolved through the function of molecular scaffolds and adaptors [7, 8]. However, such spatiotemporal interrogations are rarely and poorly addressed in plants.

Owing to the complexity of MAPK cascades, the differential selection of MAPK binding partners by the same MAPK and the transient nature of such interactions, the intracellular, activation-dependent and independent localization of MAPKs is a challenging task. Thus, it is necessary to employ flexible cloning systems allowing for the preparation of multiple constructs with alternative promoters, terminators and reporter genes. Classical restriction/ligation cloning is time consuming and labor demanding requiring the cumbersome manipulation of large plant binary vectors. At this end, MultiSite Gateway® cloning system (Life Technologies) fulfills all demands for flexible and easier cloning [9–11]. This system is based on site specific recombination. It allows the promoters, genes, terminators or any tags including reporter genes to be easily replaced and combined in simple recombination reactions. Karimi et al. [10] optimized the use of MultiSite Gateway® cloning system for plants. They created a collection of Entry clones carrying a variety of promoters, terminators, and reporter genes. Many variations and combinations are thus available. Moreover, sets of binary destination vectors with different selection markers are also available [12].

Live cell imaging has greatly advanced after the introduction of marine fluorescent proteins which could be fused to and tracked along with proteins of interest in vivo [13]. However, it was shown that N-terminal GFP fusions of MAPKs were frequently dysfunctional and mislocalized [14, 15]. Therefore it is worth to prepare both C- and N-terminal fusion proteins. So far direct localization of MAPKs is mostly studied in vivo with MAPK fusions driven by constitutive promoters (e.g., cauliflower mosaic virus 35S promoter) [17] and rarely by immunofluorescence labeling. Indirectly, cell and tissue specific MAPK localizations are inferred by visualization of MAPK promoter::GUS constructs [16, 17].

Using Multisite Gateway® technology, we prepared expression constructs with 3′ and 5′ GFP tags fused to MPK6. MPK6 gene was cloned from both genomic DNA and cDNA (for protein

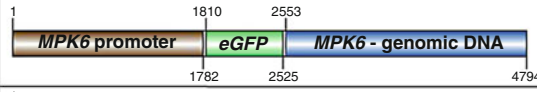
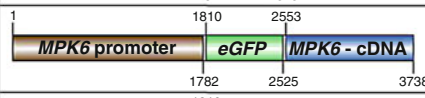
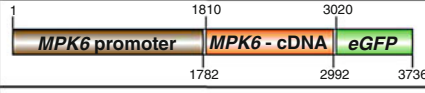
PROTEIN DESIGNATION	CONSTRUCT MAP	DESCRIPTION
GFP-gMPK6		N-Terminal fusion of *AtMPK6* with GFP cloned from genomic DNA driven by native promoter
GFP-cMPK6		N-Terminal fusion of *AtMPK6* with GFP cloned from cDNA driven by native promoter
gMPK6-GFP		C-Terminal fusion of *AtMPK6* with GFP cloned from genomic DNA driven by native promoter
cMPK6-GFP		C-Terminal fusion of *AtMPK6* with GFP cloned from cDNA driven by native promoter

Fig. 1 Detailed description of MPK6 cassettes prepared by MultiSite Gateway® cloning

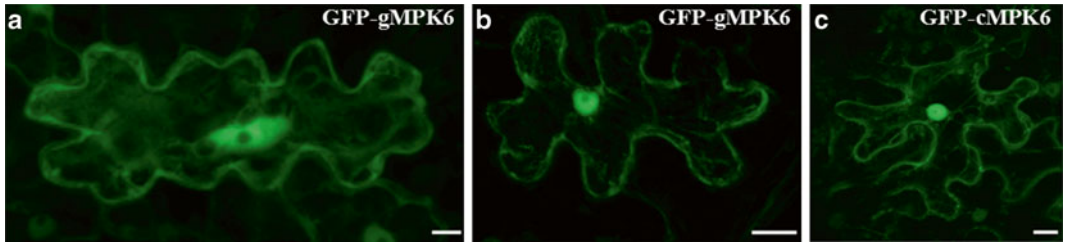


Fig 2 Transient transformation of *Arabidopsis thaliana* and *Nicotiana benthamiana*. (**a**) *Arabidopsis* cotyledon transiently expressing N-terminal fusion of AtMPK6 with GFP cloned from genomic DNA under its native promoter. (**b**) *Nicotiana* leaf transiently expressing N-terminal fusion of AtMPK6 with GFP cloned from genomic DNA under its native promoter. (**c**) *Nicotiana* leaf transiently expressing N-terminal fusion of AtMPK6 with GFP cloned from cDNA under its native promoter. Bars 5 μm (**a**), 20 μm (**b**, **c**)

designation, *see* also Fig. 1). Generated fusions are all driven by the native MPK6 promoter. So far, we have T2 generation of *Arabidopsis* plants expressing gMPK6-GFP. cMPK6-GFP, GFP-gMPK6, and GFP-cMPK6 were tested transiently (for examples, *see* Fig. 2). GFP fusions are versatile tools allowing further uses beyond in vivo localization. GFP-fusion proteins and interacting proteins can be pulled-down via co-immunoprecipitation assays using anti-GFP antibody or GFP-trap® and used for downstream biochemical analyses and proteomic identification of binding partners [18, 19].

This chapter describes how to prepare and test MAPK-FP harboring constructs driven under native promoters using MultiSite Gateway® technology system. We draw upon our experiences with cloning of MPK6 gene under its own promoter tagged with GFP. We aimed to divide the chapter into parts following the logical workflow starting from primer design and ending up with stable plant transformation.

2 Material

The laboratory should be equipped appropriately for molecular work including incubators operating at 25 °C (for *Agrobacterium tumefaciens*) or 37 °C (for *Escherichia coli*), temperature controlled shakers for liquid culture handling. All chemicals should be of analytical grade and dedicated to molecular work. Solutions should be prepared in ultrapure, RNase-free water (diethyl pyrocarbonate treated and autoclaved).

2.1 Multisite Gateway Cloning

1. PCR primers: Primers might be ordered from any supplier (*see* **Note 1**). All primers are dissolved in 100 μL of ultrapure water for molecular biology and stored at −80 °C (original tubes) or at −20 °C (working aliquots).
2. Cetyltrimethyl ammonium bromide (CTAB) isolation of genomic DNA from *Arabidopsis thaliana*: β-Mercaptoethanol (*see* **Note 2**), polyvinylpolypyrrolidone (PVPP) (*see* **Note 3**); chloroform (*see* **Note 2**), isoamyl alcohol (*see* **Note 2**), isopropanol, 80 % (v/v) ethanol (molecular biology grade), RNAse (10 mg/mL).
3. 1× TE buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0).
4. CTAB A solution (Applichem): 100 mM Tris–HCl (pH 8.0), 2 % (w/v) CTAB (20 g/L), 20 mM EDTA, 1.4 M NaCl.
5. CTAB B solution: 100 mM Tris–HCl (pH 8.0), 2 % (w/v) CTAB.

   All stock solutions should be filter sterilized.
6. PCR reaction and PCR product purification: *Arabidopsis* genomic DNA, proofreading DNA polymerase (Phusion High-Fidelity DNA polymerase, Thermo Scientific), dNTPs, betaine, DMSO, ultrapure water for molecular biology. Any kit for DNA extraction from agarose gels or Diffinity Rapid Tip 2 (Sigma-Aldrich).
7. Transformation of *E. coli* competent cells and propagation of pDONR™ vectors, destination vectors, and entry clones: *E. coli* competent cells DB3.1 (Life Technologies), *E. coli* competent cells TOP 10 (for detailed information about *E. coli* strain, *see* detailed protocol in Subheading 3).
8. Lysogeny Broth (LB) media (1 L): tryptone 10 g, yeast extract 5 g, NaCl 5–10 g, pH 7.0. For solid plates, add 1.5 % (w/v) Bacto Agar.
9. BP and LR recombinant reactions: Multisite Gateway kit (Life Technologies)—we are routinely using Multisite Gateway Three-Fragment Vector Construction Kit.

   Multisite Gateway vectors (Life Technologies, VIB-Ghent University) (*see* **Notes 4** and **5**)—for our purposes, we

used pDONR™ 221, pDONR™ 221 P4 P1r, pDONR™ 221 P2r-P3, pB7m34GW,0, pEN L1 F L2,0, pEN R2 F L3,0.

10. *E. coli* competent cells TOP10 for transformation of recombinant reactions.

2.2 Agrobacterium Handling

1. *Agrobacterium* culture
   Most *Agrobacterium tumefaciens* strains grow on minimal media containing yeast extract and carbon source. We use *A. tumefaciens* strain GV3101::pMP90 and LBA4404 (*see* **Note 6**) cultured at standard YEB media.
   YEB medium (1 L): 5 g beef extract, 1 g yeast extract, 5 g peptone, 5 g sucrose, 0.5 g $MgCl_2$, pH 7.0. Use 1.5 % (w/v) Bacto Agar for solid plates.
2. *Agrobacterium* transformation
   Agrobacterium competent cells, liquid nitrogen, YEB media, YEB plates
3. *Agrobacterium* storage
   50 % (v/v) glycerol, YEB media, deep freezer.

2.3 Transient Transformation of N. benthamiana Leaves

1. *Nicotiana benthamiana* plants.
2. Well-drained soil mix—2:1:1 mixture of sphagnum moss, 1 part of loam, 1 part of perlite.
3. Environmental chamber with programmable temperature.
4. Cocultivation media (filter sterilized): 10 mM MES, pH 5.6, 150 μM acetosyringone (*see* **Note 7**), 10 mM $MgCl_2$.
5. 2 mL needleless syringes.

2.4 "FAST" Transient Transformation of Arabidopsis Cotyledons

1. *Arabidopsis thaliana* plants, ecotypes Columbia or Wassilewskaja.
2. Well-drained soil mix—1:1:1 mixture of perlite, fine vermiculite, and sphagnum moss.
3. Environmental chamber with programmable temperature.
4. ½ MS medium (1 L): 2.15 g MS without vitamins (Duchefa), 1 % (v/v) sucrose, pH 5.8 (KOH). Use 0.8 % (w/v) Phytagel or 1 % (w/v) Phytoagar to prepare solid plates.
5. Cocultivation media (filter sterilized): 1.13 g/L MS without vitamins, 1 % (w/v) sucrose, 100 μM acetosyringone, 0.005 % (v/v) Silwet L-77, pH 6.0 (KOH).
6. Washing solution (filter sterilized): 10 mM $MgCl_2$, 100 μM acetosyringone.
7. Surface sterilization solution (filter sterilized): 0.05 % (w/v) sodium hypochlorite (*see* **Note 8**).

3 Methods

Most of the Multisite Gateway cloning methods were performed according to manufacturer's instructions (Life Technologies). Here, they are only briefly described with main emphasis on troubleshooting.

3.1 Designing attB PCR Primers

When designing PCR primers for recombinant reactions during Gateway cloning, it is necessary to incorporate *att*B sites upon primer design. In the case of Multisite Gateway Three-Fragment Vector Construction Kit, following PCR products will be flanked with three types of slightly different recombination sites depending on which DNA element will be cloned (Table 1). Rules for designing primers are step by step described in the Multisite Gateway manuals guiding customer to prepare three pairs of primers. Resulting PCR products are prepared to be recombined during BP reaction with corresponding pDONR™ vectors, as summarized in the Table 1. Recombination products after BP reaction are further called entry clones.

In general, primers for Gateway cloning are long with tendency to form hairpins and primer dimers in the PCR reaction. These secondary structures decrease specific product quantity and moreover primer dimers might recombine with pDONR™ vectors and give background after BP recombination (*see* Subheading 3.4).

For a native promoter sequence (5′ element) it is desirable to design complementary part of primers as follows: Most of the plant promoter activity has been described and proven immediately upstream of the transcriptional initiation. If the native promoter sequence of respective gene of interest is not characterized or experimentally proven, the forward primer should be situated up to 2 kp upstream from ATG codon of such gene of interest (*see* **Note 9**). Reverse primer should be situated just upstream from ATG codon of the gene of interest. Link complementary primers to *att*B sites as described in the Life Technologies manual for Multisite Gateway cloning.

Table 1
In this table, components for BP reaction are summarized. DNA elements are chosen as an example for cloning C- and N-terminal GFP fusion to your gene of interest

Forward *att*B primers	Reverse *att*B primers	DNA element	pDONR vector
*att*B4	*att*B1r	5′ element	pDONR™ 221 P4-P1r
*att*B1	*att*B2	gene or GFP	pDONR™ 221
*att*B2r	*att*B3	GFP or gene	pDONR™ 221 P2r-P3

3.2 CTAB Isolation of Genomic DNA from A. thaliana (See Note 10)

1. Add to CTAB A solution 2 % (v/v) β-mercaptoethanol and 1 % (w/v) PVPP (PVPP is optional, *see* **Note 11**), preheat the solution in the water bath to 55–65 °C (*see* **Notes 12** and **13**).
2. Homogenize 50–100 mg of fresh weight leaves to a fine powder in liquid nitrogen and put the powder into 2 mL prelabeled Eppendorf tubes (*see* **Note 14**).
3. Bind DNA to CTAB by adding 0.8 mL preheated CTAB A solution (including β mercaptoethanol and PVPP), incubate in 55–65 °C water bath for 90 min, and occasionally gently shake. After incubation, spin the cell debris by centrifugation at 12,000 × *g* for 10 min.
4. Add 800 μL of chloroform–isoamyl alcohol (24:1) and mix by gently shaking for 10–15 min. Centrifuge at 12,000 × *g* for 15 min.
5. Collect aqueous phase to the fresh 2 mL Eppendorf tube. To prevent collection of chloroform phase, leave around 1 mm of aqueous phase above the chloroform phase. Estimate volume of collected aqueous phase and add CTAB B buffer in the ratio 1:1(v/v; *see* **Note 15**)
6. (optional) Add 800 μL of chloroform–isoamyl alcohol (24:1) and mix by gently shaking 10 min. Centrifuge at 12,000 × *g* for 15 min (*see* **Note 16**). Collect aqueous phase and estimate the volume.
7. Add 60 % volume of isopropanol (room temperature), mix by inverting tubes (10–15 min), and centrifuge at 12,000 × *g* for 10 min.
8. Discard isopropanol supernatant carefully, not to dislodge the pellet.
9. Add 80 % (v/v) ethanol, invert tubes 5–10 times, centrifuge at 6,500 × *g* for 5 min. Repeat this step twice. Be careful not to dislodge the pellet.
10. Invert the tube and let it dry upside down on a clean filter paper for 10–15 min (*see* **Note 17**). If necessary, continue with drying upright (cover the tubes with filter paper).
11. Hydrate the pellet with 30–50 μL of the TE buffer supplemented with RNAse (10 μg/mL) (*see* **Notes 18** and **19**). Incubate at room temperature overnight. Store the DNA at −20 °C.

3.3 Performing PCR Reactions

1. This step depends on DNA polymerase used for proofreading. We are routinely using Phusion High Fidelity DNA polymerase (New England Biolabs) and follow the general protocol (Table 2) (*see* **Note 20**).

Table 2
General PCR conditions set up for Phusion High Fidelity DNA polymerase (New England Biolabs) (*see* Note 21)

Component	Final concentration
5× High-Fidelity buffer	1× HF buffer
10 mM dNTPs (2.5 mM each)	200 μM dNTPs (*see* **Note 16**)
DMSO (optional)	1.3 %
Betaine 5 M (optional)	1.3 M
100 μM forward primer	0.5 μM (*see* **Note 16**)
100 μM reverse primer	0.5 μM (*see* **Note 16**)
PCR template	50–250 ng/50 μL (*see* **Note 16**)
Phusion High-Fidelity DNA polymerase	1 U/50 μL
Nuclease-free water	up to final volume

2. Set up the thermocycling conditions for PCR according to DNA polymerase manufacturer's instructions taking into consideration the length of your final PCR product.

3.4 PCR Product Purification

This step is very critical for further success in the BP recombination reaction. As mentioned above, *att*B primers are due to their length prone to form dimers and hairpins. These secondary structures can compromise the specific output of the PCR reaction. Moreover, primer dimers may cause high amounts of background clones after BP recombination reaction (*see* **Note 22**). Thus, it is very important to purify the PCR product before BP recombination reaction. For this purpose any commercially available gel extraction kit may be used. A common problem arising from using gel extraction kits concerns the low final concentration of the purified DNA. However, this low yield is compensated by the acquisition of really clean DNA excluding nonspecific PCR products (*see* **Note 23**).

3.5 Transformation of E. coli Competent Cells and Propagation of pDONR™ and Destination Vectors

Transformation of *E. coli* competent cells is carried out using a heat-shock transformation protocol. It is necessary to use *E. coli* strains carrying resistance to ccdB for propagation of pDONR™ vectors or empty destination vectors with selection for ccdB gene. Such strains include DB3.1 or One ShotRccdB Survival T1R (both from Life Technologies). Conversely, for transformation of entry clones or destination vectors with genes of interest, it is necessary to use *E. coli* strains lacking resistance to ccdB gene in order to avoid background of non-transformed cells (*see* **Note 24**).

1. Thaw *E. coli* competent cells on ice (100 μL per tube).
2. Add max 1–5 μL of the standard Miniprep or 5–10 μL of BP/LR reaction mix gently by finger-tapping the Eppendorf tube.
3. Incubate on ice with occasional mixing for 30 min.
4. Heat-shock the cells at 42 °C for 50 s and place the tubes immediately on ice for at least 2 min.
5. Add 1 mL of LB to each tube.
6. Incubate for 2 h at 37 °C and shake vigorously.
7. Pellet the cells (6,000 × *g*, 1 min) and remove most of the supernatant.
8. Resuspend the cell pellet in the rest of the medium and plate it on LB agar plate containing the appropriate antibiotic.
9. Incubate the plates at 37 °C overnight.

3.6 Performing BP and LR Recombinant Reactions

Perform the BP and LR recombination reaction according to manufacturer's instructions. Each step is clearly explained in the manual for Multisite Gateway cloning. Transforming of both reactions to *E. coli* competent cells is described in previous section. Propagate prepared constructs and verify them by PCR and by restriction digest (*see* **Note 25**).

3.7 Freeze–Thaw Shock Method for Agrobacterium Transformation (See Note 26)

1. Remove *Agrobacterium* competent cells from deep freezer and let them thaw on ice for few minutes. Use 1 Eppendorf tube with 100 μL of competent cells per one transformation reaction.
2. Add 1–10 μg of plasmid DNA (standard Miniprep DNA) and mix by tapping with your finger. Incubate on ice for 10 min, occasionally mix by tapping.
3. Freeze the mixture in the liquid nitrogen for 5 min and let it thaw in the 37 °C water bath for 5 min.
4. Add 1 mL of YEB medium to each tube, mix by inverting and incubate with shaking at 28 °C for 2–4 h (*see* **Note 27**).
5. Pellet the cells by brief spinning in the minicentrifuge and plate them on solid YEB medium supplemented with appropriate antibiotics. Incubate at 28 °C for 24–48 h.
6. Test the presence of your plasmid in the colonies by standard procedures: Miniprep isolation of DNA followed by PCR, colony PCR or restriction digestion. Usually, rather low amount of DNA is isolated from *Agrobacterium tumefaciens* by Miniprep technique. Take it into account during following procedures. To increase the yield of the plasmid DNA, it is recommended to prolong cell lysis over 5 min during Miniprep procedure.
7. Make a stock solution (*see* **Note 28**). Store bacteria in the liquid YEB medium supplemented with 25 % (v/v) glycerol at −80 °C (*see* **Note 29**).

3.8 Transient Leaf Transformation of N. benthamiana Leaves by Agroinfiltration [20]

Nicotiana plants should be grown at 22 °C with 16 h light–8 h dark period. 6-week-old plants are suitable for transformation. One day before agroinfiltration, water *Nicotiana* plants properly to achieve opening of stomata. Water them also 2 h before the procedure to keep the stomata opened.

1. Inoculate 20 μL of frozen glycerol stock into 2 mL YEB medium with appropriate antibiotics. Incubate with shaking (250 rpm) at 28 °C overnight.
2. Inoculate 5 mL of fresh YEB medium with overnight saturated bacterial culture (typical inoculation is 1:100) and incubate with shaking at 28 °C up to OD_{600} 0.2–0.3.
3. Centrifuge at 3,000 × *g* for 6 min, discard the supernatant and resuspend the pellet in 5 mL of cocultivation media solution (*see* **Note 30**). Incubate at the room temperature for 2 h.
4. Fill 2 mL needleless syringe body with *Agrobacterium* solution. Infiltrate the solution by gently pressing the tip of syringe against the stomata side of the leaf. Make few spots (infiltrated area around the spot is 3–4 cm^2) per leaf separated by veins. Use one construct per one leaf. Mark infiltrated areas of leaves with waterproof marker.
5. Cover the infiltrated plants with transparent plastic bag to maintain a wet growing chamber and incubate at the room temperature for 24 h before observing in the microscope (*see* **Note 31**).

3.9 Testing the Construct by Transient Cotyledon Transformation of A. thaliana: FAST Method [21, 22]

Arabidopsis thaliana plants are grown in vitro on sterile half-strength Murashige and Skoog (1/2 MS) media without vitamins with 16 h light–8 h dark period.

1. Inoculate 20 μL of frozen glycerol stock of respective *Agrobacterium* clone into 2 mL of YEB medium with appropriate antibiotics. Incubate overnight at 28 °C, shake at 250 rpm.
2. Dilute the overnight saturated culture to OD_{600} 0.3 with the fresh YEB medium. Shake 10 mL of diluted *bacteria* solution up to $OD_{600} > 1.5$.
3. Pellet the cells by centrifugation at 4,700 × *g* for 10 min. Resuspend the pellet in 10 mL of the washing solution and repeat the centrifugation. Resuspend pellet in 1 mL of washing solution and measure OD_{600} (*see* **Note 32**).
4. Put forty 4 days-old *Arabidopsis* seedlings into 20 mL of cocultivation medium in the round Petri dish (10 cm in diameter) (*see* **Notes 33** and **34**).
5. Add Agrobacterium suspension to the plants in the cocultivation medium up to OD_{600} 0.5. Incubate the Petri dish in the dark in the plant growing chamber for 36–40 h (*see* **Note 35**).

6. After cocultivation period replace cocultivation medium by surface sterilization solution and incubate at the room temperature for 10 min (*see* **Note 36**). Wash three times with sterile tap water and one time with sterile ½ MS medium and proceed to microscope observation. Seedlings can be also transferred on solid ½ MS media for few days prior to observation (*see* **Notes 37** and **38**).

3.10 Floral Dip Stable Transformation of A. thaliana

In the case that above mentioned transient transformation assays provide sufficient fluorescence signal, you can proceed to stable transformation of *A. thaliana*. As floral dip transformation is widely used and well described [23, 24], we do not provide a detailed description here (*see* **Note 39**).

4 Notes

1. When primers exceed the length of 50 bp, it is recommended to use HPLC purification.
2. Work in the fume hood.
3. PVPP and β-mercaptoethanol are added to remove phenolic compounds from the plant extract.
4. Multisite Gateway binary destination vectors [12] and entry clones [10] for plant use are available from VIB-Ghent University (http://gateway.psb.ugent.be/search).
5. Before ordering mRFP based vector from VIB-Ghent University, Dr. Roger Tsien from University of California in San Diego should be first contacted (http://www.tsienlab.ucsd.edu/Samples.htm) for signing individual Material transfer agreement.
6. Both strains carry chromosomal resistance for rifampicin. GV3101::pMP90 has gentamicin resistance on Ti plasmid, while LBA4404 carries resistance for streptomycin. Rifampicin can be used in final dilution at 50 μL/mL, streptomycin at 30 μL/mL and gentamycin at 25 μL/mL.
7. Dissolve the stock solution of acetosyringone in DMSO or in ethanol. Store in the refrigerator for maximum of 14 days.
8. We use sterile tap water for the solution.
9. For cloning of *Arabidopsis* genes, use Sequence viewer in the TAIR database, http://www.Arabidopsis.org/. While opening the Sequence viewer, click on the Sequence ruler to open 10 kb sequence window).
10. Work in the fume hood; discard all solutions and materials according to institutional safety regulations for toxic waste disposal.

11. PVPP is not soluble. If you decide to use it, add it to each tube separately.
12. Once β-mercaptoethanol is added to the extraction buffer, the buffer is stable for max. 3 days.
13. Do not vortex the solution as the detergent should not foam.
14. Never allow thawing of powdered plant material—keep it continuously frozen under liquid nitrogen.
15. CTAB B buffer dilutes the salt in the sample by 50 %. This step is beneficial for the following isopropanol DNA precipitation.
16. This step is optional. It should be implemented if the aqueous phase is not translucent to allow for removal of undesirable protein contamination.
17. Do not let the pellet to overly dry as this will impede its dissolution while it is likely to fall off the tube.
18. RNAse stock solution is stable at the room temperature for 2 years.
19. TE buffer supplemented with RNAse should be stored in refrigerator (4–8 °C).
20. Betaine and DMSO are not necessarily but recommended PCR additives. They act by unfolding DNA secondary structures and facilitate PCR reactions with GC rich DNA templates. However in the presence of betaine and DMSO the melting temperature of the primers is significantly decreased. Thus, the annealing temperature of the primers should be properly adjusted. Moreover, DMSO at 10 % (v/v) concentration inhibits up to 50 % of DNA polymerase activity. For this reason the concentration of the DNA polymerase should be increased accordingly.
21. High concentrations of dNTPs, primers, and genomic DNA may inhibit PCR reaction.
22. When primers are in the dimer oriented with their 3′ ends against each other even with few nucleotides, resulting primer dimer is amplified during PCR reaction and provides substrate for recombination (Fig. 3).
23. In order to obtain specific PCR product it is recommended to change annealing temperature, and other PCR parameters. In this case, the BP recombination reaction can be directly applied. Alternatively use Diffinity RapidTipTM for DNA purification (Sigma-Aldrich). With this method, primer dimers and residual DNA polymerase will be removed.

Fig. 3 Example of primer dimer forming false substrate for BP recombination reaction

24. ccdB gene in pDONR™ vectors and empty destination vectors are prone to mutations. Such mutations might be propagated in standard *E. coli* strain and give false positive results after BP or LR reactions. When generating new stocks of Gateway plasmids, their growth inability should be tested in standard *E. coli* strains without resistance to ccdB effect.
25. It is highly recommended to sequence entry clones and destination vectors. Only sequencing, will reveal mutations caused by DNA polymerase errors. Likewise junctions in recombination sites originating after BP and LR reaction can be verified.
26. This method is not very effective and it yields few positive colonies. However, it is quick and not demanding.
27. The mixture can be also incubated at room temperature.
28. 300 μL of fresh overnight culture and 300 μL sterile 50 % (v/v) glycerol.
29. Label your tubes properly and make back up stocks.
30. The filter-sterilized washing solution can be stored at 4 °C in the absence of acetosyringone. Before use, acetosyringone should be freshly added.
31. While observing the leaf under the microscope, there are usually autofluorescent dead cells in the middle of the spot caused by syringe injury of the leaf surface. Use surrounding cells to observe transient transformation results.
32. For OD_{600} measuring, dilute the *bacteria* solution 10 times with washing solution not to waste the material and after measurement, calculate the OD_{600} of the original suspension.
33. Take care during handling of the seedlings and allow them to acclimate in the cocultivation media for at least 30 min.
34. This can be practiced with smaller volumes taking into account the number of seedling accordingly. Use appropriate cultivation plates (6-wells, 12-wells, 24-wells etc.) for smaller volumes.
35. Longer cocultivation period with *Agrobacterium tumefaciens* compromises viability of seedlings.
36. Carefully remove bacteria from the surface of the seedlings. Be careful while manipulating the plants because after cocultivation they are soft and might be easily damaged.
37. Before proceeding to microscope documentation, plants must be acclimated in liquid ½ MS media for at least 1 h.
38. Prepare the microscope slide chamber as follows: space a coverslip and a microscope slide with two narrow strips (ca. 3 mm each) of double-sided adhesive tape or Parafilm. Accommodate the width of the chamber according to the width of the coverslip. Pipette one drop of liquid ½ MS medium in the chamber

and mount the seedling into this drop. Cover the sample with the coverslip. Do not press the coverslip too much to avoid damage of the seedling.

39. Repeat the floral dipping twice within 1 week.

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Chapter 12

Bimolecular Fluorescent Complementation (BiFC) by MAP Kinases and MAPK Phosphatases

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Abstract

The adaptation of plants to the environment is a key property for survival. Adaptation responses to environmental cues are generated in cells by signaling initiated from cell receptors. Signal transduction is based on protein phosphorylation that is employed in mitogen-activated protein kinase (MAPK) cascades to integrate signals from receptors to cellular responses. MAPK activity is determined by phosphorylation of amino acid residues within the kinase activation loop and their dephosphorylation by phosphatases is essential to control signal duration and intensity.

Monitoring protein–protein interactions (PPIs) of MAPKs with MAPK phosphatases in vivo provides valuable information about specificity and intracellular localization of the protein complex. Here, we report studying PPIs between *Arabidopsis* MAPKs and PP2C-type MAPK phosphatases using bimolecular fluorescent complementation (BiFC) in suspension cell protoplasts. The interactions of the MAPKs MPK3, MKP4 and MPK6 with the phosphatases AP2C1 and AP2C3 have been tested.

Key words *Arabidopsis*, BiFC, Localisation, PP2C, MAPK phosphatase, Protein interaction

1 Introduction

1.1 Development of BiFC

Bimolecular fluorescence complementation (BiFC) has emerged as one of the most commonly used techniques to visualize protein–protein interactions (PPIs) [1]. The principle of BiFC is based on the reconstitution of a fluorescent complex by two non-fluorescent fragments of a fluorescent protein (e.g., GFP or its derivate YFP) where these fragments are fused to a pair of interacting proteins. BiFC enables not only the detection of PPIs but importantly also the determination of the subcellular localization of the interacting proteins.

Numerous methods have been developed to detect and verify PPIs, including yeast two hybrid assay (Y2H), immunoprecipitation (IP), Förster resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), and BiFC.

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The study of protein complementation started decades ago demonstrating reassociation of enzyme fragments in vitro [2] followed later by the rescue of inactive beta galactosidase mutants by protein fragments [3] which lead to the widely implementation of alpha-complementation as *Escherichia coli* transformation screening method in molecular cloning [4, 5].

Complementation of fluorescent proteins was first performed using the green fluorescent protein (GFP) and its derivative YFP (yellow fluorescent protein) [6] and was later expanded to other fluorescent proteins capable for protein complementation [7]. The split site for GFP and its variants is usually between the 7th and 8th ß-strands, creating new N-terminal (amino acids 1–155) and C-terminal (amino acids 156–239) non-fluorescent fragments. Also the split between residues 174 and 175 has been successfully used with an YFP variant resulting in BiFC [8].

The latest technical advance in BiFC research is the implementation of multicolor BiFC to examine complex formation of a protein with different interacting partners [9, 10].

1.2 Advantages and Disadvantages of BiFC

The major advantages of BiFC are its fast and easy performance; the technique is noninvasive and does not require application of external agents. Furthermore, the localizations of protein complexes can be visualized in the respective cellular compartments where the association takes place, avoiding creation of false positive results in case differently localized proteins are brought together during cell lysis (e.g., required during a Co-IP experiment). Analysis of BiFC can be performed in the homologous host system, so false-positive and false-negative interactions observed in heterologous expressions systems (e.g., Y2H) can be avoided [11]. BiFC requires relatively low protein expression levels enabling expression of investigated protein partners from their own promoter regions. The BiFC assay is sensitive, enables the detection of weak and transient expressions and exhibits very low background signal.

Drawbacks using BiFC are the formation of irreversible complexes preventing analysis of dissociation dynamics [6], a slow maturation of the reconstituted fluorescence protein (exception: the Venus variant of YFP), which hampers observation of changes in PPI dynamics in real time. Also very high expression levels of the fluorescent protein fragments may lead to unspecific interactions [12].

1.3 Application of BiFC

BiFC complexes have been visualized in *E. coli*, *Agrobacterium tumefaciens*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, and mammalian cells [1, 7].

The BiFC application in plants was first reported using transient expressions in onion epidermis or tobacco leaves by co-bombardment [13], infiltration of Agrobacteria harboring

binary expression vectors into leaves of *Nicotiana benthamiana* and *Arabidopsis* [14, 12], and by transient expression in protoplasts [12]. Studying PPIs in plant research using BiFC has been widely spread [1, 15, 16], including successful applications in cell signaling research [17–19].

1.4 Regulation of MAPK Signaling by MAPK Phosphatases

The transmission of environmental stimuli into cellular responses is mediated by signal transduction that involves reversible protein phosphorylation. Sequential phosphorylation of mitogen-activated protein kinase (MAPK) cascades enables rapid signal transmission, where activated (phosphorylated) MAPKs translocate from the cytoplasm into the nucleus to induce cellular responses [20, 21]. MAPK activation occurs in a transient manner, where MAPK phosphatases (MKPs) ensure proper duration and amplitude of the signal. In vertebrates mainly members of the dual-specificity phosphatases (DSPs) have been described as MKPs [22], however, in plants beside DSPs also PP2C-type Ser/Thr protein phosphatases have been identified to regulate MAPK activity [23]. Plant DSP-type phosphatases are outnumbered by type 2C (PP2Cs) family members [24, 25] and currently several members of *Medicago* and *Arabidopsis* PP2Cs have been characterized as MAPK phosphatases [26–28].

1.5 Protein–Protein Interactions of MAPK Signaling Components

PPIs in signal transduction pathways are considered as weak and transient, giving visualization of stabilized complexes by BiFC an exceptional opportunity to define the cellular compartment where the interaction takes place.

In plants interaction between DSP MKPs and MAPKs has been tested using BiFC in transiently transformed mustard hypocotyl cells [29] and by co-infiltration of *Nicotiana benthamiana* leaves with Agrobacteria [17].

In this report we detail the PPI analysis by BiFC using *Arabidopsis* MAPKs MPK3, MPK4 and MKP6 with the PP2C-type phosphatases AP2C1 and AP2C3 [27, 28]. The experiment has been performed using *Arabidopsis* cell suspension protoplasts grown in darkness. This cultivation method facilitates detection of the fluorescent protein as autofluorescence of the cells is strongly diminished.

Expression of the protein kinase and phosphatase proteins with YFP components was regulated by different versions of constitutive CaMV 35S promoter: the most commonly used version (0.8 kb fragment; 35Slong), the 0.4 kb 35S promoter of the pRT100 vector series [30] and an enhanced/doubled 35S promoter fused to a translational leader sequence (35S-TL) [31]. We found that expression of the YFP components alone driven by enhanced 35S promoter in combination with excessive amounts of transformed plasmid DNA lead to detection of YFP fluorescence (data not shown), therefore, selection of proper controls and

adjusting the plasmid DNA amounts for transformation are crucial for correct interpretation of the obtained results.

To test interaction between two proteins by BiFC, eight distinct combinations of N- or C-terminally tagged YFP fragments are possible [1], so the cloning strategy should be chosen carefully, since not all of the combinatorial protein pairs may lead to reconstitution of YFP. In our experimental set-up AP2C1 C-terminally tagged with either YFP fragment did not lead to fluorescence complementation with MAPKs (data not shown), however fusing AP2C1 with N-terminal part of either YFP fragment (YFPntd or YFPctd) lead to detection of YFP fluorescence tested with MPK6 or MPK4 (Fig. 1). Transformations of negative control plasmids (combination of pUC-SPYNE with pUC-SPYNCE) or transformation of one control plasmid together with the corresponding MAPK or PP2C BiFC expression vector did not lead to detectable YFP fluorescence (data not shown). The different promoter versions lead to comparable results of BiFC under conditions tested. However, varying intensities of reconstituted YFP protein fluorescence have been observed most probably due to different regulatory capacities of promoters (Fig. 1).

2 Materials

2.1 Plant BiFC Vectors (See Table 1)

In this work plasmids containing YFP components derived from cloning vectors originally developed by Harter and Kudla laboratories [12] have been used. In this work Expression plasmids for BiFC were created using conventional restriction enzymes. Several sources of Gateway-compatible BiFC vectors are also available [32–35]. Practically, binary BiFC vectors can be used both for transient and stable transformations, although latter have so far not been reported for successful creation of stably supertransformed plants harboring two or more expression cassettes.

Plasmids used in our experiments for transient expressions in suspension cell protoplasts are based on vectors pRT100 [30] and pGreenII [36] (*see* **Note 1**).

2.2 Cultivation of Arabidopsis Cell Suspension

1. *Arabidopsis* cell suspension medium (1 L): 4.4 g MS including B5 vitamins, 30 g sucrose, 1 mg 2,4-D, pH 5.7 (KOH), autoclaved.

2.3 Isolation of Protoplasts from Arabidopsis Suspension Cells

1. B5-S (1 L): 0.28 M sucrose, 3.16 g B5, pH 5.5 (KOH), autoclaved.
2. B5-GM (1 L): 0.34 M glucose, 0.34 M mannitol, 3.16 g B5, pH 5.5 (KOH), autoclaved.
3. Enzyme solution: 1 % (w/v) cellulase, 0.2 % (w/v) macerozyme in B5-GM (filter sterilized) (*see* **Note 2**).

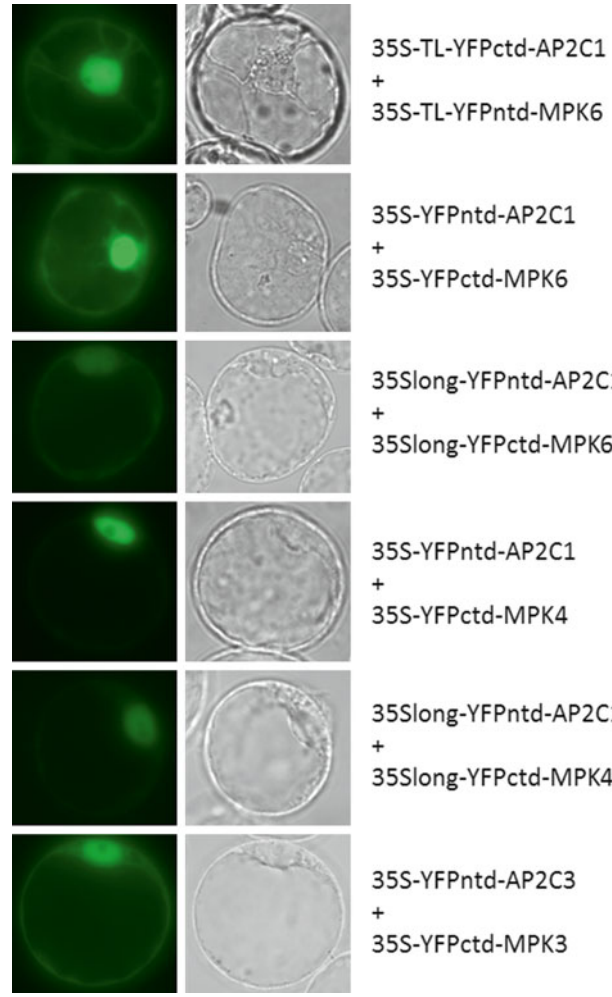

Fig. 1 Intracellular localization of AP2C1/MPK4, AP2C1/MPK6, and AP2C3/MPK3 by bimolecular fluorescent complementation (BiFC) in *Arabidopsis* protoplasts. The BiFC components (YFPntd or YFPctd) were fused N-terminal to MAPKs (MPK3, MPK4, and MPK6) and PP2C phosphatases (AP2C1, AP2C3) and indicated plasmids coexpressed under control of different CaMV 35S promoter versions. The subcellular localization of the interaction is visualized using fluorescence microscopy, the original yellow fluorescence of reconstituted YFP is converted to green signal for better definition in RGB acquisition mode

4. 0.275 M $Ca(NO_3)_2$, pH 5.6 (KOH), autoclaved.
5. Polyethylene glycol (PEG) 6000 solution: 25 % (w/v) PEG 6000, 0.45 M mannitol, 0.1 M $Ca(NO_3)_2$, pH 9.0 (KOH) (*see* **Note 3**).
6. Falcon 50-mL tubes.
7. Large petri dishes.
8. Round-bottom 12-mL tubes.

Table 1
BiFC vectors used for transient expressions

Plasmid	Notes	Cloning	References
pRT100-35Slong-YFPctd-AtMPK6[a]	Multicopy vector	MCS	Unpublished
pRT100-35S-YFPctd-AtMPK6	Multicopy vector	MCS	[27]
pGreenII0229-35S-TL-YFPntd-AtMPK6[b]	Binary vector	MCS	Unpublished
pRT100-35Slong-YFPctd-AtMPK4[a]	Multicopy vector	MCS	Unpublished
pRT100-35S-YFPctd-AtMPK4	Multicopy vector	MCS	[27]
pRT100-35S-YFPctd-AtMPK3	Multicopy vector	MCS	[28]
pGreenII0029-35S-TL-YFPctd-AP2C1[b]	Binary vector	MCS	Unpublished
pRT100-35Slong-YFPntd-AP2C1[a]	Multicopy vector	MCS	Unpublished
pRT100-35S-YFPntd-AP2C1	Multicopy vector	MCS	[27]
pRT100-35S-YFPntd-AP2C3	Multicopy vector	MCS	[28]
pUC-SPYNE	Multicopy vector	MCS	[12]
pUC-SPYCE	Multicopy vector	MCS	[12]

Abbreviations: *35S* CaMV 35S promoter, *YFPctd* YFP C-terminal domain, amino acids 156–239, *YFPntd* YFP N-terminal domain, amino acids 1–155
[a]35S promoter (0.8 kb) from pUC-SPYCE [12]
[b]Double 35S promoter with translational leader sequence from tobacco etch virus

9. Eppendorf tubes.
10. Rosenthal chamber (Hemocytometer) for counting protoplasts.
11. Inverted light microscope.
12. Rotating platform.
13. Fluorescence microscope.
14. Centrifuge with swing out rotor.

3 Methods

The method of *Arabidopsis* cell suspension protoplast transformation has been originally developed for Parsley (*Petroselinum crispum*) cells in the Dierk Scheel laboratory [37] and modified for *Arabidopsis* cells in our laboratory. The protocol has been described previously [38] and amended for the BiFC assay.

3.1 Creation of BiFC Vectors for Testing MAPK Kinase/Phosphatase Interaction in Protoplasts

In this work the N-terminal and C-terminal YFP fragments have been amplified with by PCR as *BspHI/NcoI* fragments from pUC-SPYNE and pUC-SPYCE [12]. PCR fragments have been inserted in pRT100 and pGreenII expression vectors carrying MAPKs or PP2Cs at *NcoI* sites encoding the ATG translational start codon [27, 28]. The different 35S promoter versions have been recloned

from pRTL2-GUS (35S-TL) and pUC-SPYCE (35Slong) [12, 31]. pUC-SPYNE and pUC-SPYCE vectors were used as negative controls.

3.2 Maintenance of Arabidopsis Cell Suspension

1. *Arabidopsis* suspension cells are diluted weekly 5× in *Arabidopsis* cell suspension medium (5 mL cells diluted with 20 mL medium) in 250 mL Erlenmeyer flasks under sterile conditions and propagated on a shaker at 150 rpm in darkness at 22 °C (*see* **Note 4**).

3.3 Isolation of Protoplast from Arabidopsis Suspension Cells

1. Collect 4–5-days-old *Arabidopsis* cell cultures.
2. Spin 25 mL of culture 5 min at 250 × *g* in a 50 mL Falcon tube (*see* **Note 5**)
3. Discard the supernatant by decanting (*see* **Note 6**).
4. Add 25 mL of enzyme solution to the cell pellet, resuspend gently, and fill up to 50 mL with B5-GM medium (*see* **Note 7**).
5. Split the content and place 25 mL each into two large petri dishes.
6. Add 25 mL of B5-GM medium to each petri dish.
7. Place petri dishes on the rotating platform for ~3 h (*see* **Note 8**) covered with aluminum foil.
8. Transfer the content from each dish to 50-mL Falcon tubes (*see* **Note 9**).
9. Spin the protoplast/enzyme solution in Falcon tubes for 5 min at 150 × *g* (*see* **Note 10**).
10. Decant the supernatant (*see* **Note 11**) and resuspend the pellet in 15 mL B5-S medium by carefully shaking the tube. Fill up to 35–40 mL with B5-S medium
11. Spin the tubes for 8 min at 70 × *g* (*see* **Note 12**), transfer the floating cells with a plastic wide-mouth Pasteur transfer pipette (~2–3 mL) to a new 15 mL round bottom tube, and fill up to ~10 mL with B5-S medium. Mix the cells carefully by gentle shaking the tube.
12. Spin the 15 mL tubes for 5–7 min at 70 × *g*.
13. Transfer the floating protoplasts with a plastic wide-mouth Pasteur transfer pipette to a new 15 mL Falcon tube. The protoplasts yield should be ~2–3 mL.
14. Check protoplast quality with light microscope and determine concentration of protoplast (cells/μL) using a Rosenthal chamber (*see* **Note 13**). The final protoplast concentration should be adjusted to 4×10^6 cells/mL with B5-S medium (*see* **Note 14**).

3.4 Protoplast Transformation with BiFC Plasmids

1. Use 2 μg of each plasmid per tube for transformation. Set the final DNA volume to 15 μL using sterile water in Eppendorf tubes (*see* **Note 15**).
2. Add 50 μL protoplasts ($1–2 \times 10^5$ cells) per tube, and mix by carefully ticking against the tube with the index finger (*see* **Note 16**).
3. Add 150 μL PEG solution immediately and mix well by ticking against the tube several times till the mixture appears homogenous (*see* **Note 17**).
4. Incubate the suspension 5–15 min at room temperature.
5. Add stepwise 0.275 M $Ca(NO_3)_2$ (twice 0.5 mL) to dilute PEG and mix well by inverting the tubes several times.
6. Spin tubes 7 min at $70 \times g$.
7. Remove the supernatant (*see* **Note 18**).
8. For protoplast cultivation add 0.5 mL of B5-GM and store the tube horizontally 3–14 h.

3.5 Detection of MAPK: MAPK Phosphatase Complexes by BiFC in Arabidopsis Suspension Cell Protoplasts by Fluorescence Microscopy

1. Collect protoplasts for observation of BiFC using a fluorescence microscope (*see* **Note 19**).
2. Detect reconstituted YFP expression by standard epifluorescence microscope.
3. Acquire images.

4 Notes

1. All plasmids were isolated using standard DNA isolation procedures. Plasmid DNA purification via column-based solid phase extraction is required for successful protoplast transformation. Purification columns are standard components in commercial DNA extraction kits. The stock plasmid DNA concentration is set to 1 μg/μL.
2. Fresh enzyme solution has to be prepared and stirred slowly for 1 h followed by filtering through Whatman paper and filter sterilization.
3. Stirring the PEG solution overnight is recommended; adjust the pH again before filter-sterilization.
4. Dilution rate depends on the quality of the suspension culture; use 1–3 dilution for slower growing cells.
5. Centrifugation steps in this protocol should be performed only with swing out rotor (e.g., Eppendorf Centrifuge 5810 R).

Centrifugation of suspension cells yields in approx. 10–5-mL pellets. At this step deceleration of the centrifuge may be applied using the slow brake.

6. Decant the supernatant in one movement to avoid loosening the pellet.
7. Resuspend the pellet by gentle turning the tube. You may use (electric powered) pipettes at this step but without conus to avoid damage of the cells.
8. Rotate petri dishes slowly at 30–40 rpm/min and check cells under the inverted light microscope every 30 min to decide on harvesting time of protoplasts. If approx. 80 % of the cells show a round shape (indicating that cell walls are digested), begin with the protoplast isolation procedure.
9. Pour solution gently directly from plates into Falcon tubes or use pipettes only without conus for cell transfer.
10. From this step onwards centrifugation of protoplasts should decelerate without brake.
11. Decant carefully the supernatant with one movement and watch the protoplast pellet. If the pellet loosens again (e.g., because the decantation was interrupted) repeat this centrifugation step with B5-GM medium. Here it is important to remove as much enzyme solution as possible. An electric pipette can be used also but it is more time consuming than manual decanting.
12. Intact protoplasts should float
13. Protoplasts should be single (not clumped together) and fully rounded. Calculation of protoplast concentration per microliter: number of protoplasts in 16 small squares $\times 5 \times 10^3$ (chamber with depth 0.2 mm, surface area of smallest square 0.0625 mm^2).
14. The transformation efficiency decreases with storage time due to new cell wall synthesis, therefore protoplasts have to be stored at 4 °C for transformation on the same day or stored overnight. Storage for 2–3 days is sometimes possible, dependent on the experiments.
15. Plasmid DNA was extracted and purified from *E. coli* using commercial isolation kits (Qiagen or Genomed) and the DNA concentration adjusted to 1 μg/μL with sterile water. 2 μg of each BiFC expression plasmid was used per transformation per eppendorf tube. Prior to transformation with BiFC plasmids it is recommended to perform a control transfection with a plasmid expressing full length YFP or GFP to determine the transformation efficiency. Only protoplasts isolates exhibiting >50 % transformation efficiency (= number of cells showing YFP fluorescence) should be considered for BiFC experiments.

It was observed that the transformation efficiency decreases using plasmids with over 10 kb in size, therefore usage of small multicopy plasmids or small binary vectors (e.g., pGreen) is recommended. From our experience scaling up of experiments (e.g., using 3× amount of plasmid DNA, PEG solution and washing media in a 15 mL bottom round tube) also decreases transformation efficiency, so it is recommended to combine already transformed protoplast samples rather than scaling up the sample.

16. Vortexing is not recommended at any steps in protoplast transformation.
17. Immediate mixing with PEG solution is probably the most critical step in protoplast transformation. Plasmid DNA degradation may occur if the mixing with PEG is delayed; efficiency of transformation may decrease if PEG is not mixed thoroughly.
18. Remove the supernatant without touching the cell pellet with a micropipette or aspirate with a vacuum pump.
19. Adjust tubes in vertical position and let protoplast settle down for 10–12 min (do not spin down the cells). Collect the cells for observation under microscope onto a glass slide by pipetting using wide-open tips. Carefully place the cover slip onto the cells to avoid bursting of the protoplasts.

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Part IV

Analysis of MAPK Substrates

Chapter 13

Determination of Phosphorylation Sites in Microtubule Associated Protein MAP65-1

Andrei Smertenko

Abstract

Reorganization of microtubules during cell cycle depends on the modulation of activity of microtubule-associated proteins. MAP65 is one of the main microtubule structural proteins in plants responsible for the formation of bundles of parallel and antiparallel microtubules. A member of MAP65 protein family, MAP65-1, binds to microtubules of preprophase band during early stages of cell division and later to the midzone of anaphase spindle and the phragmoplast, but exhibits no or reduced microtubule binding during metaphase. Artificially induced interaction of MAP65-1 with microtubules during metaphase promotes excessive formation of pole-to-pole microtubule bundles and causes delay of anaphase onset. The exact mechanism of this delay is not known, but it was suggested that microtubule bundles induced by MAP65 impose spatial constraints on the chromosome movement obstructing their alignment in the metaphase plate. Interaction of MAP65-1 with microtubules is controlled by phosphorylation. This chapter describes a strategy for the identification of phosphorylation residues responsible for the cell-cycle control of MAP65-1 activity.

Key words MAP65, Microtubules, Cytokinesis, Phragmoplast, Phosphorylation, Peptide phosphorylation

1 Introduction

Microtubule associated protein 65-1 (MAP65-1) belongs to the superfamily of evolutionary conserved microtubule associated proteins [1]. The main function of MAP65-1, along with several other members of MAP65 protein superfamily, was attributed to the cross-linking of microtubules by 20–25 nm long bridges in antiparallel orientation, i.e., plus- and minus-ends of individual microtubules within a bundle face the opposite directions [2–4]. The activity of the majority of members of MAP65 protein superfamily described to date is paramount for the stabilization of anaphase spindle and maintenance of the integrity of so-called anaphase spindle midzone matrix [5–11], a network of proteins responsible for

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initiation and progression of cytokinesis. Correspondingly, mutants of MAP65 proteins exhibit cytokinetic defects.

Immunofluorescence microscopy of fixed cells showed that MAP65-1 binds to the majority of interphase microtubules and to the pre-prophase band, but during metaphase becomes restricted to specific areas of the mitotic spindle adjacent to the kinetochores [12, 13]. No association of MAP65-1 with microtubules during metaphase was detected using GFP-fusions [14–16]. Both approache demonstrate that MAP65 has the weakest binding to microtubules during metaphase and suggested that interaction of MAP65 with microtubules is controlled in cell-cycle dependent manner. Initial analysis of the primary sequence suggested two mechanisms that could control MAP65-1 during cell cycle. First, MAP65-1 contains destruction box motif (D-box) characteristic for proteins degraded through the cell cycle by anaphase-promoting complex. However, degradation is unlikely to be the mechanism of MAP65 control because GFP-MAP65-1 signal in the region of metaphase spindle remains very strong [15, 16]. Second, MAP65-1 contains predicted protein kinase phosphorylation motifs suggesting that phosphorylation controls MAP65-1 interaction with the microtubules during the cell cycle.

This chapter describes a method for the identification of phosphorylation sites controlling interaction of a MAP65 protein from *Arabidopsis thaliana*, AtMAP65-1 (MAP65-1) with microtubules during the cell cycle [16]. The workflow of the method consists of four key stages:

1. *Proving that MAP65-1 is phosphorylated*. It has been shown that mitogen-activated (MAPK) and cyclin-dependent (CDK) protein kinases interact with microtubules during interphase and metaphase and hence can act on MAP65-1 [17–20]. Furthermore, MAP65-1 has a conserved CDC kinase phosphorylation motif. On the basis of these predispositions, MAP65-1 phosphorylation was confirmed using total cell protein extract from proliferating BY-2 cells as the kinase preparation under conditions favorable for the mitogen-activated and cyclin-dependent protein kinases (Fig. 1a, [16]). To prove the cell-cycle dependent nature of the phosphorylation, protein extracts from BY-2 cultures synchronized in metaphase were used in phosphorylation assays. This experiment has shown that kinase activity responsible for MAP65-1 phosphorylation peaks in metaphase, when interaction of MAP65-1 with microtubules is the weakest ([16] and Fig. 1a).
2. *Locating potential phosphorylation sites.* The protein was split in four fragments and each fragment was used in the kinase assays. All phosphorylation sites were located on the C-terminus (Fig. 1b, d). This region contains one tyrosine, 12 serine, and six threonine residues.

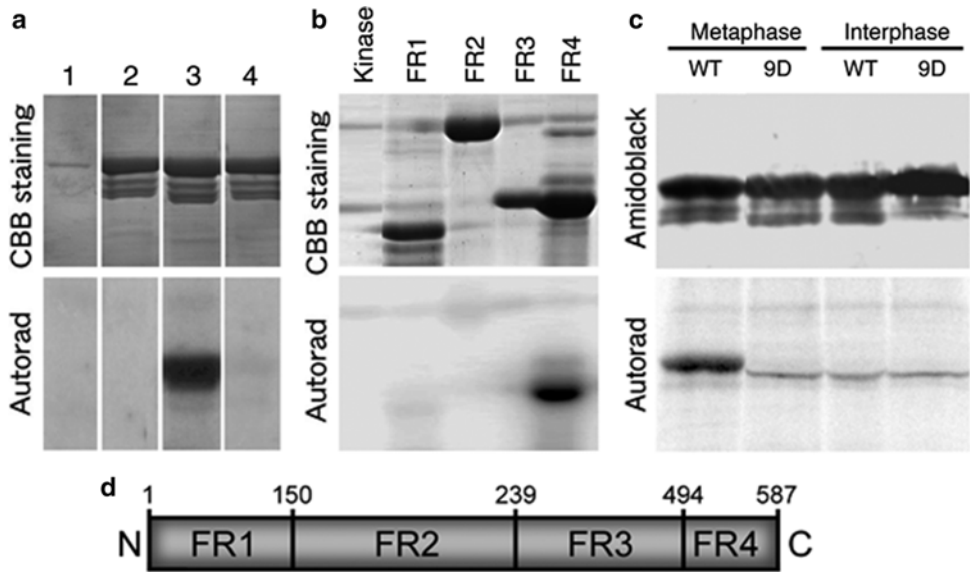

Fig. 1 Examples of protein phosphorylation results. (**a**) Phosphorylation of MAP65-1 by total protein extract from metaphase (*lane 3*) or interphase (*lane 4*) cells. *Lanes 1* and *2* are negative controls without substrate (*lane 1*) or protein extract (*lane 2*) as listed in Table 1. (**b**) Phosphorylation of four fragments of MAP65-1 (shown in **d**) by total protein extract from metaphase cells. Only fragment 4 was phosphorylated. (**c**) Phosphorylation of wild type (WT) MAP65-1 and mutant protein with all nine phosphorylation residues substituted with aspartate (9D) by protein extracts from metaphase or interphase cells. (**d**) Cartoon of MAP65-1 fragments used for phosphorylation assays

3. *Mapping of individual phosphorylation sites.* Nine peptides were designed to encompass all potential phosphorylation residues and used in the phosphorylation assays. Seven out of nine peptides were phosphorylated by the protein extract from metaphase cells. Bioinformatics analysis of these seven peptides identified nine consensus phosphorylation site motifs for MAPK, CDK, Protein kinases A and C, Aurora B kinase, Casein kinases 1 and 2, cyclic AMP- and GMP-dependent kinases (Fig. 2).
4. *Proving that identified sites are phosphorylated during metaphase.* The predicted phosphorylation residues were substituted with alanine to create non-phosphorylatable version of MAP65-1 or aspartate to mimic phosphorylated versions of MAP65-1. The phosphorylation level of aspartate mutant by protein extract from metaphase cells was the same as the phosphorylation level of wild type protein by protein extract from the interphase cells (Fig. 1c). This proves that the nine identified sites are responsible for the cell-cycle dependent regulation of MAP65-1 interaction with microtubules. In agreement with this conclusion, the aspartate mutant exhibited reduced binding to microtubules in vitro and in vivo [16].

a

EQPHVE**QESAFSTRPSPARPVSAKKTVGP**RANNGG**ANGTHNRRLSLNA**NQ**NGSRSTAK**
EAGR**RETLNR**PA**APTNYVAISK**EE**AASSPVSGA**ADH**QVPASP**

b

Peptide	Sequence	Peptide	Residue	Protein kinase	Reference
1	QESAFSTRPSPA	QESAFSTRPSPA*KKK*	S503	CDK,PKA,PKC	Scansite
2	RPVSAKKTVGP	*K*RPVSAKKTVGP	-	-	
3	ANGTHN	*KKK*ANGTHN	T526	CDK	Scansite
4	NRRLSLNA	NRRLSLNA*KKK*	S532	PKA,AuroraB, cAMK,cGMK	Scansite, Prosite
5	NGSRSTAK	NGSRSTAK*KK*	S540	CK1	Prosite
			T543	CK2,PKC	Prosite
6	RRETLNR	RRETLNR*KKK*	T552	cAMK,cGMK,PKA, PKC, AuroraB	Prosite, Scansite
7	APTNYVAISK	APTNYVAISK*KK*	-	-	
8	AASSPVSGA	AASSPVSGA*KKK*	S573, S576	CDK, Erk1 CK1	Disphos, PhosphoELM
9	QVPASP	*KKK*QVPASP	S586	CDK	Disphos, PhosphoELM

Fig. 2 Peptide design and prediction of phosphorylation residues. (**a**) Amino acid sequence of Fragment 4. Serine, threonine, and tyrosine residues are highlighted in *bold*. Regions included in the synthetic peptides are colored in *black*, regions not used in the peptides are colored in *grey*. (**b**) Design of peptides and prediction of the phosphorylation sites. Lysine residues introduced in order to facilitate binding of the peptides to P81 paper are *underlined* in the sequence shown in column "Peptides"

2 Materials

Prepare all solutions and reagents using ultrapure water (18 MΩ cm at 25 °C) and analytical grade reagents. Store materials at room temperature unless indicated otherwise. Follow health and safety regulations when working with radioactive materials.

1. Protein Extraction Buffer: 25 mM Tris–HCl, pH 7.5, 15 mM $MgCl_2$, 15 mM EGTA, 75 mM NaCl, 1 mM DTT, 0.1 mM Na_3VO_4, 1 mM NaF. Store at −20 °C. Before use add leupeptin and pepstatin A to final concentration 10 μg/mL.
2. 5× Kinase buffer: 100 mM HEPES, pH 7.5, 75 mM $MgCl_2$, 25 mM EGTA, 5 mM DTT. Store at −20 °C (*see* **Note 1**).
3. ATP mixture: 10 μL of 1 mM ATP, 9 μL of water, and 1 μL of [γ^{32}P]-ATP (10 μCi/μL). Prepare fresh on the day of experiment.
4. 50 mM Microcystin-LR (e.g., Enzo Life Sciences) in EtOH. Store at −20 °C. Microcystin-LR is very toxic, follow health and safety procedures while handling.
5. Protein Assay reagent (e.g., Bio-Rad product number 500-0006) was prepared and used according to manufacturer

recommendations. The calibration curve of protein concentration was generated using bovine serum albumin.

6. Recombinant MAP65-1 protein (accession number At5g55230) was stored at −80 °C in 25 mM PIPES, pH 6.8, 20 % (v/v) glycerol, 2 mM $MgCl_2$, 2 mM DTT. A fresh aliquot was taken for each experiment, thawed on ice and centrifuged at 150,000 × *g* to pellet protein aggregates. The concentration of MAP65-1 was measured using Bio-Rad Protein Assay.
7. 2×SDS-PAGE gel loading buffer: 0.125 M Tris–HCl, pH 6.8, 0.4 % (w/v) SDS, 20 % (v/v) glycerol, 0.1 % (v/v) Mercapto ethanol, 0.02 % (w/v) Bromophenol blue. Store at −20 °C.
8. P81 Phosphocellulose paper (strong cation exchanger; Whatman, product no. 3698-915).
9. 1.44 M orthophosphoric acid: add 75 mL of orthophosphoric acid to 925 mL of water.
10. Quick Change Multi Site-Directed Mutagenesis Kit (formerly from Stratagene, currently supplied by Agilent Technologies, Product Number 200514).

3 Methods

The first two Subheadings 3.1 and 3.2 describe a standard protein phosphorylation assay that was used to demonstrate phosphorylation of MAP65-1, determine which region of the protein contains phosphorylation sites and prove the phosphorylation sites using mutant MAP65-1 proteins in which all potential phosphorylation residues were substituted for aspartate. Subheadings 3.3 and 3.4 describe peptide design and peptide phosphorylation assay used to identify individual phosphorylation residues. Subheadings 3.5 and 3.6 describe bioinformatics analysis of phosphorylation motifs and site-directed mutagenesis.

Carry out all procedures at room temperature unless otherwise specified. Use radioactive materials in the designated areas. Follow appropriate radioactive waste disposal regulations.

3.1 Preparation of Protein Extract

Extraction of total protein from BY-2 cells and phosphorylation assays were performed according to Bögre et al. (1997) [21] with minor modifications.

1. Grind plant material in liquid nitrogen to make a fine powder (*see* **Note 2**).
2. To each 200 µL of the ground tissue powder add 300 µL of Protein Extraction Buffer.
3. Vortex vigorously and centrifuge at 20,000 × *g*, 4 °C, 3 min to pellet cell debris.

Table 1
Composition of protein phosphorylation reactions. The components were added to the tube according to their order in the table

	Negative controls		Reaction
Component	**1**	**2**	**3**
Water	Up to 20 µL	Up to 20 µL	Up to 20 µL
5× Kinase buffer	4 µL	4 µL	4 µL
MAP65	–	5 µg	5 µg
Microcystin-LR	1 µL	1 µL	1 µL
ATP mix	4 µL	4 µL	4 µL
Extract/Kinase	1 µg	–	1 µg

4. Spin at 300,000 × *g*, 4 °C, 30 min to clear the supernatant from organelles and large protein complexes (*see* **Note 3**).
5. Determine concentration of protein using Bio-Rad Protein Assay.
6. Store on ice until needed (*see* **Note 4**).

3.2 Phosphorylation Reaction

1. Set up the phosphorylation reactions as listed in Table 1.
2. To a 1.5 mL centrifuge tube add in the following order: water 5× Kinase buffer, Microcystin LR, 5 µg of MAP65 recombinant protein and ATP mixture.
3. Start reaction by adding 1 µg of total protein or an appropriate amount of kinase preparation (e.g., immunoprecipitated or chromatographically enriched fraction). The total volume of the reaction is 20 µL.
4. Set up two negative control reactions alongside the main reactions: one without substrate and another without protein extract or kinase preparation.
5. Incubate for 20 min at room temperature.
6. Stop reaction by adding 20 µL of 2× SDS-PAGE gel loading buffer.
7. Load and run 35 µL of each reaction on 10 % SDS PAGE and let the gel front run out of the gel.
8. Transfer proteins onto nitrocellulose membrane, dry and expose overnight to X-ray film or any media of choice (*see* **Note 5**). An example of phosphorylation reaction of MAP65-1 with protein extract from metaphase and interphase cells is shown in Fig. 1a.

Table 2
Composition of peptide phosphorylation reactions. The components were added to the tube according to their order in the table

	Negative controls		Reaction
Component	**1**	**2**	**3**
Water	Up to 50 µL	Up to 50 µL	Up to 50 µL
5× Kinase buffer	10 µL	10 µL	10 µL
1 mM peptide	–	5 µL	5 µL
Microcystin-LR	1 µL	1 µL	1 µL
ATP mix	10 µL	10 µL	10 µL
Extract/Kinase	1 µg	–	1 µg

3.3 Peptide Design

In order to find out which residues are phosphorylated by metaphase extract, the whole protein was split into four fragments (Fig. 1d). Out of four fragments only C-terminal fragment was phosphorylated (Fig. 1b). Fragment 4 is 100 amino acids long (Fig. 2a). Altogether, nine regions were chosen (Fig. 2b) to encompass all phosphorylation residues within this fragment (highlighted in bold in Fig. 2a). In order to facilitate binding of the peptides to negatively charged surface of the P81 paper, each peptide was designed to contain three lysine residues (Fig. 2b). Peptides were solubilised in water at concentration 10 mM.

3.4 Peptide Phosphorylation Assay

1. Set up the peptide phosphorylation reactions as listed in Table 2.
2. To 1.5 mL centrifuge tube add in the following order: water 10 µL of 5× Kinase buffer, 5 µL of 1 mM peptide (*see* **Note 6**), Microcystin-LR, ATP mixture (*see* **Notes 7** and **8**).
3. Start reaction by adding a volume containing 1 µg of total protein or an appropriate amount of kinase preparation (as in Subheading 3.2). The final volume of the reaction is 50 µL.
4. Run phosphorylation reaction for 30 min.
5. Pipet 35 µL of the reaction mixture on the 4 cm^2 square piece of P81 Phosphocellulose paper (*see* **Notes 9** and **10**).
6. Once the reaction solution has soaked into the P81 paper (*see* **Note 11**), drop them sequentially in a beaker containing 1.44 M orthophosphoric acid solution (Fig. 3; *see* **Note 12**).
7. Wash pieces three times 10 min each in the solution of orthophosphoric acid. Make sure the paper pieces stay loose and are thoroughly washed.
8. Dry with hairdryer.

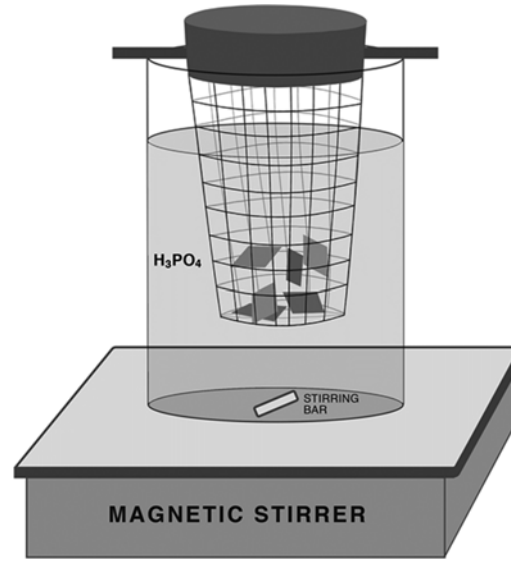

Fig. 3 Setup for washing of P81 paper squares in orthophosphoric acid. A container with plastic mesh was placed in the beaker containing 500 mL of orthophosphoric acid solution. While the stirrer is on, carefully deposit pieces of P81 paper one at a time. Set the speed of stirrer sufficiently fast to swirl the P81 pieces during the wash. Any plastic mesh can be used to make the container. We used protective mesh from empty solvent bottles

9. Place each piece into a scintillation vial containing appropriate scintillation fluid and record the counts (*see* **Note 13**).
10. Data analysis. Subtract the background counts from each reading. Normalize each reading by the total amount of [γ^{32}P]-ATP in the reaction (use reading from the 5 μL aliquot spotted on the paper). Subtract the negative controls. Calculate the average number of counts in the repeats.

3.5 Prediction of the Phosphorylation Sites

The peptide phosphorylation assay has shown that peptides 2 and 7 were not phosphorylated. So, bioinformatics was used to pinpoint which residue is likely to be targeted for phosphorylation in the seven peptides that were phosphorylated by a metaphase protein extract. The following programs were used:

Prosite (http://www.expasy.org/prosite/);

Scansite (http://scansite.mit.edu/);

Disphos (http://core.ist.temple.edu/pred/);

PhosphoELM (http://phospho.elm.eu.org/).

Altogether, nine residues were predicted to reside within conserved motives recognized by several distinct protein kinases (Fig. 2b).

3.6 Site-Directed Mutagenesis

Predicted phosphorylation residues in the C-terminal domain of MAP65-1 were substituted with neutral alanine to create the non-phosphorylatable versions of protein or with negatively charged aspartate to mimic phosphorylation event. The sited-directed mutagenesis was performed using Quick Change Multi Site-Directed Mutagenesis Kit according to the manufacturer's recommendations. Mutagenesis was confirmed by sequencing and then mutated genes were used for in vitro or in vivo assays.

4 Notes

1. Each component of Protein Extraction and 5× Kinase buffers was added from a pre-made stock solution.
2. The powder of ground cells can be stored at −80 °C.
3. Set up the phosphorylation reactions (described in Subheading 3.2) during this centrifugation step.
4. Make a fresh extract for each assay and use within 2 h of preparation.
5. A strong signal can be detected after an 80 min exposure to X-ray film.
6. Centrifuge peptide solutions.
7. Make every reaction in triplicate.
8. Negative controls: (1) for each peptide prepare control reaction lacking the protein extract or kinase preparation to check the unspecific binding of [γ^{32}P]-ATP to the peptide; (2) per each kinase preparation set up one reaction omitting the substrate to check the background phosphorylation in the kinase preparation.
9. Number the pieces of paper with soft pencil before pipetting on the phosphorylation mixture.
10. For determination of total ATP content in the reaction mixture pipet 5 μL of each phosphorylation reaction mixture on a separate piece of P81 paper. Make two repeats to take into account the pipetting error.
11. Do not let the paper dry.
12. Make sure all pieces of P81 paper are in the mesh compartment and do not stick together.
13. Record background in an empty scintillation tube.

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Chapter 14

In Vivo Phosphorylation of WRKY Transcription Factor by MAPK

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Abstract

Plants activate signaling networks in response to diverse pathogen-derived signals, facilitating transcriptional reprogramming through mitogen-activated protein kinase (MAPK) cascades. Identification of phosphorylation targets of MAPK and in vivo detection of the phosphorylated substrates are important processes to elucidate the signaling pathway in plant immune responses. We have identified a WRKY transcription factor, which is phosphorylated by defense-related MAPKs, SIPK and WIPK. Recent evidence demonstrated that some group I WRKY transcription factors, which contain a conserved motif in the N-terminal region, are activated by MAPK-dependent phosphorylation. In this chapter, we describe protocols for preparation of anti-phosphopeptide antibodies, detection of activated MAPKs using anti-phospho-MAPK antibody, and activated WRKY using anti-phospho-WRKY antibody, respectively.

Key words Anti-phosphopeptide antibody, MAPK substrate, WRKY transcription factor

1 Introduction

Mitogen-activated protein kinase (MAPK) cascades have important roles in defense-related signaling pathways. Pathogen stimuli induce activation of defense-related MAPKs in a wide range of plant species. It has been shown that tobacco (*Nicotiana* spp.) MAPKs, WIPK and SIPK, and their orthologues in other plant species are important regulators of plant defense responses [1, 2]. NtMEK2 is an upstream MAPKK activating both WIPK and SIPK [3]. In *Arabidopsis*, MPK3, MPK4, and MPK6 were identified as defense-related MAPKs. Activated MAPKs phosphorylate substrate proteins and transduce the signal to downstream components.

Recently, some group I WRKY transcription factors, which contain a conserved motif in the N-terminal region, were

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identified as MAPK substrates. NbWRKY8, a *Nicotiana benthamiana* group I WRKY transcription factor, has clustered proline-directed serines (SP cluster) in N-terminal region and these serine residues are phosphorylation targets of MAPKs [4]. MAPK-mediated phosphorylation activates NbWRKY8 and promotes the expression of downstream genes [4]. AtWRKY33, the closest *Arabidopsis* WRKY transcription factor to NbWRKY8, was also characterized as a MAPK substrate and phosphorylated at serine residues within SP cluster [5]. Furthermore, AtWRKY25 and NtWRKY1, which are in vitro substrates of MAPK, contain SP cluster [6, 7]. These findings suggest that WRKYs containing an SP cluster are potential substrates of certain MAPKs, and these WRKYs were found in diverse plant genomes [8].

Agrobacterium-mediated transient gene expression in *N. benthamiana* is a simple and reproducible experimental system, and widely utilized for functional analysis of several proteins [9]. For the phosphorylation analysis of WRKY proteins, the expression system is also quite useful. We employed the expression system to activate specific MAPK for the phosphorylation analysis of NbWRKY8 in vivo [4]. Popescu et al. [10] showed the in vivo phosphorylation of *Arabidopsis* WRKY proteins by expressing target WRKY with *Arabidopsis* MAPKK-MAPK module in *N. benthamiana*. The expression system is applicable to the phosphorylation analysis of WRKYs not only of *N. benthamiana* but also of other plants.

Recent advances in mass spectrometry (MS) have allowed highly sensitive detection of phosphorylation sites. However, there are some limitations to applying this methodology to the phosphoprotein analysis. For example, it can be difficult to detect phosphorylated residues, in case that a phosphorylation site is not located in MS-friendly peptides upon cleavage by a protease. In contrast, immunoblot analysis using anti-phosphopeptide antibody can be broadly applicable and overcome the limitations of the MS methodology. Although it is necessary to make specific antibody against each phosphorylation site, these powerful antibodies allow quick and simplified detection of the phosphorylation sites.

2 Materials

Unless otherwise noted, deionized water (Millipore Elix) and basic molecular biology reagents and equipment are used to prepare all of the following solutions. Unless other temperatures are specified, chilled solutions can be kept on ice on the bench.

2.1 Immunoblot Detection of Phosphorylated MAPKs

2.1.1 Agrobacterium-Mediated Transient Expression in N. benthamiana

1. 3- to 4-week-old *N. benthamiana*.
2. *Agrobacterium tumefaciens* strain GV3101 containing kinase-inactive 35S:HA-MEK2^{KR} or constitutively active 35S:HA-MEK2^{DD} mutants in pEL2 binary plasmid [11].
3. LB media.
4. Suspension buffer: 10 mM MES/NaOH, 10 mM $MgCl_2$, pH 5.6.
5. 150 mM acetosyringone dissolved in DMSO, aliquot, and store at −20 °C.

2.1.2 Protein Extraction from Plant Tissues

1. 0.5 M HEPES/NaOH, pH 7.5, sterilize by filtration.
2. 1 M $MgCl_2$, sterilize by filtration.
3. 0.5 M EGTA/NaOH, pH 8.0, sterilize by filtration.
4. 4 M NaCl, sterilize by filtration.
5. 1 M DTT, sterilize by filtration, aliquot, and store at −20 °C.
6. 0.5 M NaF, sterilize by filtration, aliquot, and store at −20 °C.
7. 0.1 M Na_3VO_4, sterilize by filtration, aliquot, and store at −20 °C (*see* **Note 1**).
8. β-Glycerophosphate.
9. Protease inhibitor cocktail for plant cell extracts (Sigma).
10. Nonidet P40.
11. Protein extraction buffer: 20 mM HEPES-NaOH, pH 7.5, 10 mM $MgCl_2$, 15 mM EGTA, 100 mM NaCl, 2 mM DTT, 1 mM NaF, 0.5 mM Na_3VO_4, 30 mM β-glycerophosphate, 1 % (v/v) protease inhibitor cocktail for plant cell extracts (Sigma), 0.1 % (v/v) Nonidet P40.
12. Bradford assay kit (e.g., Bio-Rad Protein Assay Dye Reagent Concentrate).

2.1.3 Detection of Phosphorylated MAPKs

1. Standard equipment for SDS-PAGE and semi-dry transfer.
2. TBS-T: TBS buffer containing 0.05 % (v/v) Tween 20.
3. Hybond™-ECL™ nitrocellulose membrane (0.45 μm) (GE Healthcare).
4. MemCode™ reversible protein stain kit (Pierce).
5. Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb (Cell Signaling Technology).
6. Albumin, from bovine serum (BSA).
7. Anti-rabbit IgG, HRP-linked whole Ab donkey (e.g., GE Healthcare).

8. Skimmed milk powder (e.g., WAKO).
9. Enhanced chemiluminescence (ECL) reagent: SuperSignal West Dura Extended Duration Substrate (e.g., Pierce).
10. Chemiluminescence imager (ATTO).
11. CS Analyzer 3.0 to analyze chemiluminescence signals (ATTO).

2.2 Agrobacterium-Mediated Transient Expression in N. benthamiana

1. 3- to 4-week-old *N. benthamiana*.
2. *Agrobacterium* strain GV3101 containing 35S:WRKY8-HA-StrepII, 35S:FLAG-MEK2^{KR}, 35S:FLAG-MEK2^{DD} or 35S:p19 (silencing suppressor) (*see* **Note 2**).
3. LB media.
4. Suspension buffer: 10 mM MES/NaOH, 10 mM $MgCl_2$, pH 5.6.
5. 150 mM acetosyringone dissolved in DMSO, aliquot, and store at −20 °C.

2.3 Preparation of Anti-phosphopeptide Antibodies

1. Phosphorylated and non-phosphorylated synthetic peptides (*see* **Note 3**).
2. Hitrap NHS activated HP, 1 mL (GE Healthcare).
3. Coupling buffer: 0.2 M $NaHCO_3$, pH 8.3, 0.5 M NaCl, filter through 0.45 μm filter.
4. 1 mM HCl.
5. Buffer A: 0.5 M ethanolamine-HCl, pH 8.3, 0.5 M NaCl, filter through 0.45 μm filter.
6. Buffer B: 0.1 M sodium acetate, pH 4.0, 0.5 M NaCl, filter through 0.45 μm filter.
7. Buffer C: 20 mM Tris–HCl, pH 7.4, 0.5 M NaCl, filter through 0.45 μm filter.
8. Elution buffer: 0.2 M glycine–HCl, pH 2.5, filter through 0.45 μm filter.
9. Antisera of rabbits immunized with phosphorylated peptide (*see* **Note 4**).
10. Millex-HV Syringe Filter Unit, 0.45 μm, PVDF (Millipore).
11. TBS buffer: 50 mM Tris–HCl, pH 7.5, 150 mM NaCl.
12. 1.5 M Tris–HCl, pH 8.8.
13. BSA solution (20 mg/mL).
14. Absorption spectrometer.

2.4 Immuno-detection of Phosphorylated WRKY Protein Using Anti-phosphopeptide Antibodies

1. 3- to 4-week-old *N. benthamiana*.
2. *Agrobacterium* strain GV3101 containing 35S:WRKY8-HA-StrepII, 35S:FLAG-MEK2^{KR}, 35S:FLAG-MEK2^{DD} and 35S:p19 constructs.
3. LB media.

4. Suspension buffer: 10 mM MES/NaOH, 10 mM $MgCl_2$, pH 5.6.
5. 150 mM acetosyringone dissolved in DMSO, aliquot, and store at –20 °C.
6. Standard equipment for SDS-PAGE and semi-dry transfer.
7. TBS-T: TBS buffer containing 0.05 % (v/v) Tween 20.
8. Hybond™-ECL™ nitrocellulose membrane (0.45 μm) (GE Healthcare).
9. Anti-rabbit IgG, HRP-linked whole Ab donkey (e.g., GE Healthcare).
10. ECL reagent: SuperSignal West Dura Extended Duration Substrate (Pierce).
11. Chemiluminescence imager (ATTO).
12. CS Analyzer 3.0 to analyze chemiluminescence signals (ATTO).

2.5 Affinity Purification of StrepII-Tagged WRKY Proteins

1. Lysis buffer: 20 mM HEPES/NaOH, pH 7.5, 10 mM KCl, 10 mM $MgCl_2$, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, 40 mM beta-glycerophosphate, 5 mM DTT, 1 % (v/v) Protease inhibitor cocktail for plant cell extracts.
2. 5 M NaCl, sterilize by filtration.
3. Sonicator.
4. Bradford assay kit (Bio-Rad Protein Assay Dye Regent Concentrate; Bio-Rad).
5. Avidin solution (5 mg/mL).
6. Wash buffer: 20 mM HEPES-NaOH, pH 7.5, 400 mM NaCl, 5 mM DTT, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100.
7. MagStrep type 2 Beads (IBA).
8. Magnet stand.

3 Methods

3.1 Detection of Phosphorylated MAPKs

3.1.1 Agrobacterium-Mediated Transient Expression in N. benthamiana

1. Inoculate one single colony of *Agrobacterium* containing the 35S:HA-MEK2^{KR}, 35S:HA-MEK2^{DD} constructs in 5 mL LB with appropriate antibiotics overnight at 28 °C, shaking at 140 rpm.
2. Use 2 mL of the overnight culture to inoculate 20 mL LB with same antibiotics and incubate for 5 h at 28 °C, shaking at 140 rpm.
3. Precipitate the *Agrobacterium* (3,000 × *g*, 15 min), then resuspend the pellet in 10 mL of suspension buffer.

4. Precipitate the *Agrobacterium* (3,000 × *g*, 15 min), then resuspend the pellet in 5 mL of the suspension buffer containing 150 μM acetosyringone.
5. Measure the optical density (OD) at 600 nm, and adjust OD_{600} to 0.5.
6. Incubate the suspension at room temperature for 2 h before infiltration.
7. Infiltrate the suspension into the underside of the leaf using 1 mL syringe without a needle.

3.1.2 Protein Extraction for Phosphorylated MAPKs from N. benthamiana Leaves

1. Weight out 160 mg of leaf for each treatment, grind leaf sample in liquid nitrogen and thaw in 1 mL protein extraction buffer. Let stand on ice for 10 min.
2. Centrifuge the mixture at 20,000 × *g* for 20 min at 4 °C.
3. Transfer supernatants carefully to clean chilled microcentrifuge tube. Measure protein concentrations by Bradford assay.

3.1.3 Immuno-detection of Phosphorylated MAPKs

1. Separate extracted 30 μg samples by electrophoresis on a 10 % SDS-polyacrylamide gel and transfer to a nitrocellulose membrane.
2. Block the membrane with 5 % (w/v) BSA in TBS-T for 1 h at room temperature (*see* **Note 5**).
3. Incubate with anti-phospho-p44/42 MAPK antibody diluted 1:5,000 in 5 % (w/v) BSA/TBS-T for overnight at 4 °C.
4. Wash three times with TBS-T (3× 10 min).
5. Incubate with HRP-conjugated anti-rabbit IgG diluted 1:5,000 in 5 % (w/v) skimmed milk/TBS-T for 1 h at room temperature.
6. Wash three times with TBS-T (3× 10 min).
7. Immunoreactive signals were visualized using ECL reagents and chemiluminescence imager.
8. An example of immunoblotting showing phosphorylation of SIPK and WIPK by MEK2 is shown in Fig. 1.

3.2 Preparation of Anti-phosphopeptide Antibodies

3.2.1 Peptide Coupling to the Medium

1. Dissolve 3 mg of the peptides in 1 mL of coupling buffer.
2. Apply 6 mL of ice-cold 1 mM HCl onto the column (*see* **Note 6**).
3. Immediately apply 1 mL of the peptide solution onto the column.
4. Seal the column and incubate it for 30 min at 25 °C.
5. Apply 6 mL of buffer A onto the column.
6. Apply 6 mL of buffer B onto the column.
7. Apply 6 mL of buffer A onto the column.

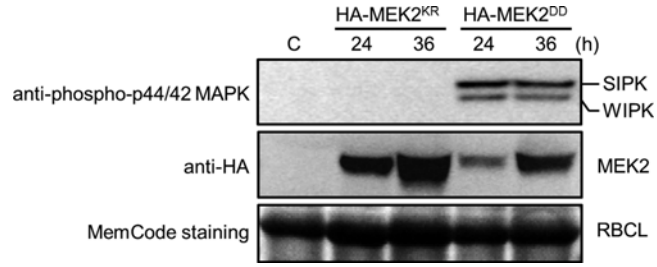

Fig. 1 Detection of phosphorylated MAPKs using anti-phospho-p44/42 MAPK antibody in *N. benthamiana* leaves. Extracted proteins (30 μg) from the leaves transiently expressing 35S:MEK2^{KR} and 35S:MEK2^{DD} after agroinfiltration were separated in a 10 % SDS-polyacrylamide gel. Blotted nitrocellulose membrane was stained by MemCode staining kit, and the bands corresponding to ribulose-1,5-bisphosphate carboxylase large subunit (RBCL) are shown. After destaining, phosphorylated MAPKs and MEK2 variants were detected by anti-phospho-p44/42 MAPK antibody and anti-HA antibody, respectively. No phosphorylated MAPK was detected in control (C; without agroinfiltration) and 35S:MEK2^{KR} expressed leaves. Phosphorylated SIPK and WIPK were detected in 35S:MEK2^{DD} expressed leaves with molecular masses of 48 and 44 kDa, respectively

8. Leave the column for 30 min in room temperature.
9. Apply 6 mL of buffer B onto the column.
10. Apply 6 mL of buffer A onto the column.
11. Apply 6 mL of buffer B onto the column.
12. Apply 2 mL of buffer C.

3.2.2 Affinity Purification of Anti-phosphopeptide Antibodies

1. Apply 3 mL of buffer C onto the column.
2. Apply 3 mL of elution buffer onto the column.
3. Apply 10 mL of buffer C onto the column.
4. Dilute 10 mL antiserum with equal amount of buffer C and filter the mixture through a 0.45 μm filter.
5. Apply the diluted antiserum onto the column.
6. Apply 10 mL buffer C onto the column.
7. Apply 5 mL the elution buffer onto the column and collect the fractions and immediately neutralize the eluting fractions by 1.5 M Tris–HCl, pH 8.8 (approximately 70 μL per 1 mL of eluate).
8. Add 100 μL of BSA solution (20 mg/mL) to 1 mL of the eluate and dialyze against TBS buffer at 4 °C.
9. Apply the dialyzed antibody solution onto the non-phosphopeptide-coupled column equilibrated with TBS buffer for absorption of anti-non-phosphopeptide antibodies and pass through the column twice. Collect the pass-through fraction.

10. Measure absorbance of the flow-through fraction at 280 nm. At 280 nm an absorbance of 1.0 is equivalent to an immunoglobulin concentration of 0.74 mg/mL.
11. Confirm the reactivity against phosphopeptide and non-phosphopeptide by immunoblot.

3.3 Characterization of Anti-phosphopeptide Antibodies by Dot Blotting

1. Dissolve peptides in TBS-T to final concentrations of 50 and 5 μg/mL.
2. Spot 2 μL of the peptide solutions onto a nitrocellulose membrane.
3. Let the membrane dry.
4. Block the membrane with 3 % (w/v) BSA in TBS-T for 1 h at room temperature or overnight at 4 °C (*see* **Note 5**).
5. Incubate with primary antibody (0.1–10 μg/mL for purified antibodies) dissolved in TBS-T for 30 min at room temperature.
6. Wash three times with TBS-T (3× 10 min).
7. Incubate with HRP-conjugated secondary antibody at the desired dilution for 1 h at room temperature.
8. Wash three times with TBS-T (3× 10 min).
9. Immunoreactive signals were visualized using ECL reagent as shown in Fig. 2.

3.4 Immunodetection of Phosphorylated WRKY Protein using Anti-phosphopeptide Antibodies

3.4.1 Agrobacterium-Mediated Transient Expression in N. benthamiana

1. Prepare *Agrobacterium* containing the 35S:WRKY-HA-StrepII, 35S:FLAG-MEK2 or 35S:p19 construct as described in Subheading 3.1.1 [1–4].
2. Measure the optical density (OD) at 600 nm, and adjust OD_{600} to 0.5. Mix the *Agrobacterium* suspensions containing the 35S:WRKY-HA-StrepII, 35S:FLAG-MEK2 or 35S:p19 construct in a 4:1:5 ratio.
3. Incubation and infiltration as described in Subheading 3.1.1 [6, 7].

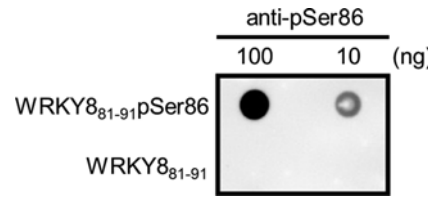

Fig. 2 Dot blot analysis of anti-phosphopeptide antibody. Non-phosphorylated peptide of WRKY8 (WRKY8$_{81-91}$) and Ser86-phosphorylated WRKY8$_{81-91}$ (WRKY8$_{81-91}$pSer86) were spotted on nitrocellulose membrane. Immunoblot analysis was performed using anti-pSer86 antibody. The WRKY8$_{81-91}$pSer86 was only detected by the antibody

3.4.2 Affinity Purification of StrepII-Tagged WRKY Proteins

1. Grind 500 mg leaves in liquid nitrogen with a mortar and pestle and thaw in 1 mL lysis buffer.
2. Incubate the homogenate at 4 °C for 10 min, and then add 5 M NaCl to a final concentration of 400 mM.
3. Incubate the mixture at 4 °C for 1 h with gentle mixing and then sonicate to reduce viscosity.
4. Centrifuge the mixture at 20,000 × *g* for 10 min at 4 °C, and collect the supernatant.
5. Measure the protein concentration in the extract by Bradford assay, and then equalize the protein concentration of all extracts.
6. Transfer a 1 mL aliquot of the extract into a 1.5 mL microcentrifuge tube and add 5 μL Avidin solution (5 mg/mL) (*see* **Note 7**). Incubate the mixture on ice for a few minutes.
7. Add 40 μL 50 % slurry of pre-equilibrated MagStrep type 2 Beads with wash buffer to the mixture and incubated overnight at 4 °C with gentle shaking.
8. Wash the resin three times with 1 mL of wash buffer, and then resuspend in 30 μL of 2× SDS-PAGE sample buffer.

3.4.3 Immuno-detection of Phosphorylated WRKY Proteins

1. Separate eluted samples by electrophoresis on a 10 % SDS-polyacrylamide gel and transferred to a nitrocellulose membrane.
2. Block the membrane with 3 % (w/v) BSA in TBS-T for 1 h at room temperature or overnight at 4 °C (*see* **Note 5**).
3. Incubate with primary antibody (0.1–10 μg/mL for purified antibodies) dissolved in TBS-T for 1 h at room temperature.
4. Wash three times with TBS-T (3× 10 min).
5. Incubate with HRP-conjugated secondary antibody at the desired dilution for 1 h at room temperature.
6. Wash three times with TBS-T (3× 10 min).
7. Immunoreactive signals were visualized using ECL reagent.
8. An example of immunoblotting showing phosphorylate WRKY8 by MEK2-activated SIPK/WIPK is shown in Fig. 3.

4 Notes

1. Preparation of 0.1 M Na_3VO_4 solution: Dissolve Na_3VO_4 in water and adjust to pH 10 with HCl. Boil until solution turns colorless then cool to room temperature and adjust the pH to 8. Repeat boil/cool/pH step until solution remains at pH 8. Store in aliquots at –20 °C. Just before use, boil at 100 °C for 5 min, then on ice.

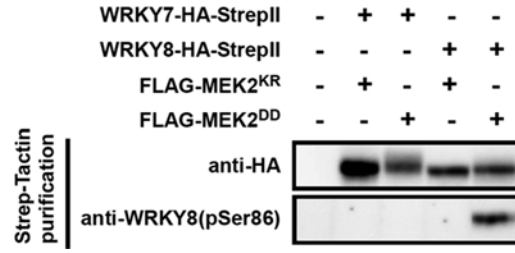

Fig. 3 In vivo detection of phospho-Ser86-WRKY8. HA-StrepII-tagged WRKYs were expressed with $MEK2^{DD}$ or $MEK2^{KR}$ in *N. benthamiana* leaves by agroinfiltration. After Strep-Tactin purification, immunoblot analyses were done using anti-HA and anti-WRKY8 (pSer86) antibodies. HA-StrepII-tagged WRKY7 and WRKY8 showed mobility shift, which indicates phosphorylation, only when the WRKYs were coexpressed with $MEK2^{DD}$. The anti-WRKY8 (pSer86) antibody specifically reacts with phosphorylated WRKY8

2. The p19 protein of Tomato bushy stunt virus is a suppressor of RNA silencing. Coexpression of p19 prevents the onset of RNA silencing and allows high-level transient expression of transgenes [12].
3. We generally use a peptide, which consists of the phosphoserine/threonine and four or five residues on both the N- and C-terminal sides. Cysteine residue is added to the N-terminus of the peptide to conjugate with a carrier protein, KLH (Keyhole Limpet Hemocyanin).
4. We usually utilize custom antibody production services, including peptide synthesis. Recently, many companies provide such services. Of course, it is also possible to produce antibodies in own laboratory. The procedure of antibody production is well described in the literature [13].
5. BSA is a better blocking reagent for phosphoprotein detection. Because milk contains a phosphoprotein, casein, blocking with milk may produce a high background when blotting with anti-phosphopeptide antibodies.
6. The column can be operated with a syringe, peristaltic pump, or liquid chromatography system.
7. StrepII-tagged proteins bind with high affinity to Strep-Tactin, an engineered form of streptavidin. However, biotinylated proteins are also bound to Strep-Tactin and produce background. Avidin prevents endogenous biotinylated proteins from binding to Strep-Tactin coated MagStrep type 2 Beads during protein purification.

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Chapter 15

Rapid Mutagenesis-Based Analysis of Phosphorylation Sites in Mitogen-Activated Protein Kinase Substrates

Lennart Eschen-Lippold, Nicole Bauer, Julia Löhr, Mieder A.T. Palm-Forster, and Justin Lee

Abstract

In eukaryotes, mitogen-activated protein kinases (MAPKs) are one of the best studied pathways for posttranslational modification-mediated regulation of protein functions. Here, we describe a rapid in vitro method to screen potential protein phosphorylation sites targeted by MAPKs. The method is based on PCR-mediated mutagenesis together with a type IIs restriction digest. Screening for the successfully mutated clones is further facilitated through introduction of a second diagnostic restriction site. Besides time-saving, this reduces the cost for sequencing confirmation of the positive clones, which are used for subsequent recombinant protein production and kinase assay validation.

Key words MAPK substrates, Phosphorylation, Mutagenesis

1 Introduction

Phosphorylation events play an important role in signal transduction since they are major posttranslational modifications (PTM) regulating protein folding, activity, and stability. Like in other eukaryotes, plant mitogen-activated protein kinase (MAPK) cascades are important cellular signaling modules involved in stress responses, growth and development [1, 2]. They typically comprise three hierarchically organized kinases, a MAP triple kinase (MAPKKK) which activates a MAP kinase kinase (MKK) by phosphorylation and a MAP kinase (MAPK) that, in turn, is phosphorylated by the MKK. Activated MAPKs then phosphorylate a variety of substrate proteins that orchestrate the appropriate pathway-specific response [3]. For the understanding of phosphorylation-mediated control of protein functions in such complex regulatory signaling networks, the characterization of the phosphorylation pattern introduced by MAPKs is a prerequisite. While mass spectrometric analysis is a powerful tool for the identification of

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relevant phosphorylation sites, it is not always accessible for some laboratories and even then, identified phospho-sites need to be further validated. Here, we describe a simple method to create mutations in individual and multiple phosphorylation sites, which permits rapid MAPK target site identification, thus providing the basis for subsequent in vivo analysis of the impact of PTM on cellular signaling.

The method is based on plasmid re-circularization by ligation of the PCR-amplified linear plasmid after digestion (*see* Fig. 1). Mutagenic primers are designed with a type IIs restriction-site, such as Eco31I that cuts outside the bound sequence, at their 5′-ends. The chosen type IIs restriction site should preferably be absent elsewhere in the sequence to be amplified. Otherwise, multi-fragment ligation can be attempted if there is no interference between the cohesive ends of the different restriction sites. To further facilitate screening for the correctly mutated clones, a second diagnostic restriction site can be introduced into the primers. For instance, mutagenesis of MAPK-targeted (Ser/Thr)-Pro to Ala-Pro creates a *Kas*I diagnostic restriction site if the wobble base of the codon of the amino acid preceding Ala is a guanine (i.e., if the amino acid at the −1 position relative to Ala-Pro is Leu, Met, Val, Ser, Pro, Thr, Ala, Gln, Lys, Glu, Trp, Arg, or Gly). Table 1 lists two alternatives (and limitations) if such conditions are not fulfilled. Furthermore, rather than Ala-Pro, one may mutate to Gly-Pro, Glu-Pro, or Arg-Pro (thus adding *Apa*I, *Ban*II, or *Stu*I restriction sites, respectively) without imposing any further requirements for the sequence flanking the phosphorylation site. The small uncharged glycine is a good alternative to alanine for conversion into a non-phosphorylatable amino acid. By contrast, the Glu-Pro can serve as a negatively charged phospho-mimic, while an Arg-Pro mutation allows the evaluation of a positive charge at the potential phosphorylation site on protein function. Thus, mutation of Ser/Thr to other amino acids might be considered to address the experimental purposes. For illustration, the protein encoded by *Arabidopsis thaliana* gene At1g04330, which carries two putative phosphorylation sites, was chosen. The coding sequence was mutated (Fig. 1), transferred to a bacterial expression vector and the recombinant protein used in an in vitro kinase assay to identify MAP kinase-relevant phosphorylation sites. S44 appears to be the main phospho-site targeted by MPK3 or MPK6 since phosphorylation of S44A mutated proteins is strongly reduced (Fig. 2).

2 Materials

In general, all cloning reactions are set up with autoclaved ultrapure H_2O (dH_2O; 18.2 MΩ cm at 25 °C, TOC < 10 ppb).

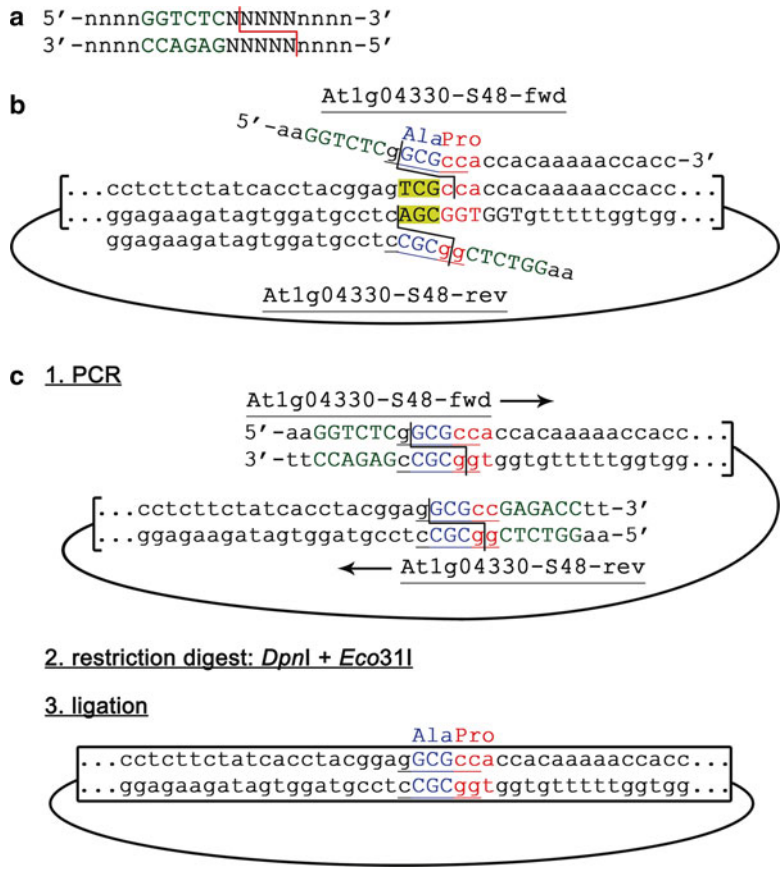

Fig. 1 Primer design and experimental outline for mutating the MAPK target site. (**a**) Recognition sequence of type IIs restriction enzyme Eco31I (*green characters*). The cleavage site is indicated as a *red line*. (**b**) A putative phosphorylation site of At1g04330 (S48) is depicted with both mutagenic primers containing the 5′-Eco31I recognition sequence (*green*), and the alanine-coding nucleotides (*blue*) are shown adjacently to the double-stranded template DNA. Proline-coding nucleotides are marked *red*, while serine-coding nucleotides in the parental plasmid are highlighted in *yellow*; Eco31I cleavage sites are indicated as *black lines*, and the diagnostic *Ssp*DI restriction site is *underlined*. (**c**) "Whole plasmid amplification" was achieved with PCR using the indicated primers (*step 1*). After purification of the PCR product, restriction digest with DpnI and Eco31I (*step 2*) was used to remove any remaining parental template plasmid and to produce the sticky ends for ligation, respectively. Ligation was then used to re-circularize the plasmid (*step 3*) before transformation into competent bacteria cells. Note that *step 2* and *step 3* can also be performed simultaneously (*see* Subheading 3.4 for details). ("Reproduced from Palm-Forster et al. [4] with permission from Academic Press")

2.1 PCR Components

1. Template plasmid: small sub-cloning vector carrying the gene to mutate (*see* **Note 1**).
2. Primers: standard primers dissolved in water (100 pmol/μL). Store at −20 °C.

Table 1
Alternatives for creation of diagnostic restriction sites in mutagenized phosphorylation sites ("Reproduced from Palm-Forster et al. [4] with permission from Academic Press")[a]

Diagnostic (second) enzyme	–1 Amino acid position	Ser/Thr	Pro	+1 Amino acid position	
Alternative 1		*Ala* *GCx*	*Pro* *CCx*		*Limitations*
*Kas*I (*Ssp*DI)	xxG Leu/Met/Val/ Ser/Pro/Thr/Ala/ Gln/Lys/Glu/ Trp/Arg/Gly	GCG	CCx		Not suitable in position –1 are: Phe/Ile/ Tyr/His/ Asn/Asp/Cys
*Hae*II	xxR Leu/Met/Val/Ser/ Pro/Thr/Ala/ Gln/Lys/Glu/ Trp/Arg/Gly/Ile	GCG	CCx		Not suitable in position –1 are: Phe/Tyr/ His/Asn/ Asp/Cys
*Hgi*AI	xGW Cys/Arg/Ser/Gly	GCW	Ccx		Constraints at position –1
Alternative 2		*Ala* *GCx*	*Pro* *CCx*		*Limitations*
*Nae*I		gcG	CCG	GCx Ala	Constraints at position +1
*Bsp*EI		gcT	CCG	Gax Asp/Glu	Constraints at position +1
*Sma*I/*Xma*I		gcC	CCG	GGx Gly	Constraints at position +1
*Bpm*I (cuts 14/16 nt away from recognition site)		gCT	CCA	Gxx Val/Ala/Asp/ Glu/Gly	Constraints at position +1
Alternative 3		*Gly*	*Pro*		*Limitations*
*Apa*I or *Bsp*120I		GGG	CCC		None
Alternative 4		Glu	Pro		
*Ban*II		GAG	CCC		None
Alternative 5		*Arg*	*Pro*		
*Stu*I		AGG	CCT		None

[a]Mutagenesis of (Ser/Thr) to Ala preceding Pro allows creation of different diagnostic restriction sites. Possible codons of Ala-Pro and adjacent positions (–1/+1) are listed in Alternatives 1 and 2, including the resulting variable amino acids and their limitations. Alternatives 3–5 list mutations of (Ser/Thr) to other amino acids without imposing any requirements on flanking sequences (nucleotides required for diagnostic restriction analysis are *underlined*; abbreviations for nucleotides: W = A or T, R = A or G)

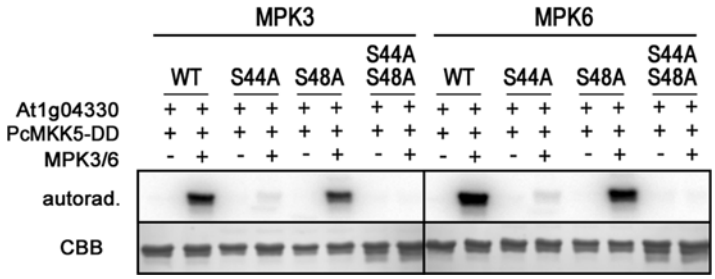

Fig. 2 Phosphorylation assays. Purified recombinant proteins were used in kinase phosphorylation assays as described [4]. Autoradiogram shows the phosphorylation of the At1g04330 substrate variants by MPK3 or MPK6, which were activated by inclusion of a constitutively active PcMKK5-DD upstream kinase. Note that specificity of the reaction is shown by the fact that active PcMKK5-DD did not phosphorylate the At1g04330 proteins. *Autorad.* autoradiography of SDS-gels, *CBB* Coomassie Brilliant Blue R-250 stain. ("Reproduced from Palm-Forster et al. [4] with permission from Academic Press")

3. PCR-amplification: Phusion High-Fidelity DNA Polymerase (Thermo Scientific; www.thermoscientific.com). Store at −20 °C.
4. dNTPs: aqueous solution (10 mM each), store at −20 °C.
5. PCR cycler: use any standard PCR cycler.

2.2 Gel Electrophoresis and Purification of PCR Products

1. Preparative agarose-gels: use gel electrophoresis-grade agarose cast in Tris-acetate-EDTA (TAE) buffer, where 50× TAE = 2 M Tris base, 17.5 % (v/v) glacial acetic acid, 50 mM EDTA (pH 8.0) (*see* **Notes 2** and **3**).
2. Gel electrophoresis: use any small-size gel electrophoresis device and standard gel staining procedures.
3. DNA purification from agarose gels: scalpels to cut out bands from the gels; use any commercially available kit (important, *see* **Note 3**).

2.3 Restriction Digest and Ligation

1. Restriction enzymes: DpnI, Eco31I, and *Ssp*DI (Thermo Scientific; *see* **Note 4**). Store at −20 °C.
2. Ligation: T4 DNA Ligase (Thermo Scientific; *see* **Note 5**). Store at −20 °C.

2.4 Transformation and Growth of Bacteria

1. Bacterial strain: use any standard competent *Escherichia coli* cloning strain, e.g., DH5 and standard transformation protocols (*see* **Note 6**).
2. Selection media: grow transformed bacteria on solid Lysogeny Broth (LB; [5]) media-containing petri dishes with selective antibiotics. For screening purposes, overnight cultures of individual colonies are grown in liquid LB (2 mL) containing the appropriate antibiotics (*see* **Note 7**).

2.5 Plasmid DNA Purification

1. Use any commercially available kit (*see* **Note 8**).

2.6 DNA Sequencing

1. Use any sequencing service available.

2.7 Expression of MAPK-Substrate Candidate Proteins in E. coli

1. Expression vector: use any suitable expression vector. We routinely use the pDEST-N110 vector (N-terminal 10× His-tag; [6]).
2. Gateway® LR Clonase® II Enzyme Mix (Life Technologies, www.lifetechnologies.com).
3. Bacterial strain: use any standard competent *Escherichia coli* expression strain suitable for the expression vector. We use the strain KRX (Promega, www.promega.com) for T7-promoter-driven expression.
4. Protein purification resin: depends on the tag of the protein. Here, Ni^{2+}-NTA agarose (Ni^{2+}-nitrilotriacetic acid (NTA)-agarose; Qiagen, www.qiagen.com) is used.

2.8 Kinase Assays and Phosphorylation Detection

1. Purified (mutated) recombinant MAPK substrate protein (*see* Subheading 3.6).
2. Kinases: His-tagged constitutively active MAP kinase kinase 5 from parsley (PcMKK5; [7]), GST-tagged MAP kinases 3 and 6 from *A. thaliana* (AtMPK3 and AtMPK6; [8]).
3. Kinase reaction buffer (5× concentrated): 100 mM HEPES (pH 7.5), 75 mM $MgCl_2$, 25 mM EGTA, 5 mM DTT, 50 µg/mL leupeptin, 50 µg/mL aprotinin (*see* **Note 9**). If autoradiography is used to detect phosphorylation, 0.1 µL of [γ^{32}P] ATP (specific activity = 3,000 Ci/mmol) is added. If nonradioactive detection is preferred, 20 µM ATP is used (*see* **Note 10**).
4. SDS-PAGE and Coomassie staining: standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis equipment is used. Gels are stained with Coomassie Brilliant Blue R-250 according to standard protocols.
5. Autoradiography (visualization of phosphorylation): use any suitable imaging machine or X-ray films. Alternatively, for nonradioactive visualization, Pro-Q Diamond phosphoprotein stain (Invitrogen, www.invitrogen.com) may be used.

3 Methods

3.1 Primer Design

For every putative phosphorylation site, a pair of primers is designed that consist of the following features (5′–3′ orientation): 2–6 variable nucleotides (*see* **Note 11**), the type IIs restriction enzyme recognition site (*see* **Note 12**), (parts of) the mutated

version of the phosphorylation site including the diagnostic restriction site (*see* **Note 13**) and 13–20 nucleotides complementary to the template for annealing (*see* Fig. 1).

3.2 PCR

1. For every mutagenesis reaction, 1–10 ng of template plasmid carrying the gene to mutate is mixed with Phusion High Fidelity DNA Polymerase buffer HF (final conc. 1×), 0.5 μM of both mutagenic primers, 200 μM dNTPs, and 0.02 U Phusion DNA Polymerase in a total volume of 50 μL.
2. The cycling conditions are as follows: initial denaturation at 98 °C for 30 s; followed by three cycles at 98 °C for 10 s, [annealing temperature I] (*see* **Note 14**) for 20 s, 72 °C for 90 s (*see* **Note 15**); followed by 15 cycles at 98 °C for 10 s, [annealing temperature II] (*see* **Note 16**) for 20 s, 72 °C for 90 s; followed by a final elongation step at 72 ° C for 5 min.

3.3 Purification of the PCR Products

1. Run the PCR products on 1 % (w/v) agarose gels, stain gels.
2. Check for the presence of bands representing the full-length plasmid. If the PCR worked, proceed to **step 3**, otherwise consult the Phusion DNA polymerase manual for troubleshooting.
3. Cut out the bands representing the full-length plasmid and isolate DNA with a kit according to the manufacturer's protocol.

3.4 Digestion–Ligation Step

1. Use standard PCR-tubes: mix a 17 μL aliquot of the eluted PCR product with 2 μL of 10× concentrated restriction buffer G (Thermo Scientific) and 10 U DpnI. Incubate for 2 h at 37 °C (or overnight; *see* **Note 17**).
2. To every mutagenesis reaction add (in a final volume of 25 μL) 0.5 μL 10× restriction buffer G, 5 U Eco31I, 2 U DpnI and 5 U T4 DNA Ligase. Incubate in a PCR cycler with the following program: 37 °C for 5 min, then 22 °C for 5 min, ten cycles (*see* **Note 18**).

3.5 Transformation and Screening of E. coli

3. Use a 5 μL aliquot of the digestion–ligation product to transform standard competent *E. coli* (*see* **Notes 6** and **19**).
4. Pick single colonies, transfer to a master plate and inoculate 2 mL liquid LB containing the appropriate antibiotics. Grow overnight at 37 °C.
5. Purify plasmid DNA and digest with diagnostic *Ssp*DI according to the vendors' protocols. Check for the presence of expected bands in agarose gels (*see* **Note 2**) and sequence positive clones.

3.6 Protein Expression

1. Transformation of competent KRX *E. coli* cells with plasmid DNA of a correct clone and growth of liquid expression cultures is performed according to the vendor's protocol.
2. Protein purification is performed using Ni^{2+}-NTA agarose according to the vendor's protocol.
3. Check purified protein for correct size and purity by SDS-PAGE and Coomassie stain before proceeding to kinase assays.

3.7 Kinase Assays and Phosphorylation Detection

1. Set up kinase reactions in a total volume of 20 µL: 4 µL 5× kinase reaction buffer, kinases, and MAPK substrate in a ratio of 1:1:10 (0.2 µg PcMKK5; 0.2 µg AtMPK3/6; 2 µg MAPK substrate). Incubate for 30 min at 37 °C.
2. Stop reactions by addition of 5 µL SDS sample buffer, boil for 5 min at 95 °C and perform SDS-PAGE.
3. Stain the gel with Coomassie, dry and expose to X-ray films or equivalent imaging technologies (*see* Fig. 2).

4 Notes

1. In this example, the pENTR™/D-TOPO® vector (Life Technologies; www.invitrogen.com) carrying the At1g04330 open reading frame is used. Note that pENTR/D-TOPO does not contain Eco31I sites in the plasmid backbone.
2. For preparation of agarose gels, 1× TAE buffer is used. We routinely use 1 % (w/v) agarose gels, but the gel concentration should be adjusted to accommodate optimal fractionation for the expected fragment size.
3. Purification of the PCR products is mandatory to dispose of the Phusion DNA polymerase, which otherwise would create blunt ends by filling-in the cohesive ends created in the combined digestion–ligation procedure (*see* Subheading 3.4).
4. DpnI is used to digest parental methylated plasmid, Eco31I generates cohesive ends necessary for ligation and *Ssp*DI is used to screen transformants for the presence of the diagnostic site (*see* Subheading 3.5).
5. T4 DNA ligase and restriction enzymes are used from the same vendor to allow a combined digestion–ligation approach using the type IIs restriction enzyme-specific buffer.
6. For standard protocols describing procedures to create competent bacterial cells and bacterial transformation, *see* ref. 9.
7. The pENTR™/D-TOPO® vector (*see* **Note 1**) mediates resistance to kanamycin. We use a working concentration of 50 µg/mL in both solid and liquid media.

8. A commercial column-based plasmid DNA purification is recommended to remove any contaminants that might inhibit DNA sequencing.
9. The 5× concentrated reaction buffer is diluted to 1× working concentration in the final kinase assays (*see* Subheading 3.7).
10. In this example, autoradiography is used to detect phosphorylation. Nonradioactive detection requires the Pro-Q® Diamond Phosphoprotein Gel Stain (Life Technologies).
11. The 5′-variable nucleotides are necessary for type IIs restriction enzyme binding in the combined digestion–ligation procedure (*see* Subheading 3.4).
12. Type IIs restriction enzymes cut outside their recognition sequence, thereby creating cohesive ends of any sequence. In this example, Eco31I is used, which cuts in the following manner: GGTCTC (N)1^…3′/(N)5^…5′ (*see* Fig. 1).
13. Typically, phosphorylation sites (serine/threonine–proline, S/T-P) are mutated to alanine–proline (A-P). Different codons coding for the same amino acid enable the incorporation of a diagnostic type II restriction site which is later used to screen transformants for the desired mutation. In this example, a diagnostic *Ssp*DI-site is incorporated spanning nucleotides coding for the mutated phosphorylation site (A-P) and the amino acid in −1 position. For further diagnostic site variants *see* Table 1. Individual primers may contain only parts of the site to mutate (including the diagnostic site), since the combined digestion–ligation approach creates the correct sequence features (*see* Fig. 1).
14. Annealing temperature I for the respective primer pair is calculated on the basis of only the 13–20 nucleotides complimentary to the template. (Be sure to use the recommended Tm Calculator for Phusion DNA Polymerase, http://www.thermoscientificbio.com/webtools/tmc/).
15. In this example, the size of the plasmid is 2.9 kb. According to the vendor's protocol, the Phusion DNA Polymerase needs 15–30 s/kb in the elongation phase, so we used 90 s. The elongation time needs to be adapted according to the template plasmid length used.
16. Annealing temperature II is calculated on the basis of the full primers. Often, this annealing temperature is above 72 °C, thus, a two-step cycling can be used: 98 °C for 10 s, followed by 72 °C for 90 s (*see* **Note 15**).
17. Do not shorten incubation time or use lower amounts of DpnI enzyme, since digestion of parental methylated plasmid has to be complete to avoid growth of bacteria carrying the non-mutated wild-type plasmid.

18. More cycles can be run to increase the amount of bacterial colonies.
19. If colony number is very low, the remaining digestion–ligation reaction mix can be evaporated, then dissolved in a small volume of dH_2O and transformed completely.

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Part V

Large Scale Studies on MAPKs, Substrates, and Interactions

Chapter 16

Yeast Two-Hybrid System for Dissecting the Rice MAPK Interactome

Raksha Singh, Sarmina Dangol, and Nam-Soo Jwa

Abstract

Protein–protein interactions are a preliminary but fundamental key to many biological systems. Identification of proteins that interact with particular bait not only contributes to a deeper understanding of bait protein function but also provides much information for the discovery of larger-scale interaction networks (interactome). Therefore, protein–protein interaction mapping is regarded as a widely accepted standardized functional genomics technique that provides comprehensive functional interpretation of previously uncharacterized proteins. A commonly used approach to detecting novel protein–protein interactions is the yeast two-hybrid system. In this chapter we describe in detail the protocols used to dissect the rice MAPK interactome, including the bait protein auto-activation test, identification of a rice MAPK interacting protein, confirmation of interaction by retransformation assay and characterization of the novel interacting protein.

Key words Yeast two-hybrid, Retransformation assay, Auto-activation test, Interactome, Protein–protein interaction

1 Introduction

Completion of rice genome sequencing has revealed a number of novel genes, including genes involved in the mitogen activated protein kinase (MAPK) cascade [1–4]. MAPK cascades are serine/threonine-specific protein kinases, which are conserved from yeast to mammals, and composed of MAP kinase kinase kinase (MAP3K), MAP kinase kinase (MAP2K), and MAPK family module [5]. MAPK cascades are involved in mediating a diverse array of extracellular responses by interaction with other receptors, kinases, transcription factors, and regulatory proteins [6–10]. Numerous MAPK genes have been reported in rice, without any specific functional clues [9–11]. Few rice MAPK genes have been functionally characterized [12–17]. The physical interaction between novel and well-studied proteins may provide information regarding the function of the novel protein. Therefore, identification of potential interacting

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partners and dissection of the interactome is a prerequisite for functional genomics [18].

Several studies have identifies potential interacting proteins: small-scale analyses of a small number of bait proteins to large-scale analyses containing more than 500 proteins, which yielded a comprehensive interactome [6, 19, 20]. Although identification of interactions between two proteins was initially carried out using various biochemical techniques, such as proteomics (LC-MS/MS), co-immunoprecipitation, cross-linking, and co-fractionation by chromatography, in recent years yeast two-hybrid systems have become the standard genetic tool for interaction mapping [21–24]. Interaction mapping using the yeast two-hybrid system is more convenient, versatile and less time-consuming than other technically difficult biochemical techniques because the yeast two-hybrid system utilizes the properties of the *Saccharomyces cerevisiae* transcriptional activator GAL4 protein [21, 23]. However, it should be noted that the yeast two-hybrid system is not suitable for identification of all predicted interactions for various reasons, such as protein folding, different subcellular compartmentalization of bait and prey proteins, nonspecific interactions, etc.

The yeast two-hybrid system is based on reporter gene activation by the physical interaction between the Gal4 transcription factor and upstream activating sequences (UAS) [21, 24]. The Gal4 transcription factor consists of two domains, one that binds to a specific DNA sequence, known as the DNA-binding (DB) domain, and another transactivation domain, known as the activation domain (AD), which activates the RNA polymerase to express the reporter gene [21, 23]. For interaction screening, bait and prey proteins are fused with DNA-binding and activation domains, respectively [23]. If bait containing the DNA-binding domain and prey containing the activation domain interact, transcriptional activation is reconstituted and the reporter gene is expressed [23].

Here, we describe in detail the step-by-step protocol used to generate the rice MAPK interaction map using specific yeast two-hybrid screening and library screening. For library screening, we developed a modified version of The ProQuest™ Two-Hybrid System (Catalog nos. PQ10001-01 and PQ10002-01) [25]. To generate the rice MAPK interaction map, we used two yeast strains: the AH109 strain for specific yeast two-hybrid screening and the MaV203 strain for yeast two-hybrid library screening [6]. Furthermore, we focused on the bait protein auto-activation test, which removes any nonspecific interactions, and a retransformation assay, which excludes any false-positive interaction and confirms the loss of interaction due to prey protein mutations. The yeast two-hybrid techniques developed to generate the rice MAPK interaction MAP will provide a basic scheme for generation of a complete rice MAPK interactome.

2 Material

All chemicals should be of analytical purity and solutions should be prepared in Milli-Q water. Handling and disposal of chemicals should comply with institutional safety regulations and good laboratory practice. At any moment standard equipments (balances, vortexers, benchtop centrifuges, water bath, pH meters, horizontal electrophoretic assembly, etc.) should be at hand and properly maintained. For the culture of bacteria and yeast it is essential to maintain: 30 °C incubator, 30 °C shaking incubator, 37 °C incubator, and a 37 °C shaking incubator.

2.1 Materials

2.1.1 Yeast Strains and Reporter Genes

1. The two yeast strains used were AH109 and MaV203. AH109 was MATα with genotype (trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL2UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-lacZ) [26]. MaV203 was MATα with genotype (leu2-3, 112, trp1-901, his3Δ200, ade2-101, gal4Δ, gal80Δ, SPAL10::URA3, GAL1::lacZ, HIS3UASGAL1::HIS3@LYS2, can1R, cyh2R) [27].
2. We used three reporter genes for each strain. AH109 and MaV203 featured three reporter genes –(GAL1::ADE2, GAL2::HIS3 and MEL1::lacZ) and –(GAL1::lacZ, GAL1::HIS3 and SPO13::URA3), respectively [25, 26]. Each reporter gene showed a different interaction phenotype upon its activation. GAL1::ADE2 reporter gene expression showed strong nutritional selection, whereas GAL2::HIS3 showed growth phenotype in the selection media with 3-aminotriazole (3-AT) [26]. The lacZ reporter gene, which encodes the β-galactosidase gene, results in strong blue/white staining upon activation in the presence of the X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) indicator [25]. SPO13:: URA3 reporter gene expression allows both positive and negative selection [25]. URA3 reporter gene induction results in conversion of 5-fluororotic acid (5-FOA) to the toxic compound, 5-fluorouracil [25]. Hence, positive selection of URA3 is accomplished on medium lacking uracil, whereas negative selection can be performed on medium lacking 5-FOA.

2.1.2 Media

1. YPD (Yeast extract Peptone Dextrose) Media preparation (solid agar plates, 1 L): 10 g yeast extract, 20 g Bacto Peptone, 40 mL 50 % (w/v) glucose, 20 g agar powder (*see* **Notes 1–4**).
2. YPD media preparation (liquid media, 1 L): 10 g yeast extract, 20 g Bacto Peptone, 40 mL 50 % (w/v) glucose (*see* **Note 5**).

3. SC-LEU-TRP media preparation (solid agar plates, 1 L): 6.7 g YNB with ammonium sulfate, 0.64 g CSM-LEU-TRP, 40 mL 50 % (w/v) glucose, 20 g agar powder (*see* **Notes 1–4**).
4. SC-LEU-TRP media preparation (liquid media, 1 L): 6.7 g YNB with ammonium sulfate, 0.64 g CSM-LEU-TRP, 40 mL 50 % (w/v) glucose (*see* **Note 5**).
5. SC-TRP media preparation (solid agar plates, 1 L): 6.7 g YNB with ammonium sulfate, 0.74 g CSM-TRP, 20 g agar powder, 40 mL 50 % (w/v) glucose (*see* **Notes 1–4**).
6. SC-TRP media preparation (liquid media, 1 L): 6.7 g YNB with ammonium sulfate, 0.74 g CSM-TRP, 40 mL 50 % (w/v) glucose (*see* **Note 5**).
7. SC-LEU media preparation (solid agar plates, 1 L): 6.7 g YNB with ammonium sulfate, 0.69 g CSM-LEU, 40 mL 50 % (w/v) glucose, 20 g agar powder (*see* **Notes 1–4**).
8. SC-LEU media preparation (liquid media, 1 L): 6.7 g YNB with ammonium sulfate, 0.69 g CSM-LEU, 40 mL 50 % (w/v) glucose (*see* **Note 5**).
9. SC-LEU-TRP-HIS + 3-AT (3-Amino-1,2,4-triazole) media preparation (solid agar plates, 1 L): 6.7 g YNB, 0.62 g CSM-LEU-TRP-HIS with ammonium sulfate, 40 mL 50 % (w/v) glucose, 20 g agar powder, 3-AT (*see* **Note 6**).
10. SC-LEU-TRP-URA media preparation (solid agar plates, 1 L): 6.7 g YNB with ammonium sulfate, 0.62 g CSM-LEU-TRP-URA, 40 mL 50 % (w/v) glucose, 20 g agar powder.
11. SC-LEU-TRP + 0.2 % (w/v) 5-FOA (5-Fluoroorotic Acid) media preparation (solid agar plates, 1 L): 6.7 g YNB with ammonium sulfate, 0.64 g CSM-LEU-TRP, 40 mL 50 % (w/v) glucose, 20 g agar powder, 2 g 5-FOA (*see* **Note 7**).
12. Luria–Bertani (LB) antibiotics-free media preparation for *E. coli* transformations (solid agar plates, 1 L): 15 g NaCl, 10 g tryptone, 5 g yeast extract, 15 g of agar powder (*see* **Note 8**).
13. LB antibiotics-free media preparation for *E. coli* transformation (liquid media, 1 L): 15 g NaCl, 10 g tryptone, 5 g yeast extract.
14. LB with selective antibiotics media preparation for *E. coli* transformation (solid agar plates, 1 L): 15 g NaCl, 10 g tryptone, 5 g yeast extract, 15 g agar powder, 1 mL of 100 mg/mL of selected antibiotics (*see* **Note 9**).
15. LB with selective antibiotics media preparation for *E. coli* transformation (liquid media, 1 L): 15 g NaCl, 10 g tryptone, 5 g yeast extract, 1 mL of 100 mg/mL of selected antibiotics (*see* **Note 9**).
16. 50 mL LB liquid media for *E. coli* transformation (Antibiotics free): 0.75 g of NaCl, 0.5 g of tryptone, 0.25 g of yeast extract.

2.1.3 Solutions

1. LiSORB solution for specific yeast transformation: 100 mM lithium acetate (LiAc), 1 M sorbitol in TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Prepare by diluting 2 M LiAc, 1 M Tris–HCl pH 8.0, 0.5 M EDTA pH 8.0, 5 M Sorbitol. Sterilize by autoclaving (*see* **Note 10**).
2. Salmon Sperm DNA (ssDNA): 2 mg/mL, dilute from 10 mg/mL stock in ultrapure water and denature in boiling water bath (*see* **Note 11**).
3. Polyethylene Glycol (PEG)-3400: 50 % (w/v) PEG-3400 in ultrapure water (*see* **Note 12**). Sterilize by autoclaving and store at room temperature.
4. 10× Lithium Acetate (LiAc): 10.201 g LiAc in 100 mL ultrapure water. Sterilize by autoclaving.
5. 10× TE: 1 M Tris–HCl pH 7.5, 0.5 M EDTA pH 8.0. Sterilize by autoclaving (*see* **Note 13**).
6. Z-buffer for β-galactosidase assay specific screening: 3.22 g of $Na_2HPO_4 \cdot 7H_2O$, 1.1 g of $NaH_2PO_4 \cdot 7H_2O$, 0.15 g of KCl, 0.0492 g of $MgSO_4 \cdot 7H_2O$ in 200 mL ultrapure water. Sterilize by autoclaving and store at 4 °C.
7. X-gal (5-bromo-5-chloro-3-indolyl-β-D-galactoside): 20 mg/mL X-gal powder in *N*,*N*-dimethylformamide (DMF). Mix 0.02 g of X-gal in 0.98 mL of *N*,*N*-dimethyl formamide (DMF) in the sterile eppendorf tube. Adjust volume to 1 mL with DMF. Aliquot and store at –20 or –80 °C (*see* **Note 14**).
8. 1× LiAc/40 % (w/v) PEG-3350/1× TE: add 40 g of PEG-3350 in minimal amount of prewarmed ultrapure water, dilute accordingly 10× LiAc and 10× TE, bring final volume to 100 mL with ultrapure water and filter sterilize.
9. 0.1 M $CaCl_2$.

2.1.4 Materials for Nucleic Acid Isolation and Purification

1. Wizard® plus SV Miniprep DNA purification system (Promega, Catalog no. A1460).
2. Yeast Plasmid DNA isolation Kit (MP Biol, Catalog no. 2069-400).
3. Accuprep® PCR purification Kit (Bioneer, Catalog no. K-3034).

3 Methods

3.1 Generation of DB-X (Bait) and AD-Y (Prey) Constructs

Bait (DB-X) and prey (AD-Y) plasmids need to be generated before yeast transformation. (DB-X) and (AD-Y) are generated by cloning gene of interest (X and Y) into Gateway compatible vectors (pDEST™ 32 and pDEST™ 22) following Gateway recombination reaction. Here, X and Y represent the bait gene of interest (X) and prey gene of interest (Y) respectively.

3.1.1 DB-X (Bait) Construction

For this, gene of interest needs to be cloned in-frame to downstream of GAL4 DBD (DNA binding domain).

1. Amplify gene of interest ORF with adaptive primer containing attB1 and attB2 sites (*see* **Note 15**).
2. Perform a BP recombination reaction between the amplified product with attB1 and attB2 sites and a donor vector (pDONR™ 221) (*see* **Note 16**).
3. Transform into *E. coli* and choose the correct clone (*see* **Note 17**).
4. Confirm the reading frame of entry clone (gene of interest in pDONR™ 221 vector) before inserting into pDEST™ 32 vector (*see* **Note 18**).
5. Perform a LR recombination reaction between the purified entry clone with attL1 and attL2 sites and bait plasmid pDEST™ 32 (*see* **Note 19**).
6. Transform into *E. coli* and choose the correct clone (*see* **Note 17**).

3.1.2 DB-Y (Prey) Construction

For this, gene of interest needs to be cloned in-frame to downstream of GAL4 AD (Activation Domain).

1. Amplify gene of interest ORF with adaptive primer containing attB1 and attB2 sites (*see* **Note 15**).
2. Perform a BP recombination reaction between the amplified product with attB1 and attB2 sites and a donor vector (pDONR™ 221) (*see* **Note 16**).
3. Transform into *E. coli* and choose the correct clone (*see* **Note 17**).
4. Confirm the reading frame of entry clone (gene of interest in pDONR™ 221 vector) before inserting into pDEST™ 22 vector (*see* **Note 18**).
5. Perform a LR recombination reaction between the purified entry clone with attL1 and attL2 sites and bait plasmid pDEST™ 22 (*see* **Note 19**).
6. Transform into *E. coli* and choose the correct clone (*see* **Note 17**).

3.2 Specific Yeast Transformation

Experimental outline of this method is given as a flowchart in Fig. 1.

1. Streak AH109 yeast cells on an YPD plate and incubate for 3 days at 30 °C (*see* **Note 20**).
2. Pick the single colony using a sterile toothpick and culture in 10 mL YPD liquid media for 2 days at 30 °C.
3. Resuspend 10 mL yeast cells (cultured for 2 days at 30 °C) in 250 mL YPD to obtain an OD_{600} of ~0.2 and culture for 4–5 h at 30 °C in shaking incubator (220 rpm). The OD_{600} should reach ~0.8.

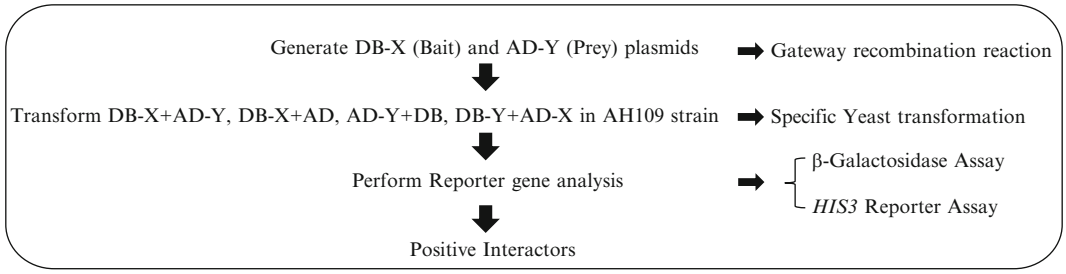

Fig. 1 Experiment outline for specific yeast two-hybrid screening

4. Harvest the cells in six 50 mL Falcon tube by centrifugation at 3,000 rpm (890 × *g*) for 10 min at room temperature.
5. Discard the supernatant and wash the cells by adding 5 mL sterile water in each tube and collect all of the cells from six tubes to one tube and make final volume 50 mL with sterile water. During resuspension do not use vortex but instead gently resuspend with pipette.
6. Centrifuge the cells at 3,000 rpm (890 × *g*) for 10 min at room temperature.
7. Discard the supernatant and gently resuspend the cells first in 5 mL LiSORB solution and finally make total volume 20 mL with LiSORB solution.
8. Incubate in 30 °C incubator for 30 min.
9. Centrifuge the cells at 3,000 rpm (890 × *g*) for 10 min at room temperature.
10. Discard the supernatant and gently resuspend the cells by pipetting in 250 μL LiSORB solution. Finally make the volume 500 μL with LiSORB solution (yeast competent cells).
11. In a separate 1.5 mL eppendorf tube, make a mixture (500 μL) of 100 μL salmon sperm DNA (2 mg/mL) and 400 μL LiSORB solution.
12. Add 500 μL of the mixture (salmon sperm DNA and LiSORB solution) to the competent cells from **step 10**.
13. Make 5 mL solution of (50 % (w/v) PEG 3400/10× LiAc/10× TE) by adding 4 mL 50 % (w/v) PEG 3400, 0.5 μL 10× LiAc, and 0.5 μL 10× TE (*see* **Note 21**).
14. Add 5 mL of 50 % (w/v) PEG 3400/10× LiAc/10× TE solution to the solution from **step 12**.
15. Aliquot 1 mL of the resulting solution from **step 14** to 1.5 mL eppendorf tubes and add 100 ng of bait (DB-X) and prey (AD-Y) plasmids of interest. For swapping, add 100 ng (DB-Y) and (AD-X) plasmids. For negative controls, add 100 ng of bait (DB-X) or prey (AD-Y) plasmids with 100 ng of empty vectors (pDEST™ 22, AD).

16. Invert.
17. Incubate for 30 min at 30 °C.
18. Heat-shock for 20 min at 42 °C.
19. Quick centrifuge for 5 s and discard the supernatant.
20. Resuspend the cells in 100 μL sterile water and spread on the appropriate selection plates (*see* **Note 22**).
21. Incubate for 3–4 days at 30 °C (*see* **Note 23**).
22. Check the colonies.

3.3 Reporter Gene Analysis

3.3.1 β-Galactosidase Assay

1. Pick colonies from **step 22** in 2 mL eppendorf tubes containing 1 mL SC-LEU-TRP broth and culture overnight in 30 °C shaking incubator.
2. Dilute the cultured cells to an OD_{600} of ~0.2 with sterile water and drop 12 μL on YPD plates (*see* **Note 24**).
3. Incubate YPD plate for 2 days at 30 °C incubator.
4. Prepare 2 mL solution of Z buffer and X-gal. For 2 mL solution, add 1.96 mL Z buffer, 5.4 μL β-mercaptoethanol and 33.4 μL X-gal (20 mg/mL).
5. For each plate, stack one sterile Whatman filter paper in an empty petri plate (15 cm).
6. For each plate, use 1.8 mL of Z buffer solution from **step 4**.
7. Pour the Z buffer (1.8 mL) to the petri plate containing the Whatman paper; make sure the entire paper is soaked in Z buffer.
8. Remove air bubbles if any with forceps.
9. Place ~200 mL liquid nitrogen into an ice box with lid and cover the box with lid.
10. Using forceps, carefully attach the nitrocellulose membrane to the surface of the YPD plate from **step 3** until all of the cells from plate attached to the membrane.
11. Carefully remove the membrane containing yeast patches from the surface of the YPD plate with forceps and immediately freeze for 20 s in liquid nitrogen.
12. Thaw the membrane at room temperature by holding with forceps for few seconds (*see* **Note 25**).
13. Carefully place the membrane on top of the soaked Whatman paper with yeast patches upside. Remove any air bubbles.
14. Remove excess buffer by slightly slanting the petri plate.
15. Incubate at 37 °C at slanting position; check frequently for the development of blue color over 24 h.
16. Score the interaction strength depending on the intensity of the blue color change. Usually, strong interactors show blue

color within a 1–24 h period. Weak interactors show very dim blue or remain white until 96 h. However, in many cases weak interactors remain white on β-galactosidase Assay but show growth on SC-LEU-TRP-HIS (3-AT) plates (*see* **Note 26**).

3.3.2 HIS3 Reporter Gene Assay

1. Prepare (SC-LEU-TRP-HIS + 3-AT) selective plates without leucine, tryptophan and histidine with 5 mM 3-AT.
2. Pick colonies from **step 22** in 2 mL eppendorf tubes containing 1 mL SC-LEU-TRP liquid media and culture overnight in 30 °C shaking incubator.
3. Dilute the cultured cells to an OD_{600} of ~0.2 with sterile water and drop 12 μL on SC-LEU-TRP-HIS + 3-AT (5 mM) plates.
4. Incubate SC-LEU-TRP-HIS + 3-AT (5 mM) plate for 4 days at 30 °C incubator.
5. Check the growth pattern on fourth day.
6. Score the interaction strength based on the growth pattern of the yeast cells.
7. Strong interactors show excellent growth pattern within 4 days of incubation whereas weak interactors show normal or low growth within 6 days of incubation. However, while scoring weak interactors it is highly recommended to compare the growth pattern with negative controls (Bait or Prey proteins with empty vectors). Distinguishable growth patterns must be seen between interactors and the negative controls (*see* **Note 27**).

3.4 Swapping Interactors

Swap the protein of interest (X or Y) from DB to AD plasmids and vice versa. Perform all of the steps as mentioned in Subheading 2.

3.5 Yeast Two-Hybrid Library Screening

Experimental outline of this method is given as a flowchart in Fig. 2.

3.5.1 Small Scale Yeast Transformation

1. Streak MaV203 yeast cells on an YPD plate and incubate for 3 days at 30 °C (*see* **Note 20**).
2. Pick the single colony using a sterile toothpick and culture in 10 mL YPD liquid media overnight at 30 °C.
3. Resuspend 10 mL yeast cells (cultured for overnight at 30 °C) in 250 mL YPD to obtain an OD_{600} of ~0.2 and culture for 4–5 h at 30 °C in shaking incubator (220 rpm). The OD_{600} should reach ~0.5.
4. Centrifuge the cells in six 50 mL Falcon tubes at 3,000 rpm (890 × *g*) for 5 min at room temperature.
5. Discard the supernatant and resuspend the pellet in 40 mL 1× TE (*see* **Note 28**).
6. Centrifuge the cells at 3,000 rpm (890 × *g*) for 5 min at room temperature.

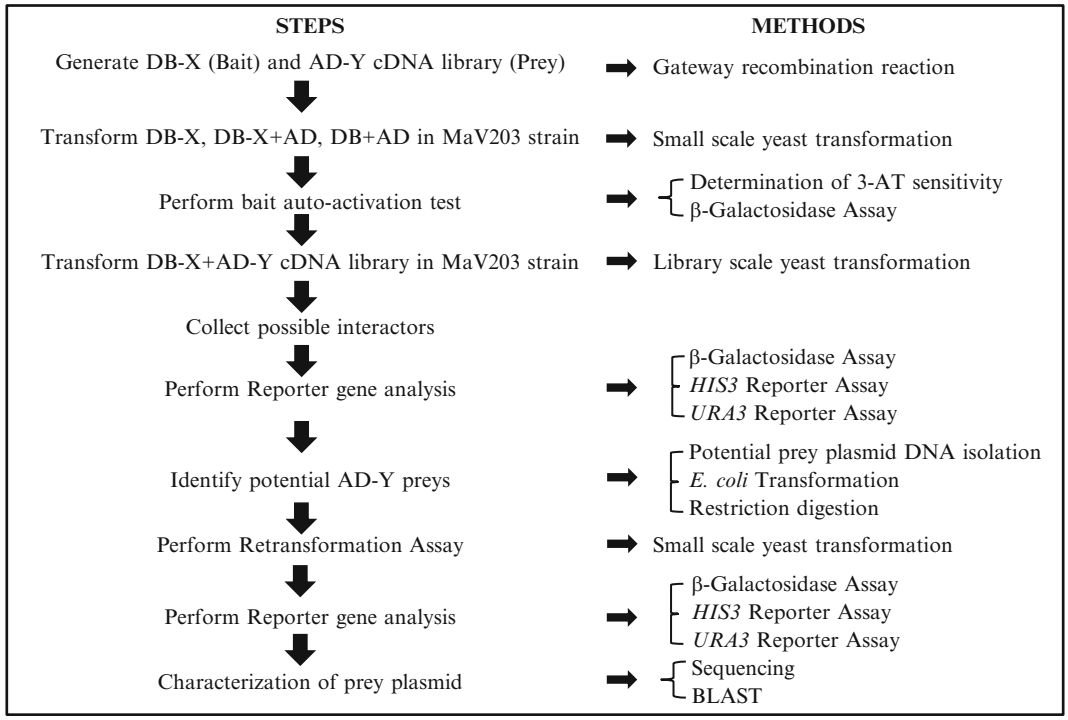

Fig. 2 Experiment outline for library scale yeast two-hybrid screening

7. Discard the supernatant and gently resuspend the cells first in 1 mL 1× LiAc/0.5× TE and finally make total volume 2 mL with 1× LiAc/0.5× TE (*see* **Note 29**).
8. Incubate the cells at room temperature for 10 min.
9. Immediately use the yeast competent cells from **step 8** for transformation.
10. Aliquot 100 µL of the yeast suspension from **step 8** to 1.5 mL eppendorf tubes and add 1 µg of plasmids [(DB-X), (AD-Y), (DB-X+AD-Y), (DB-X+AD), (AD-Y+DB), (DB+AD)] of interest with 10 µL salmon sperm DNA (10 mg/mL) (*see* **Note 11**).
11. Add 700 µL of 1× LiAc/40 % (w/v) PEG-3350/1× TE to the solution from **step 9** and mix well (*see* **Note 30**).
12. Incubate for 30 min at 30 °C.
13. Add 88 µL DMSO solution.
14. Heat-shock for 10 min at 42 °C.
15. Quick centrifuge for 10 s and discard the supernatant.
16. Resuspend the cells in 1 mL 1× TE.
17. Quick centrifuge for 10 s and discard the supernatant.
18. Resuspend the cells in 100 µL 1× TE.

19. Spread on appropriate selection plates (*see* **Note 31**).
20. Incubate for 3–4 days at 30 °C.
21. Check the colonies and proceed to next step.

3.5.2 Bait Auto-activation Test with Determination of 3-AT Sensitivity

1. Prepare (SC-LEU-TRP-HIS + 3-AT) selective plates without leucine, tryptophan and histidine containing 0, 10, 25, 50, 75 and 100 mM 3-AT.
2. Pick colonies from **step 21** (DB-X + AD, DB + AD) in 1.5 mL eppendorf tubes containing 1 mL SC-LEU-TRP liquid media and culture overnight in 30 °C shaking incubator.
3. Dilute the cultured cells to an OD_{600} of ~0.2 with sterile water and drop 12 μL on SC-LEU-TRP-HIS + 3-AT (0, 10, 25, 50, 75 and 100 mM) plates.
4. Incubate for 4 days at 30 °C incubator.
5. Analyze the growth pattern. If bait plasmid with empty vector (DB-X + AD) grow on all SC-LEU-TRP-HIS + 3-AT (0, 10, 25, 50, 75 and 100 mM) plates, it is not suitable for yeast two-hybrid screens due to auto-activation property. If bait plasmid show growth pattern until 25 mM 3-AT but growth inhibition at 50 mM 3-AT, than 50 mM 3-AT can be used as an optimum concentration for library screening. Representative figure of growth inhibition by 3-AT is presented in Fig. 3. Depending upon the bait protein, optimum 3-AT concentration for screening is different (*see* **Note 32**).

3.5.3 Bait Auto-activation Test with β-Galactosidase Assay

1. Pick colonies from **step 21** (Subheading 3.5.1) in 1.5 mL eppendorf tubes containing 1 mL SC-LEU-TRP liquid media and culture overnight in 30 °C shaking incubator.
2. Dilute the cultured cells to an OD_{600} of ~0.2 with sterile water and drop 12 μL on YPD plates.

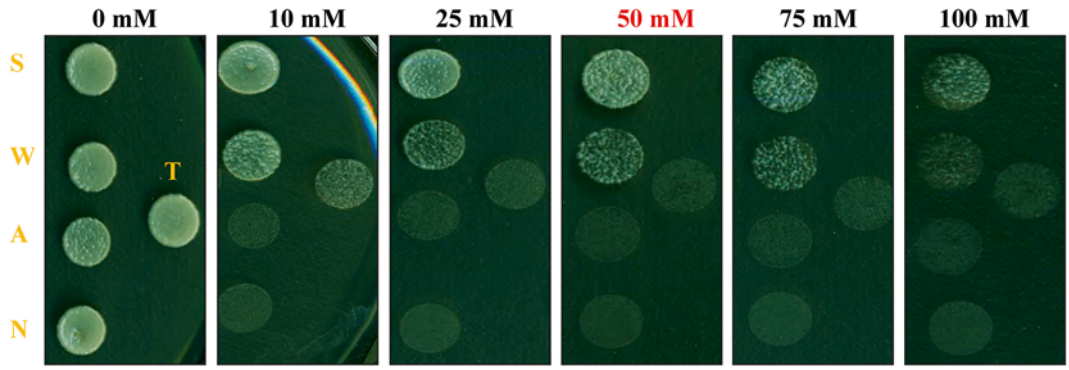

Fig. 3 Determination of 3-AT sensitivity. The optimum concentration of 3-AT for bait T screening is shown in *red font* (50 mM). Control plasmid pairs: S (strong), pEXP™ 32/Krev1 + pEXP™ 22/RalGDS-wt; W (weak), pEXP™ 32/Krev1 + pEXP™ 22/RalGDS-m1; and A (absent), pEXP™ 32/Krev1 + pEXP™ 22/RalGDS-m2 are used as ref. 25. N represents combination of DB + AD empty vectors and T represents DB-X bait

3. Incubate YPD plate overnight in 30 °C incubator.
4. Prepare 10 mL solution of Z buffer and X-gal. For 10 mL solution, combine 100 μL X-gal (100 μg/μL), 60 μL β-mercaptoethanol and 10 mL Z buffer (*see* **Note 33**).
5. For each plate, place two sterile Whatman filter papers in an empty petri plate (15 cm).
6. For each plate, use 10 mL of Z buffer solution from **step 4**.
7. Pour the Z buffer (10 mL) to the petri plate containing the Whatman paper; make sure the entire paper is soaked in Z buffer.
8. Remove air bubbles if any with forceps. Place ~200 mL liquid nitrogen into an ice box with lid and cover the box with lid.
9. Using forceps, carefully attach the nitrocellulose membrane to the surface of the YPD plate from **step 3** until all of the cells from plate attached to the membrane.
10. Carefully remove the membrane containing yeast patches from the surface of the YPD plate with forceps and immediately freeze for 20 s in liquid nitrogen.
11. Thaw the membrane at room temperature by holding with forceps for few seconds (*see* **Note 25**).
12. Carefully place the membrane on top of the soaked Whatman paper with yeast patches upside. Remove any air bubbles.
13. Remove excess buffer by slightly slanting the petri plate.
14. Incubate at 37 °C with slanting position; check frequently the appearance of blue color over 24 h.
15. Analyze the phenotype of the yeast patches. If bait plasmid with empty vector (DB-X + AD) shows blue color, it is not suitable for yeast two-hybrid screens due to auto-activation property.

3.6 Library Scale Yeast Transformation

1. Streak MaV203 yeast cells transformed with bait plasmid on an SC-LEU plate and incubate for 3 days at 30 °C (*see* **Note 20**).
2. Pick the single colony using a sterile toothpick in 20 mL SC-LEU liquid media and grow overnight at 30 °C.
3. Mix 20 mL yeast cells (cultured overnight at 30 °C) in 300 mL SC-LEU to obtain an OD_{600} of ~0.1 and culture for 4–5 h at 30 °C in shaking incubator (220 rpm). The OD_{600} should reach ~0.5.
4. Harvest the cells in six 50 mL Falcon tubes at 3,000 rpm (890 × *g*) for 5 min at room temperature.
5. Discard the supernatant and resuspend the pellet in 30 mL sterile water (*see* **Note 34**).
6. Centrifuge the cells at 3,000 rpm (890 × *g*) for 5 min at room temperature.

7. Discard supernatant and gently resuspend the cells first in 600 µL 1× TE/1× LiAc and finally adjust volume to 1.5 mL with 1× TE/1× LiAc (*see* **Note 35**).
8. Immediately use the yeast competent cells from **step 7** for transformation.
9. Prepare thirty 1.5 mL sterile eppendorf tubes. Aliquot 50 µL of the yeast suspension from **step 7** to each 30 tubes. Combine 1 µg library DNA and 5 µL salmon sperm DNA (10 mg/mL) to each tube (*see* **Note 36**).
10. Add 300 µL of 1× LiAc/40 % (w/v) PEG-3350/1× TE to each tube and mix by inverting the tube (*see* **Note 30**).
11. Incubate for 30 min at 30 °C shaking incubator.
12. Add 40 µL DMSO solution.
13. Heat-shock for 10 min at 42 °C.
14. Keep in ice for 20–30 min (*see* **Note 37**).
15. Quick centrifuge for 10 s and discard the supernatant.
16. Resuspend the pellet in 300 µL 0.9 % saline.
17. Divide 300 µL on two SC-LEU-TRP-HIS (+3-AT) plates and spread (*see* **Note 38**).
18. Incubate until 7 days at 30 °C.
19. Check the colonies regularly and proceed to next step.

3.7 Collection of Possible Interactors

1. Pick the colonies that appear until 7 days from **step 19** and streak on SC-LEU-TRP plates (*see* **Note 39**).
2. Precede confirmation of interactors by various reporter assays.

3.8 Reporter Gene Analysis

3.8.1 β-Galactosidase Assay

1. Pick colonies of possible interactors from Subheading 3.6 in 1.5 mL eppendorf tubes containing 1 mL SC-LEU-TRP liquid media and culture overnight in 30 °C shaking incubator.
2. Follow all the steps from Subheading 3.5.3.
3. Score the interaction strength depending upon the intensity of the blue color change. Usually, strong interactors show blue color within 1–24 h period. Weak interactors show very dim blue or remain white until 96 h. However, in many cases weak interactors remain white on β-Galactosidase Assay but show growth on SC-LEU-TRP-HIS (3-AT) plates.

3.8.2 HIS3 Reporter Assay

1. Prepare (SC-LEU-TRP-HIS + 3-AT) selective plates without leucine, tryptophan, and histidine containing (0, 10, 25, 50, 75, and 100 mM) 3-AT (*see* **Note 6**).
2. Pick colonies of possible interactors from Subheading 3.6 in 1.5 mL eppendorf tubes containing 1 mL SC-LEU-TRP liquid broth and culture overnight at 30 °C shaking incubator.

3. Dilute the cultured cells to an OD_{600} of ~0.2 with sterile water and drop 12 μL on SC-LEU-TRP-HIS + 3-AT (0, 10, 25, 50, 75 and 100 mM) 3-AT plates.
4. Incubate above plate from **step 3** for 4 days at 30 °C incubator.
5. Check the growth pattern on fourth day.
6. Score the interaction strength depending upon the growth pattern of the yeast cells.
7. Strong interactors show excellent growth pattern within 4 days of incubation whereas weak interactors show normal or low growth within 6 days of incubation. However, while scoring weak interactors it is highly recommended to compare the growth pattern with negative controls (Bait or Prey proteins with empty vectors). Distinguishable growth patterns must be seen between interactors and the negative controls.

3.8.3 URA3 Reporter Assay (Positive Selection)

1. Prepare (SC-LEU-TRP-URA) selective plates without leucine, tryptophan and uracil.
2. Pick colonies of possible interactors from Subheading 3.6 in 1.5 mL eppendorf tubes containing 1 mL SC-LEU-TRP liquid media and culture overnight in 30 °C shaking incubator.
3. Dilute the cultured cells to an OD_{600} of ~0.2 with sterile water and drop 12 μL on (SC-LEU-TRP-URA) plates.
4. Incubate above plate from **step 3** for 4 days at 30 °C incubator.
5. Check the growth pattern on fourth day.
6. Score the interaction strength depending upon the growth pattern of the yeast cells.
7. Strong interactors show excellent growth pattern within 4 days of incubation whereas weak interactors show normal or low growth within 6 days of incubation. However, while scoring weak interactors it is highly recommended to compare the growth pattern with negative controls (Bait or Prey proteins with empty vectors). Distinguishable growth patterns must be seen between interactors and the negative controls.

3.8.4 URA3 Reporter Assay (Negative Selection)

1. Prepare (SC-LEU-TRP + 0.2 % (w/v) 5-FOA) selective plates without leucine, tryptophan and with 0.2 % (w/v) 5-FOA as mentioned in Subheading 3.
2. Pick colonies of possible interactors from Subheading 3.6 in 1.5 mL eppendorf tubes containing 1 mL SC-LEU-TRP liquid media and culture overnight in 30 °C shaking incubator.
3. Dilute the cultured cells to an OD_{600} of ~0.2 with sterile water and drop 12 μL on (SC-LEU-TRP + 0.2 % (w/v) 5-FOA) plates.

4. Incubate above plate from **step 3** for 4 days at 30 °C incubator.
5. Check the growth pattern on fourth day.
6. Score the interaction strength depending upon the growth pattern of the yeast cells.
7. Strong interactors show no growth whereas weak interactors show growth within 6 days of incubation (*see* **Note 40**).

3.9 Identification of Potential AD-Y Preys

1. Transfer the colonies showing positive test for at least one or two reporter gene assays onto fresh SC-LEU-TRP plates (*see* **Note 41**).
2. Grow each colony from **step 1** in 5 mL SC-TRP liquid media overnight in 30 °C shaking incubator.
3. Isolate potential prey (AD-Y) plasmid DNA using plasmid DNA isolation kit (MP Bio, Catalog no. 2069-400) (*see* **Note 42**).
4. Proceed with *E. coli* transformation of the isolated yeast plasmid DNA (*see* Subheading 3.9.1. below for detail protocol (*see* **Note 43**).

3.9.1 E. coli Transformation (Yeast Plasmid DNA)

1. Prepare LB antibiotics-free plates.
2. Streak *E. coli* (DH5α) on LB antibiotics-free plate and incubate overnight at 37 °C (*see* **Note 44**).
3. Pick single colony with sterile tooth pick in 4 mL LB antibiotics-free broth and grow overnight at 37 °C.
4. Transfer 1 mL overnight *E. coli* culture into 50 mL LB antibiotics free in 250 mL conical flask.
5. Grow cells at 37 °C for 90 min until $OD_{600} \sim 0.5$.
6. Place sterile empty 50 mL Falcon tube in ice. Also place 0.1 M $CaCl_2$ solution in ice (*see* **Note 45**).
7. Transfer the cells from **step 5** in chilled 50 mL Falcon tube from **step 6** and store in ice for 20–30 min.
8. Harvest the cells at 3,000 rpm ($890 \times g$) for 5 min at 4 °C.
9. Discard supernatant and resuspend the cells in 5 mL chilled 0.1 M $CaCl_2$ solution and adjust the volume to 10 mL with 0.1 M $CaCl_2$.
10. Store the solution from **step 9** on ice for 20–30 min (*see* **Note 46**).
11. Centrifuge the cells at 3,000 rpm ($890 \times g$) for 5 min at 4 °C.
12. Decant supernatant and resuspend pellet in 1–2 mL chilled 0.1 M $CaCl_2$ solution.
13. Immediately use the competent cells from **step 12** for transformation.

14. Place 1.5 mL sterile eppendorf tubes in ice before transformation.
15. Aliquot 100 µL of *E. coli* competent cells from **step 12** to each tube in ice. Combine 8 µL of yeast plasmid DNA from step to each tube (*see* **Note 47**).
16. Slightly tap at the bottom of the tube.
17. Incubate for 20 min in ice.
18. Heat-shock for 40–90 s at 42 °C.
19. Keep in ice for 5 min (*see* **Note 48**).
20. Transfer the cells into 1 mL LB free antibiotics liquid media in test-tube.
21. Incubate in 37 °C shaking incubator for 1 h.
22. Transfer the cells in 1.5 mL sterile eppendorf tube and centrifuge for 1 min at 3,000 rpm (890 × *g*) at room temperature.
23. Discard the supernatant and resuspend the pellet in 200 µL of remaining supernatant.
24. Spread the cells in LB plates with appropriate antibiotic (*see* **Note 49**).
25. Incubate the plate overnight at 37 °C.
26. Next day, pick colonies from the LB plate from **step 25** with sterile tooth pick and culture overnight in 4 mL LB liquid media with appropriate antibiotics at 37 °C.
27. Isolate plasmid DNA using Wizard® Plus SV Minipreps DNA Purification System (*see* **Note 50**).
28. Perform restriction digestion of the isolated DNA from **step 27** (*see* **Note 51**).
29. Use the potential prey plasmid with specific restriction patterns and bait plasmid for yeast retransformation assay (*see* **Note 52**).

3.10 Retransformation Assay

3.10.1 Small Scale Yeast Transformation

1. Streak transform bait plasmid in MaV203 cells on an YPD plate and incubate for 3 days at 30 °C.
2. Transform combination of bait and potential prey plasmids as well as negative controls.
3. For transformation, follow **steps 2–21** as mentioned above in Subheading 3.9.
4. Check the colonies and proceed to next step.

3.10.2 Reporter Gene Analysis

Sequence analysis (Prey plasmids).

1. Positive prey plasmid DNA showing positive test for at least one or two reporter gene assays are subjected to sequence analysis (*see* **Note 53**).

2. For sequencing, use GAL4 DNA Activation Domain forward sequencing primer (TAT AAC GCG TTT GGA ATC ACT) and reverse sequencing primer (AAG CCG ACA ACC TTG ATT GGA GAC) (*see* **Note 54**).
3. Blast the resulting sequence in NCBI site against *Oryza sativa* genome database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?).

4 Notes

1. While preparing any solution, do not dissolve all solute directly in full volume of solvent, i.e., if you are preparing 1,000 mL of solution; first add about 800 mL of water to solute completely dissolve the solute and add remaining amount of water. It is because when large amount of solute is added solution volume increases. Other solutions should be similarly prepared.
2. Before autoclaving make sure that no lumps are remaining in the solution.
3. Prepare 50 % (w/v) glucose in separate bottle, autoclave and store at RT. After autoclaving, media should be opened only inside a clean laminar flow bench and also addition of remaining solution should be done inside clean bench in order to prevent contaminations.
4. Solid agar plates can be stored at 4 °C for maximum 1 month.
5. Liquid media can be stored at 4 °C for maximum 1 month.
6. After autoclaving SC-LEU-TRP-HIS media, let it cool till 65 °C in RT and then add appropriate amount of 3-AT inside clean bench. For specific screening, use 5 mM 3-AT and for library screening 3-AT concentration depends upon the growth inhibition of bait protein by 3-AT. The amount of 3-AT for different concentration; 0.1051 g for 5 mM, 0.210 g for 10 mM, 0.526 g for 25 mM, 1.051 g for 50 mM, 1.577 g for 75 mM and 2.102 g for 100 mM.
7. Liquid 5-FOA can also be used. 5-FOA is light sensitive. Prevent it from light while preparing 5-FOA agar plates.
8. LB powder mix can also be used instead of individual components. For LB-powder mix 25 g in 1 l distilled water.
9. Prepare 100 mg/mL stock solution of antibiotics and aliquot and store at −20 °C. Antibiotics should be added after autoclaving of the media and only after temperature is tolerable by touch.
10. Prepare 5 M sorbitol by adding 91.085 g sorbitol in 100 mL ultrapure distilled water and autoclave at 121 °C for 15 min and store in room temperature. Prepare 2 M LiAc by adding 20.4 g LiAc in 100 mL and autoclave at 121 °C for 15 min and

store in room temperature. To prepare 1 M Tris–HCl pH 8.0 add 12.114 g of Trizma-base in 100 mL ultrapure distilled water and then maintain the pH to 8.0 by adding 0.1 M HCl. Sterilize the solution by autoclaving at 121 °C for 15 min and store in room temperature. To prepare aqueous solution of 0.5 M EDTA pH 8.0 weigh 18.612 g of EDTA in 100 mL ultrapure distilled water and mix well. While mixing, add 0.1 M of NaOH to adjust the pH 8.0 and allow dissolution of free acid EDTA. Sterilize the solution by autoclaving at 121 °C for 15 min.

11. Denature the ssDNA at 100 °C in water bath for 10 min before use. Aliquot the stock solution and store at –20 or –80 °C for long term.
12. Do not add 50 g PEG-3400 at one time while dissolving. Dissolve it in aliquot volumes to make it dissolve faster.
13. To prepare 1 M Tris–HCl pH 7.5 weigh 12.114 g of Tris-Base (Trizma-base) in 100 mL ultrapure distilled water and mix well and then maintain the pH to 7.5 by adding 0.1 M HCl. Sterilize the solution by autoclaving at 121 °C for 15 min and store in room temperature.
14. X-gal is light sensitive so store in dark.
15. Make gene specific primer with attB1 and attB2 sites.

    Gene specific attB1: AA AAA GCA GGC TNN NNN NNNNNNNNNNNNNN

    Gene specific attB2: AGA AAG CTG GGT NNN NNNNNNNNNNNNNN NN

    N: Gene specific primer sequence.

    Perform the first PCR with gene specific attB1 and attB2 primers. Dilute first PCR product to 1/100 with sterile water and perform second PCR with attB1 and attB2 primers.

    attB1: GGG GAC AAG TTT GTA CAA AAA AGC AGG CT
    attB2: GGG ACC ACT TTG TAC AAG AAA GCT GGG T

16. Purify the second PCR product with Accuprep® PCR purification kit and set up BP reaction as follows:

Purified second PCR product (50–100 ng/rxn)	1–7 µL
Donor vector (100 ng/µL)	1 µL
BP clonase enzyme	1 µL
Sterile water	To 8 µL

    Mix well by pipetting, quick-centrifuge, and incubate the above reaction mixture at 25 °C overnight.

17. Perform *E. coli* transformation of the BP reaction mixture. Isolate plasmid DNA and perform restriction digestion to confirm the correct clone.

18. Confirm the frame of entry clone in donor vector (pDONR™ 221) with pDONR F and pDONR R sequencing primer.

    pDONR F: TTT GAT GCC TGG CAG TTC CCT

    pDONR R: TGG CTC ATA ACA CCC CTT GTA.

19. Set up LR reaction as follows:

Entry clone in pDONR™ 221 (50–100 ng/rxn)	1–7 μL
pDEST™ 32 (100 ng/μL)	1 μL
LR clonase enzyme	1 μL
Sterile water	to 8 μL

    Mix well by pipetting, quick centrifuge and incubate the above reaction mixture at 25 °C overnight.

20. Use fresh streak yeast cells (within 3–7 days).
21. Make mixture of 50 % (w/v) PEG-3400/10× LiAc/10× TE just before use for better efficiency.
22. Use SC-LEU-TRP plates for spreading.
23. Colonies start to appear from day 3.
24. Do this step in clean bench.
25. Hold the membrane for few seconds only to prevent mixing of yeast patches.
26. Use previously confirmed interacting proteins as positive controls and non-interacting proteins or empty vectors as negative controls.
27. Weak interactors show growth from 4 to 6 days of incubation whereas negative controls do not show any growth.
28. After resuspension, transfer all solution to one Falcon tube and finally make total volume with 1× TE.
29. Prepare fresh 1× LiAc/0.5× TE solution from stock before use.
30. Prepare fresh 1× LiAc/40 % (w/v) PEG-3350/1× TE solution from stock before use. Sterile the solution by filtering with syringe filter with pore size 0.2 μm.
31. Spread bait protein (DB-X) in SC-LEU plate, prey protein (AD-Y) in SC-TRP plate, and bait and prey protein together in SC-LEU-TRP plate.
32. Test 3-AT sensitivity for all baits before screening.
33. Prepare Z-buffer with X-gal solution just before use. Bring Z-buffer to room temperature first from 4 °C.
34. After resuspension, transfer all solution to one Falcon tube and finally make total volume with ultrapure distilled water.
35. Prepare fresh 1× LiAc/1× TE solution from stock before use.

36. Aliquot 1 μg cDNA library and 50 μg denature salmon sperm DNA to each 30 tubes. Mix by tapping at the bottom of the tube and add 50 μL yeast competent cell.
37. This step is recommended to increase the transformation efficiency.
38. The optimum concentration of 3-AT should be determined before library transformation.
39. Colonies start to appear from 3 days. Colonies that appears within 3–7 days are regarded as potential interactors.
40. URA3 reporter gene activation results in conversion of 5-FOA to the toxic compound, 5-fluorouracil.
41. Potential strong interactors must show positive test for all three reporter gene assays. However, potential weak interactors might show positive test for at least one or two reporter gene assays.
42. Need to be very careful while isolating yeast plasmid DNA due to low copy number. Final elution volume needs to be decreased to 50 μL to increase the concentration of isolated yeast plasmid DNA. Except this follow the protocol as mentioned in the kit.
43. Due to low concentration of isolated yeast plasmid DNA, it is difficult to confirm the correct clone by restriction digestion. Hence, it is necessary to transform the yeast plasmid DNA into *E. coli* to increase copy number and efficient transformation.
44. It is highly recommended to use fresh streaked *E. coli* colony to increase transformation efficiency.
45. It is important to use chilled Falcon tube and chilled 0.1 M $CaCl_2$ solution for high transformation efficiency.
46. This step is optional.
47. For transformation, use >6 μL yeast plasmid DNA for successful transformation.
48. This step will increase the transformation efficiency.
49. For prey (AD-Y) spread the cells in ampicillin (100 μg/mL) plates. Use gentamycin (15 μg/mL) plates for bait (DB-X).
50. Follow the protocol as mentioned in the kit.
51. For restriction digestion use 1 μL of 100 ng/μL DNA in 10 μL volume. Use pDEST™ 22 empty vector (AD) as a reference for prey restriction mapping.
52. There is possibility of presence of more than one restriction patterns. Therefore, it is recommended to choose two or more candidate interactors with specific restriction pattern for retransformation assay.

53. In retransformation assay, if interactors show positive tests for all three reporter gene analysis than they are categorized as strong interactors, whereas if interactors show positive tests for one or two reporter gene analysis, they are characterized as weak interactors. And non-interactors show negative tests for all three reporter gene analysis.
54. Identified prey plasmid should be in frame with GAL4 DNA activation domain to be considered as real interacting protein.

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Chapter 17

Experimental and Analytical Approaches to Characterize Plant Kinases Using Protein Microarrays

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Abstract

Comprehensive analysis of protein kinases and cellular signaling pathways requires the identification of kinase substrates and interaction partners using large-scale amenable approaches. Here, we describe our methods for producing plant protein microarrays (PMAs) and discuss various parameters critical to the quality of PMAs. Next, we describe methods for detecting protein-protein interactions and kinase activity including auto-phosphorylation and substrate phosphorylation. We have provided a short video demonstrating how to conduct an interaction assay and how to properly handle a protein microarray. Finally, a set of analytical methods are presented as a bioinformatics pipeline for the acquisition of PMA data and for selecting PMA candidates using statistical testing. The experimental and analytical protocols described here outline the steps to produce and utilize PMAs to analyze signaling networks.

Key words Protein microarrays, Kinase substrates, Protein interaction, Phosphorylation assays, Statistical decision

1 Introduction

Understanding the function of proteins, both individually and in protein interaction networks, is the next great challenge of the post-genomic era. Protein Microarrays (PMAs) represent a relatively new approach that has significantly contributed to our understanding of protein interaction networks in model organisms [1–5]. PMA technology consists of depositing minute quantities of full-length or truncated proteins on a modified microscope slide [6, 7]. Functional PMAs can contain thousands of proteins and are used for high-throughput probe–protein interaction screens and kinase activity screens in vitro (Fig. 1). PMAs assays are more rapid,

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George Komis and Jozef Šamaj (eds.), *Plant MAP Kinases: Methods and Protocols*, Methods in Molecular Biology, vol. 1171,
DOI 10.1007/978-1-4939-0922-3_17,

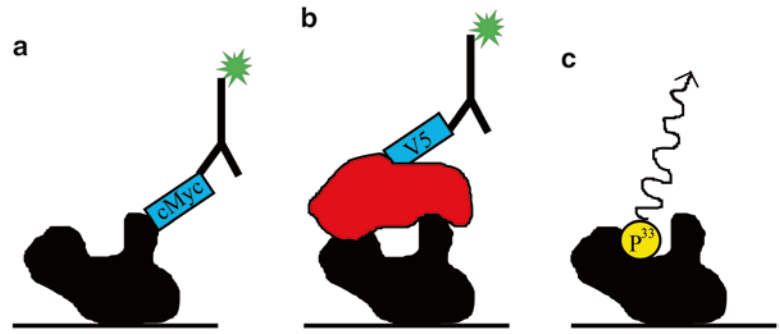

Fig. 1 Protein microarrays can be used in three types of assays; in an immunoassay, in an interaction assay and in a kinase assay. (**a**) In an immunoassay, the printed cMyc-tagged proteins on the PMA are detected by probing with an anti-cMyc Cy3-conjugated antibody. The signal intensity from the Cy3 fluorophore is detected and used to approximate the amount of protein printed on the slide. (**b**) In an interaction assay, the PMA is incubated with a V5-tagged probe protein followed by an anti-V5 Cy3-conjugated antibody. The resulting signal indicates probe–protein interaction provided the spotted protein does not show signal with the anti-V5 antibody alone (control slide). (**c**) If the spotted protein on the array is a kinase, autophosphorylation or substrate phosphorylation can be measured by providing the kinase with cofactors and radioactive [γ^{33}P]-ATP, washing and detecting the bound ^{33}P using film

precise and accurate for determining probe–protein interactions than in vivo methods such as the yeast-two-hybrid assay [8, 9].

PMAs can be a powerful resource to generate hypotheses regarding a protein of interest or a network of protein interactions. To produce reliable results, several parameters should be optimized prior to experimentation. A first critical factor that needs consideration is the quality of the proteins used for PMA probing or printing. The model organism used to produce the purified recombinant proteins may have an effect on the protein activity, particularly in the case of kinases [3]. To circumvent this problem, plant proteins should be purified when possible from plant tissue as described below. Another important parameter is the slide surface chemistry on which the PMA is printed. Commercial glass slides are overlaid with various chemicals that allow efficient protein immobilization. The most commonly used slide surfaces for producing PMAs include nitrocellulose, silanes, aldehydes, resins, silicons, and polyacrylamide [10, 11]. The type and thickness of the slide surface will determine the quantity of protein which can be immobilized, the amount of background signal and the activity of the proteins on the slide. To illustrate the importance of considering slide surface chemistry in designing a PMA experiment, we compared the performance of three commercially available slides in protein immobilization and in retaining the activity of immobilized proteins. Three types of slides, UltraGAPS, PATH and FAST, were

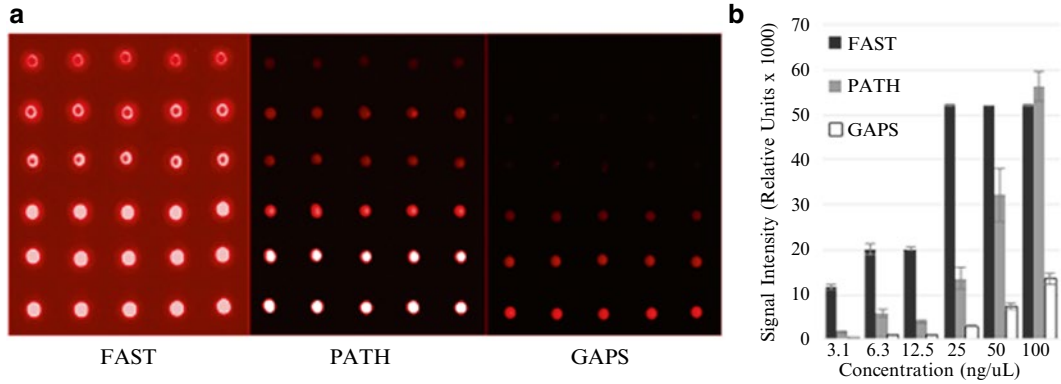

Fig. 2 Protein binding capacity in commercially available PMA slides. (**a**) Signal intensity was measured for five replicate spots of Cy-3 labelled anti-V5 antibody in a dilution series (100, 50, 25, 12.5, 6.3, 3.1 ng/μL) printed on FAST, PATH and GAPS slides. The range of florescence is visualized from low to high by *black* to *red* to *white* coloration in slides scanned at 50 % PMT gain immediately following printing. (**b**) Quantification of the signal on arrays shown in (**a**). Background signal was subtracted from the median signal of five replicate spots. The resulting intensities were averaged and graphed for each dilution level of the antibody along with the standard deviation

used to demonstrate differences in immobilization of a dilution series of Cy5-labelled Anti-V5 antibodies (Fig. 2a). UltraGAPS have an amino silane-covered hydrophobic surface (Corning), while PATH and FAST slides are covered with a proprietary nitrocellulose polymer (Grace Bio-Labs, Schleicher & Schuell BioScience). The FAST slides have the thickest slide surface, and bind the most protein as demonstrated by significantly higher signal in all six concentrations of printed antibody (Fig. 2b). However, the FAST slides also showed the highest level of background, visualized by the red color surrounding the spots in Fig. 2a. PATH and UltraGAPS slides have thinner slide surfaces compared to FASTs, and demonstrated lower protein binding and signal intensity with the printed antibody. These slides also have the best non-saturated range of detection with minimal background and are recommended for the assays described below.

Slide surface chemistry also has a direct impact on the outcome of either an interaction assay or a kinase assay. To illustrate this effect, PMAs containing 558 *Arabidopsis* protein preparations, including over 300 kinases, were printed on GAPS II and UltraGAPS amino silane-covered slides, Full Moon 3-D polymer covered slides (Full Moon Biosystems) and FAST slides. The PMAs were used in kinase autophosphorylation assays and the number of phosphorylated proteins was compared in Fig. 3a. We found that 42 % of the immobilized kinases displayed autophosphorylation activity on GAPS, 28 % on Full Moon, and 14 % on FAST (Fig. 3b). Quantification of signal intensity showed that kinases immobilized on GAPS slides exhibited a higher activity overall compared to the other types of slides (Fig. 3c). These results indicate that GAPS,

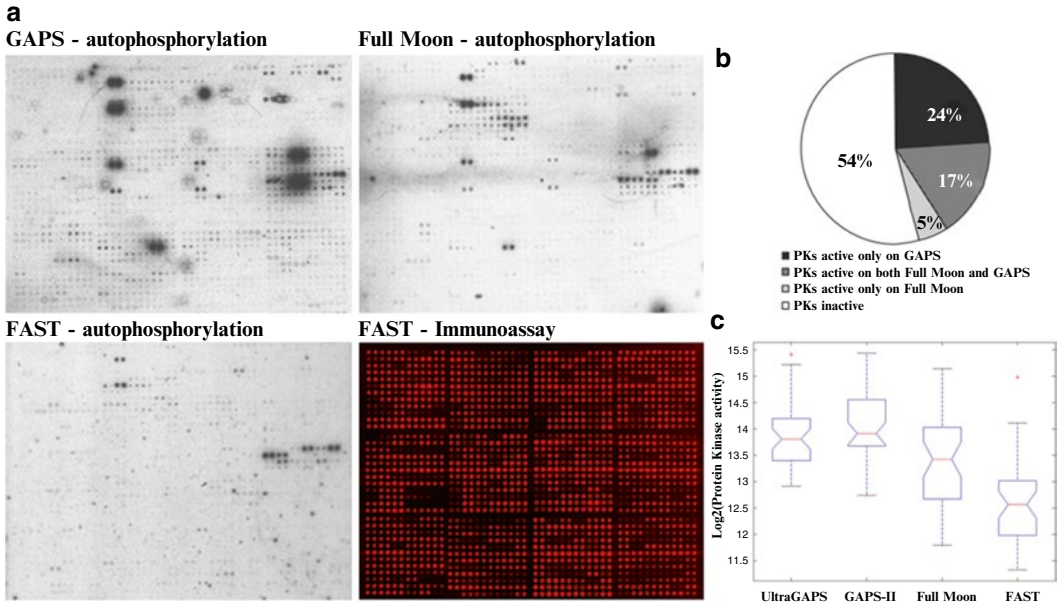

Fig. 3 Variation of MAPK activity as a function of slide surface chemistry. (**a**) Auto-phosphorylation activity assays were performed to assess kinase activity on protein microarrays (PMAs). The PMAs containing 558 *Arabidopsis* proteins were printed in duplicate on two types of GAPS, Full Moon and FAST slides. A representative PMA area is shown for kinase assays and immunoassays, performed as described in the protocols. For the immunoassays, the PMAs were probed with anti c-Myc primary antibody, a Cy5-labeled secondary antibody, and scanned at 633 nm at PMT gain 400. (**b**) Quantification of the results from the kinase assays performed on various PMAs. (**c**) Quantification of the activity of *A. thaliana* MAPKs on PMAs. The *box plots* show the distribution of MAPK activity on the four types of slides

followed closely by the Full Moon slides, have the best kinase activity detection properties among the tested slides. Thus, while GAPS bind less protein, their surface chemistry is conducive for kinase assays. This may be due to the better availability of active protein domains for biochemical reactions or to preservation of the native folding of the proteins.

A number of other parameters can be optimized for PMA assays including minimizing non-specific binding of antibodies to proteins on the array, assay conditions and probe concentration. These parameters have been discussed elsewhere and should be optimized depending on the probe and experimental design [12].

The protocols described below have been designed to be compatible with a broad range of plant protein kinases. Protein kinases are core components of signal transduction pathways that mediate cellular communication and fundamental processes such as growth, development and the immune response. Phosphorylation of protein substrates by kinases is one the best understood post-translational modifications and is a universal regulatory mechanism of enzymatic function. Applications of PMAs in this area are numerous and

include identification of kinase phosphorylation substrates, interaction partners and substrate specificity [3, 13–16].

Phosphorylation is a chemically simple enzymatic reaction catalyzed by a kinase, in which a phosphoryl group from the donor molecule ATP is transferred to specific serine, threonine or tyrosine residues in the protein substrate [17]. The reaction requires divalent metal ions, usually Mg^{2+}, which correctly position the ATP in the kinase active site and facilitate the phosphoryl transfer reaction [17]. An active protein kinase overlaid on the PMA is able to complete the transfer of the phosphoryl group from the [γ^{33}P]-ATP donor molecule to the immobilized proteins on the slide. In general, kinases and their substrates establish transient and/or low affinity interactions, and thus kinase–substrate complexes are difficult to detect on PMAs or when using other classical molecular biology approaches such as co-immunoprecipitation. To identify putative kinase substrates on the PMAs, the enzymatically modified proteins labeled with P^{33} are visualized by exposing the probed PMA to X-ray film. Autophosphorylation can be visualized with our without a probe of interest depending on the experimental design. Interaction assays can be used to detect protein complexes formed between a probe (kinase) and the proteins immobilized on PMAs.

Here we present a comprehensive guide for generating plant PMAs, characterizing protein–protein interactions and kinase activity using PMAs, and analyzing PMA data.

2 Materials

Prepare all materials using ultrapure water and analytical grade reagents at room temperature unless indicated otherwise. Of great importance to the workflow is a solid plan and good organization. For example, ensure that the proteins to be spotted on the PMA contain an appropriate tag (V5, cMyc, HA) which is different from the tag used on the probe protein. In the Popescu lab, we use the pYL436 vector (GenBank: AY737283.1) to purify the proteins to be printed on the PMA (cMyc tag) and the SPDK1433 vector to purify the protein probe (V5 tag) [16]. Both vectors were developed from the pLIC vector backbones and are identical except for the cMyc or V5 tag. All clones should be transformed into Agrobacterium GV2260 and single colonies should be selected by plating transformants on antibiotic-containing plates. Protocols describing infiltration of *N. benthamiana* with clones for transient protein expression have been described elsewhere [1, 18]. All of the PMA assays described below have been optimized to test a wide range of protein kinases. Buffer components can be added or excluded depending on the proteins of interest.

2.1 Protein Purification

1. Extraction buffer: A stock of 0.1 M Tris–HCl pH 7.5, 0.1 M NaCl, 0.005 M EDTA, 0.005 M EGTA, 0.1 % (v/v) Triton-X 100, 10 % (v/v) glycerol, can be made and stored at 4 °C for up to 6 months. Add 0.001 M phenyl methyl sulfonyl fluoride (PMSF) dissolved in ethanol, 0.0001 M sodium orthovanadate (Na_3VO_4), 0.01 M β-glycerophosphate, protease inhibitor, 0.1 % (v/v) β-mercaptoethanol and 0.01 M NaF immediately preceding the extraction. Na_3VO_4 must also be activated prior to use. Keep the buffer on ice.
2. Wash buffer: A stock of 0.1 M Tris–HCl pH 7.5, 0.5 M NaCl, 0.005 M EDTA, 0.005 M EGTA, 0.1 % (v/v) Triton-X 100, 10 % (v/v) glycerol, can be made and stored at 4 °C for up to 6 months. Add 0.001 M PMSF dissolved in ethanol, 0.0001 M Na_3VO_4, 0.01 M β-glycerophosphate, protease inhibitor, 0.1 % (v/v) β-mercaptoethanol and 0.01 M NaF immediately preceding the extraction. Keep the buffer on ice.
3. Cleavage buffer: 0.05 M Tris–HCl pH 7.0, 0.15 M NaCl, 0.001 M EDTA, 0.001 M DTT, 0.1 % (v/v) Triton-X. Keep the buffer on ice.
4. TURBO 3C cleavage protease (Accelagen).
5. IgG Sepharose 6 Fast Flow beads (GE Healthcare). Wash the beads three times with extraction buffer and resuspend the beads with the buffer at a 1:1 ratio. Use immediately.
6. Zirconia or silica beads (0.5 mm).
7. Glutathione-conjugated beads (e.g., Pierce).

2.2 Printing the Protein Microarrays

1. Slides: use PATH slides for immunoassay or interaction assays (Grace Bio-Labs), and UltraGAPS for kinase assays (Corning).
2. Purified protein preparations in 20 % (v/v) glycerol, stored at −80 °C.
3. Microarray printing instrument.
4. Buffer for cleaning microarrayer pins: 0.1 % (w/v) SDS.
5. Negative controls: 30 % (w/v) BSA in 20 % (v/v) glycerol.
6. Positive control: 30 μg/mL Cy3 or Cy5-conjugated antibodies in 20 % (v/v) glycerol.
7. 96- or 384-well microarray sample plates (Genetix).

2.3 PMA Immunoassay

1. Protein microarray on a PATH slide.
2. Clean, empty tip box.
3. Incubation chamber; a square tissue culture plate or other small container that has 2–3 layers of wetted blotting paper lining the bottom.
4. Blocking buffer: PBS buffer, 0.1 % (v/v) Tween 20, 1 % (w/v) BSA.

5. Washing buffer: PBS buffer, 0.1 % (v/v) Tween 20.
6. Primary and labeled secondary antibody against the spotted protein tag.
7. Scanner and detection software.

2.4 Screening for Kinase–Protein Interactions on PMAs

1. All the materials listed in Subheading 2.4.
2. Probing buffer: 0.01 M Tris–HCl pH 6, 0.0002 M ATP (e.g., Cell Signaling), 0.002 M $MgCl_2$, 0.002 M $MnCl_2$, 0.0001 M $CaCl_2$, 0.2 % (w/v) BSA, 0.0002 M DTT. It may be desirable to make a concentrated (4×) buffer in order to not dilute the purified probe protein.
3. Slide cover slips—HibriSlip hybridization covers (Sigma). The cover slips are only necessary when less than 300 μL of probing buffer will be used on the arrays, to prevent drying of the slide.
4. Purified probe protein. V5-tagged protein probes should be used at a final concentration of 10 nM to 1 μM, and some experimentation with different concentrations of probe may be necessary.
5. Primary and fluorescently-labeled secondary antibody against the probe protein tag.
6. Scanner and detection software.

2.5 PMA-Based Kinase Phosphorylation Assay

1. Protein microarray printed on a GAPS slide.
2. Clean, empty pipette tip box.
3. Incubation chamber; a square tissue culture plate or other small container which has two to three layers of wetted blotting paper lining the bottom.
4. Purified probe protein.
5. Blocking buffer: PBS buffer, 0.1 % (v/v) Tween 20, 1 % (w/v) BSA.
6. Kinase buffer: 0.05 M Tris–HCl pH 7.5, 0.01 M $MgCl_2$, 0.01 M $MnCl_2$, 0.001 M $CaCl_2$, 1 % (w/v) BSA, 0.001 M DTT, purified probe protein, 0.5 % [γ^{33}P]-ATP.
7. Washing buffer: 0.05 M Tris–HCl pH 7.5, 0.5 % (w/v) SDS.
8. X-ray Film (e.g., Kodak).

3 Methods

3.1 Generating PMAs Using Plant Proteins

In this section, we describe the production of protein preparations using a plant-based expression system, generation of PMAs by contact printing onto commercial slides and visualization of the printed proteins on the array.

3.1.1 Protein Purification

1. Cool centrifuges to 4 °C and prepare extraction buffer on ice.
2. Grind leaf tissue expressing the protein of interest very finely in liquid nitrogen and add tissue to the 0.5 mL mark on a 1.5 mL microcentrifuge tube. Fill to the 1.0 mL mark with extraction buffer and vortex immediately. Add 0.2 g of chilled zirconia beads. Incubate on ice.
3. Place the tubes in a rack under a layer of paper towels and shake using a paint shaker to further disrupt the tissue. Shake 4–5 times for 1 min each followed by a 1 min incubation on ice until large pieces of tissue are macerated.
4. Centrifuge for 10 min at maximum speed at 4 °C. Transfer supernatant to a new tube and centrifuge again for 5 min. Transfer supernatant to a new tube taking care **not to transfer any tissue**.
5. Add 40 μL of IgG Sepharose beads and invert the tube 3–4 times to mix. Incubate for 2 h at 4 °C in a rotator.
6. Centrifuge for 5 min at 1,000 rpm (94 rcf) at 4 °C and remove supernatant. Wash the beads with 0.5 mL of wash buffer three times.
7. Wash the beads with 1 mL of cleavage buffer without cleavage protease, centrifuge (5 min, 1,000 rpm, 4 °C) and remove supernatant.
8. Add 50 μL of cleavage buffer containing the cleavage protease (40 U/mL) and incubate overnight in a rotator at 4 °C.
9. Optional: To remove the cleavage protease, centrifuge and add 1/10 of the total volume of glutathione-conjugated beads to the supernatant. Incubate in a rotator at 4 °C for 1 h.
10. Harvest the supernatant and add sterile glycerol to a final concentration of 20 % (v/v). Aliquot the purified protein into 15–30 μL samples, flash freeze the protein in liquid nitrogen and store at –80 °C until use.
11. Run a Western to visualize the purity and abundance of your protein of interest in the total extract, on the IgG Sepharose beads and in the final purified sample. A size difference should be apparent between the cleaved (+12 kDa) and uncleaved protein (+25 kDa).
12. Perform a Bradford assay to determine the approximate amount of protein in the sample.

3.1.2 Printing the Protein Microarrays

1. Prior to printing the PMA, determine the parameters and layout. For example, it is necessary to know the number of proteins to be printed, the number of replicate protein spots within a slide and the number of slides to be printed. It is useful to include a positive and negative control at a specific location within each block of spotted proteins. These controls help with data normalization between slides and provide a reference

point during scanning. We typically use a fluorescently labeled antibody as a positive control and BSA as a negative control but other controls may be more appropriate, depending on the experiment.

2. Create an excel file with the name of the protein and the placement of the sample on the sample source plate. This is an example of an excel layout file:

Plate	Row	Column	Name	ID
1	A	1	Antibody	None
1	A	2	BSA	None
1	B	1	MPK4	At4G01370

3. Create a layout file using the excel spreadsheet as input into the microarray printing software. Open the output file to visualize where samples are going to be printed on the PMA based on the parameters that you have specified (replicate spot number, number of pins, number of blocks, and distance between spots).
4. Adjust your setup as necessary to print the PMAs on an appropriate number of slides. Typically, there is variation both within and between slides and thus the experimenter should consider using a minimum of three replicate slides per treatment and three replicate control slides within an experiment. Each microarray printer is different and thus a number of parameters need to be optimized ahead of printing such as the height of the printing pins during sample transfer and the number and pattern of pins that are used. Below are some of our general recommendations:

Parameter	Recommendation
Spacing between spots	0.3 mm
Number of duplicate spots within a block	2–5
Margin of unprinted area at the slide periphery	3 mm
Speed of contact between pin and surface	Slow, use soft touch if possible (0.5 mm)
Frequency of pin washes	After each sample printing
Room humidity	>50 %
Room temperature	18 °C

5. Create a GAL file and save that file for future data analysis.
6. Place samples in a 96 or 384-well plate in the order you specified in the Excel file.

7. Print the PATH or GAPS slides using the instructions provided by the microarray manufacturer.
8. Clean the pins following printing with 0.1 % (w/v) SDS solution and sonication for 30 min.
9. Carefully place your microarrays in slide holders and allow them to set overnight at 4 °C prior to long-term storage at −80 °C.

3.1.3 Immunoassay

Please watch the video associated with this chapter to see how to correctly handle the slides during an immunoassay.

1. Defrost the PMA by placing it on ice for 5–10 min.
2. Block the slides in SuperBlock buffer by carefully submerging each slide such that the buffer covers the slide all at once. Incubate for 1–2 h, without shaking, at 4 °C.
3. Remove the slide using tweezers by grasping on the barcode edge and drain excess liquid by tapping one side of the slide onto a paper towel. Do not allow the slide to dry.
4. Apply 300 μL of a 1:1,000 dilution of primary antibody in blocker reagent to the slide by dripping the solution onto the slide without touching the surface. Incubate in the incubation chamber at 4 °C for 1 h without shaking.
5. Drain excess liquid as before and wash slides three times in PBS-T for 1 min each, with shaking at 50 rpm at 4 °C.
6. Apply 300 μL of a 1:1,000 dilution of Cy3 or Cy5-conjugated secondary antibody in blocker reagent to the slide by dripping the solution onto the slide without touching the surface. Incubate in the incubation chamber at 4 °C for 1 h without shaking.
7. Drain excess liquid as before and wash slides three times in PBS-T for 1 min each, with shaking at 50 rpm at 4 °C.
8. Wash once by dipping the slide into a 50 mL tube containing deionized water.
9. Drain excess liquid in a 50 mL Falcon tube with a Kimwipe at the bottom, and centrifuge for 3 min at 800 × *g*. Allow the array to dry completely (1–2 min).
10. Scan for fluorescence at an appropriate PMT gain and resolution. Alter the PMT gain in order to minimize the number of saturated spots, and scan all slides at the same PMT gain from each experiment.

3.2 PMA-Based Methods for Characterizing Kinases

Once PMAs have been produced or obtained commercially, they can be used in the two assays described below. The kinase assay is used to test PMA-printed kinase auto-phosphorylation, identify candidate phosphorylation substrates of a kinase probe. The interaction assay is used to test for protein–protein interactions with a

kinase probe of interest. Please refer to that attached video for a demonstration of the interaction assay.

3.2.1 Kinase Phosphorylation Assay

1. Block the slides in SuperBlock buffer by carefully submerging the slide such that the buffer covers the slide all at once. Incubate for 1–2 h without shaking at 4 °C.
2. Remove the slide using tweezers by grasping on the barcode edge and drain excess liquid by tapping one side of the slide onto a paper towel. Do not allow the slide to dry. Apply 300 μL of kinase buffer containing (or not, for the negative control) the kinase probe of interest, without touching the surface of the slide.
3. Incubate in a wet chamber at 30 °C for 1 h without shaking. Make sure the slides are flat and not tilted. Check back periodically to make sure that the slides are not drying on one side if a coverslip is not being used.
4. Drain excess liquid and wash slides three times in PBS-T for 1 min each (50 rpm, 4 °C).
5. Wash once by dipping the slide into a 50 mL tube containing deionized water.
6. Centrifuge at 800 rpm for 1 min in a 50 mL Falcon tube, with a tissue paper at the bottom of the tube. Allow the array to dry completely (1–2 min).
7. Place slides in a cassette on top of a piece of paper, cover carefully with Saran wrap (no wrinkles) and tape entire unit down so nothing moves. Do not re-adjust the Saran wrap as this will dislodge the proteins on the slide.
8. Expose the slides to film for 1, 3, and 7 days in an exposure cassette.

3.2.2 Protein–Protein Interaction Assay

1. Defrost PMA placing on ice for 5–10 min.
2. Block the slides in SuperBlock buffer by carefully submerging the slide such that the buffer covers the slide all at once. Incubate for 1–2 h without shaking at 4 °C.
3. Remove the slide using tweezers by grasping on the barcode edge and drain excess liquid by tapping one side of the slide onto a paper towel. Do not allow the slide to dry. Apply 300 μL of probing buffer containing the V5-labelled purified protein probe (10 nM to 1 μM probe final concentration) by dripping the solution without touching the surface of the slide. Cover the slide with a coverslip by touching the end of the slide with the edge of the coverslip and allowing the coverslip to fall on to the surface of the buffer.
4. Incubate in the incubation chamber for 90 min at 4 °C without shaking.

5. Wash the slide three times in PBS-T for 1 min each, with shaking at 50 rpm at 4 °C. Remove the coverslip.
6. Drain excess liquid and apply 300 µL of a 1:1,000 dilution of primary antibody in blocker reagent. Incubate in incubation chamber at 4 °C for 1 h without shaking.
7. Drain excess liquid and wash slides three times in PBS-T for 1 min each (50 rpm, 4 °C).
8. Apply 300 µL of a 1:1,000 dilution of Cy3 or Cy5-conjugated secondary antibody in blocker reagent. Incubate in incubation chamber at 4 °C for 1 h without shaking.
9. Drain excess liquid and wash slides three times in PBS-T for 1 min each (50 rpm, 4 °C).
10. Wash once by dipping the slide into a 50 mL tube containing deionized water.
11. Drain excess liquid and centrifuge in 50 mL Falcon tube with a Kimwipe at the bottom for 3 min at 800 × *g*. Allow the array to dry completely (1–2 min).
12. Scan for fluorescence at an appropriate PMT gain and resolution. Alter the PMT gain in order to minimize the number of saturated spots, and scan all slides from the same experiment at the same PMT gain. Refer to **Note 1** for more information on scanning.

3.3 Protein Microarray Data Processing and Analysis

Here we describe the statistical methods needed to analyze protein microarray data. These methods can be used to identify kinase-binding proteins from interaction assay data and phosphorylation substrates from kinase assay data.

PMAs can measure thousands of protein–protein interactions in parallel. However each interaction is measured a limited number of times, necessitating a robust method for minimizing detection error and selecting interactors which perform consistently. Here, we present methods for PMA analysis including both data acquisition and statistical analysis for candidate selection. Data acquisition includes scanning the PMA, aligning features to the scanned image, filtering outliers and generating the results report. Using the results report, a number of statistical steps can be applied including statistical modeling, statistical decision (candidate selection), and error analysis. Many of the acquisition and preprocessing steps are automated in commercially available software suites such as GenePix and ScanArray/QuantArray. Statistical decision methods are made available as free software, written in R (Bioconductor), as data analysis software (MATLAB Bioinformatics Toolbox) and as independent tools such as ProtoArray® Prospector v5.2 (Invitrogen), ProCAT [19] and ProMAT [20] and other PMA software [21–23]. Recommendations regarding each step in the analysis of PMA assay output are outlined below. Step 4 in 3.2.1 is optional and protocols 3.2.2, 3.2.3 and 3.2.4 are alternative protocols.

3.3.1 Protein Microarray Data Acquisition and Processing

1. Select the wavelength for the scanning laser according to the fluorophore used for detection (i.e. 633 nm for Cy5 and 543 for Cy3, etc.); pre-scan the probed PMAs at low resolution, optimizing photomultiplier (PMT) gain such that the positive controls do not saturate. In most cases that corresponds to an intensity between 50 and 90 % of the maximum. Scan the PMA at high resolution (typically 10 μm), at the same PMT gain for each slide within an experiment. Save the image as a TIFF (.tif) file for further analysis and export JPG (.jpg) images for graphic illustration of the probed PMA.
2. Load the layout file (.gal file for GAL GenePix format) used to map PMA features to protein identifiers. The layout file can be generated from the clone list file describing the content of the samples used for array printing using the robotic printing software, the scanning software (i.e. Array List Generator), custom scripts, or it is simply provided by the PMA vendor. The layout includes printing details such as position of printed blocks, rows, column, printed feature size, distance between pins, etc.
3. Align the layout features on the PMA using GenePix: for best results align all blocks first, then detect and align features in each block (pay attention to correct setting of the printing head parameters such as feature size and pin distance). Depending on the noise on the arrays and the quality of the printing, it is best to constrain the variation of the feature size to 50 % from the spot diameter indicated in the layout file. Alternatively, one can use free feature segmentation (irregular shape). Review the alignment and manually adjust feature position and size if needed.
4. Perform the quality control of detected features. Validate detected features and mark features corresponding to slide artifacts (use the provided QC code, i.e. Good/Bad/Absent/Not Found). Use the provided tools to identify and mark additional outliers (i.e. features where the background is too high) if necessary. Save the GPS (.gps) file containing the optimal layout alignment for each PMA slide.
5. Generate the GPR report file (.gpr) after selecting the relevant data columns (i.e. signal intensity mean, median, standard deviation, background mean, median, standard deviation, flags, log ratios, other statistics).

3.3.2 Statistical Decision for Candidate Selection

1. There are many statistical decision methods for microarray (DNA, peptide and protein) data analysis available in the bioinformatics/biostatistics literature. Many can be adapted to PMA data analysis after developing the statistical model according to the experimental design. Several basic methods are available as part of PMA data analysis software (Prospector Analyzer) or have been developed and published with large scale PMA

screens [1, 3]. The first steps in all methods are data normalization and outlier removal.

Protein Microarray Data Normalization

1. Read the GPR data using the statistical analysis software routines (R, Matlab, and Excel).
2. Perform outlier removal: for the *Arabidopsis* PMA, compute the distribution of the difference of signal intensity between duplicate spots. Eliminate as outliers the data points where the absolute value of signal intensity difference of duplicate spots is larger than three times the standard deviation of this intensity difference computed for the whole array. For the remaining data points, compute the intensity average of duplicate spots (from this point on we refer to a single protein measurement, which is the average intensity on duplicate spots). Eliminate from the analysis all features marked as bad, absent or not found in the QC report above.
3. Perform intensity-based normalization in sets of probe-control experiments using one of many available methods ([24–27]; *see* **Note 1**).
   - 3.1 Scaling: Subtract background median intensity from signal median intensity. Scale the signal between min—negative controls on the array and max—positive controls.
   - 3.2 Perform robust multi-array normalization (if testing series of technical replicates).

     Regress each PMA slide vs. a reference control PMA slide; apply a linear transformation (using the regression coefficients) to each PMA dataset.
4. Perform concentration-based normalization (*see* **Note 2**).
   - 4.1 Proportional concentration dependence model (the method used in ProCAT).

     Estimate concentration of proteins printed on the microarray from the immunoassay data; normalize PMA data by dividing intensities by concentrations when above a noise threshold.
   - 4.2 Concentration-dependent analysis (the method used in ProMAT). Select the set of proteins with concentrations between 0.75 and 1.25. Restrict decision method to analysis for candidate protein j.

Statistical Testing for Identification of Kinase Substrate Candidates

Here, we assume that n replicated probed kinase assays and n control (phosphorylation) assays are performed. The statistical testing method for kinase substrate identification is as follows:

1. Read the GPR data of the probed and control series of experiments using the statistical analysis software (R, Matlab, and Excel).
2. Perform normalization procedures as described in Subheading 3.3.2.1 (*see* **Note 3**).

3. Perform a one-sided *t*-test with the null hypothesis: mean signal intensity in probed microarrays is no larger than the mean signal intensity of the control microarrays.

   The test statistic is:

   $t = \frac{\bar{Y}_P - \bar{Y}_C}{S_{\bar{Y}_P - \bar{Y}_C}}$, where $S_{\bar{Y}_P - \bar{Y}_C}$ is the standard error of the difference between the means; p: probe, c: control. A typical significance level for rejection of null hypothesis is 5 % (default in most implementations). Given the small sample size, "equal" variance for the two probed and control populations is recommended. Alternative testing methods can be applied if deviation from normal distribution of probed and control signal intensity is expected.

4. Save the *P* values and the decision values *H* corresponding to each tested PMA protein in a results table (.xls).

Bayesian Decision for Identification of Protein Binding Candidates

1. Read the GPR data of the probed and control series of experiments using the statistical analysis software (R, Matlab, and Excel).
2. Perform normalization procedures as in Subheading 3.3.2.1.
3. Estimate the mean $\mu(C_i)$, $i = 1, 2$ and standard deviation $\sigma(C_i)$, $i = 1, 2$ for each of the two classes after selecting representatives (assume two classes of proteins—C_a active proteins and Ci—inactive proteins). Use the control dataset for the inactive class. Use the probed PMA dataset for the active class, including spots which have intensities three standard deviations larger than the background mean $s(J_a) - \mu(bk) > 3 \times \sigma(bk)$, where J_a are the indices of proteins selected for the estimation of active protein class parameters. The background mean and standard deviation are computed on the entire array.
4. Classify proteins as active when:

$$s(J) > \mu(C_i) + \sigma(C_i) \times \frac{\mu(C_a) - \mu(C_i)}{\sigma(C_a) + \sigma(C_i)},$$

   $J = 1 \ldots N$, where $s(J)$ is the normalized signal intensity of protein J, and N is the total number of proteins printed on the slide. Estimate the probability of error associated with each decision. Save the classification in a results table (.xls; *see* **Note 4**).

Z-factor Method for Protein Binding Candidate Identification

1. Read the GPR data of the probed and control PMAs using the statistical analysis software (R, Matlab, and Excel).
2. Perform normalization procedures as in Subheading 3.3.2.1.
3. Select as putative candidates the data points whose intensity is larger than three standard deviations above microarray mean signal: $s(J) - \mu_{PMA} > 3 \times \sigma_{PMA}$, where J is the index of microarray probed protein, $J = 1 \ldots N$.
4. Save the decision in a results table (.xls; *see* **Note 5**).

3.4 Error Analysis and Multiple Testing Corrections

Large-scale screening of protein interactions or protein kinase activity requires additional corrections for multiple testing. Controlling the false discovery rate is necessary in very large screens, in particular when building hypotheses that involve multiple candidates from the statistical decision outcome as well as when analyzing pathways, signaling cascades or when performing Interactome analysis. Depositing the results of large PMA screens in public databases such as BioGRID [28, 29], IntAct [30] and others, imply their potential use in analyzing signaling pathways and interactomes, and therefore requires multiple testing corrections of candidate selection. We recommend that the decision results from Subheading 3.2 be corrected using the Storey's direct FDR method [31]or the Benjamini and Hochberg FDR [32] both implemented in R/Bioconductor and MATLAB Bioinformatics Toolbox.

3.5 Summary and Concluding Remarks

The protocols presented here provide detailed instructions to produce and use PMAs to examine protein kinase activity and interactions in plants. Commercially available *Arabidopsis* PMAs contain approximately 50 % of the proteome (http://abrc.osu.edu/protein-chip), providing the field of plant science with an invaluable resource. Using these PMAs, the experimenter is able to probe thousands of proteins within 1 day, using resources that are available in a typical molecular biology lab. PMA-based assays provide testable hypotheses for the cellular function of individual proteins and produce valuable insights into the system-level properties of kinases and cellular signaling. However, sub-optimal PMA probing conditions and inappropriate slide chemistry may facilitate spurious phosphorylation events or interactions. Like other in vitro methods, PMAs may produce false positive or false negatives since interactions are being measured outside of context of the cell where post-translational modifications, compartmentalization or additional protein interactions may have an influence [5–7, 33]. Further verification experiments using complementary methods followed by in vivo functional validation are required to establish the functional relevance of the PMA-obtained hits.

Moving forward, there is a need in the plant research community for shared proteomic resources such as PMAs for not only model organisms, but important crops as well. Continued production of PMAs and availability of results will enhance our understanding of protein networks and signal transduction in plants.

4 Notes

1. There are a number of intensity normalization methods available, developed for DNA microarrays, including quantile normalization, multidimensional-scaling, LOWESS, RMA and

LIMMA, which can be adapted for PMA normalization [24–27].

2. Using biochemical modeling requires concentrations of reactants of both printed and probing proteins. To estimate reaction parameters one would need to sample several concentrations, typically by probing the PMA with a geometric series of concentrations of the analyzed protein. However, using simple linear models of concentration-based normalization may improve the true positive rate for candidate selection when the concentration of proteins printed on microarray has a large variation, even in the absence of a comprehensive experimental design and of the corresponding biochemical analysis.
3. It is imperative to normalize the data before using statistical testing, as pairs of (probed, control) experiments may exhibit large differences of mean and variance due to technical factors such as concentration of reactants, labeling, washing and scanning procedures. This method requires at least three replicates of each probed and control protein microarrays.
4. This method can be applied with only pairs of probe and control experiments available. The method can also be applied with the controls printed on the same array with the proteins probed.
5. This decision is based on computing the *Z*-factor (signal to noise ratio), where the noise statistics is estimated from all PMA data points. The method can be used when technical replicates of PMAs are not available. When a control PMA is available, the noise mean and standard deviation can be computed from pooled probe and control data. *Z*-score, *Z*-factor and Chebyshev's inequality *P* value are available in Prospector Analyzer.

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Chapter 18

Protein Complexes Characterization in *Arabidopsis thaliana* by Tandem Affinity Purification Coupled to Mass Spectrometry Analysis

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Abstract

Proteins are major elements participating in all the key functions of the cells. They rarely fulfill their physiological roles in an autonomous way but rather act as part of more complex cellular machines. Indeed they can bind different types of molecules (proteins, nucleic acids, metabolites, etc.), via stable or transient interactions, depending on their nature and functions. The identification of the molecular partners of a given protein is hence essential to better understand its roles, regulation, and mechanisms of action.

This chapter describes the use of a tandem affinity purification approach followed by mass spectrometry analysis to try to identify and characterize the proteins involved in protein complexes in *Arabidopsis thaliana* and decipher some mechanisms of regulation of the modules. Important elements to consider in such an approach are first extensively exposed in the introduction. This technique, in combination with complementary approaches like yeast two-hybrid and bimolecular fluorescence complementation, can be an interesting source of data to identify and characterize in vivo protein complexes.

Key words *Arabidopsis thaliana*, Protein complexes, Tandem affinity purification, Mass spectrometry, Posttranslational modifications

1 Introduction

Affinity-based purification methods were proven quite efficient for the enrichment and further analysis of protein complexes in several organisms, including plant species. These methods can be separated into two main groups: epitope-tagging (allowing single-step affinity purification) and tandem affinity purification (TAP) (two-step affinity purification). The TAP method was initially developed to isolate and identify protein complexes in the yeast *Saccharomyces cerevisiae* [1]. In the TAP method, a bait protein is fused to a combination of tags which are sequentially used to isolate a highly purified bait protein and its associated protein partners. In the first TAP version, three tags were used: two IgG-binding units of

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protein A of *Staphylococcus aureus* (ProtA), a tobacco etch virus (TEV) protease cleavage site and a calmodulin-binding peptide (CBP). This method, applied in large-scale studies, led to numerous protein complex identifications in yeast [2, 3]. Due to its success TAP-tagging was rapidly implemented to protein interaction studies in other organisms including bacteria [4], mammalian cells [5], insect cells [6], and plants [7]. Adaptations of the technique mainly included the development and use of different combinations of tags as previously detailed [8].

In our work, a strategy based on TAP followed by mass spectrometry (MS) analysis was developed to identify in vivo protein partners of our proteins of interest. The stability of signaling complexes might be weaker as the interactions are expected to be transient; in this case, it makes the TAP approach rather challenging as this two-step affinity purification requires numerous incubations and washings. Alternatively, TAP protocols allow the purification of better purified protein complexes compared to single-step procedures; subsequent identifications of proteins usually have a higher confidence. Moreover, this strategy can allow the identification, of some posttranslational modifications (PTMs) of the bait and interacting proteins. This particularly interesting aspect will be discussed in the following sections of the chapter.

We designed a new combination of tags allowing a faster TAP protocol. This combination of tags, named PC2, is fused to the carboxy-terminal part of the protein. It is composed of a nine-Myc (EQKLISEEDL) peptide repeat followed by an eight-histidine (8×His) stretch and a Strep-tag II (WSHPQFEK) peptide (Fig. 1a). The relatively small size of the whole tag (about 19 kDa) reduces the risk of steric hindrance. To our knowledge this combination of tags has not been previously reported in the literature. As shown in Fig. 1b, the plant protein extract containing the TAP-tagged complexes is first bound on a resin bearing metal ions using the chelation interaction with the 8×His tag, like in an Immobilized Metal Affinity Chromatography (IMAC). After washes and elution using imidazole, the simplified protein extract is incubated with a Strep-Tactin resin. Strep-Tactin is a streptavidin mutant allowing enhanced interaction with the Strep-tag II, itself a variant of the original Strep-tag [9]. After washes and subsequent elution using desthiobiotin, proteins are precipitated and prepared for MS analysis. The 8×His motif, with an affinity constant for IMAC resin in the order of 10^{-13} M [10] allows for efficient capture of the diluted TAP-tagged protein in the initial protein extract. Oppositely, the Strep-tag II/Strep-Tactin interaction requires higher concentration of the bait but is much more specific than IMAC and represents an ideal second step in the TAP procedure. No protease cleavage, often the limiting step, is necessary in this purification process. The nine-Myc-peptide repeat can be used as an efficient epitope for western blotting. It can also serve as a step in the

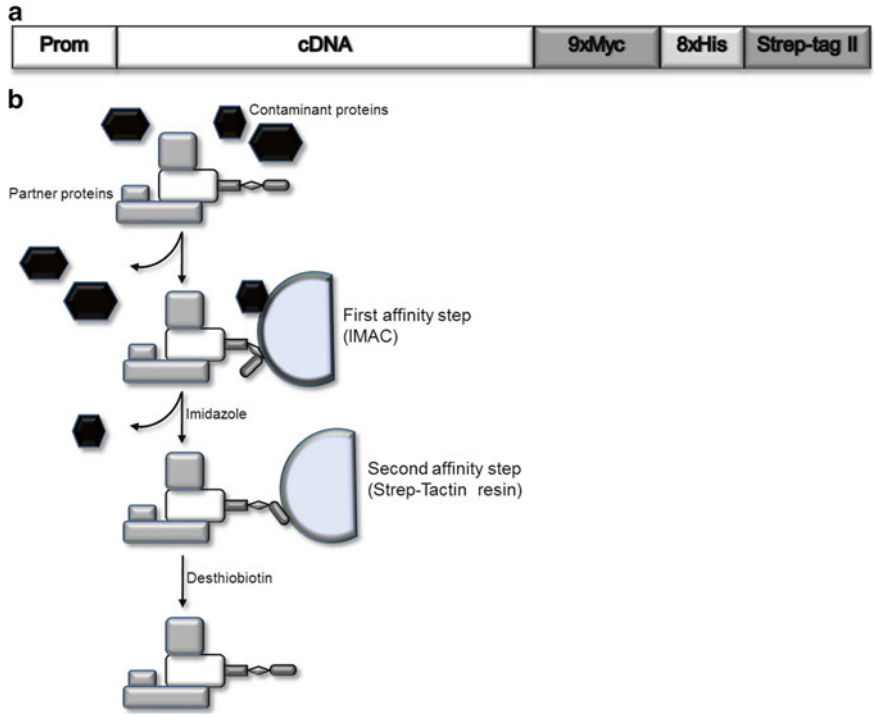

Fig. 1 The gene of interest fused to the PC2 cassette and the TAP strategy. (**a**) Structure of the TAP PC2 cassette where the various tags are indicated. (**b**) Overview of the TAP strategy. *Grey boxes* represent partner proteins of the TAP-tagged bait protein and *black boxes* represent contaminant proteins; the *white box* and the three small *grey boxes* correspond to the TAP-tagged bait protein

purification process, preferentially as the last step because the corresponding elution necessitates stringent conditions, such as low pH or high concentration of detergents, which would disrupt non-covalent protein–protein interactions.

We originally designed a binary Gateway vector allowing the expression of the bait protein merged in frame with the PC2 tag under the control of the strong CaMV 35S promoter. This transgene did not always allow the generation of viable plant lines and is often silenced in the progeny. Even when the CaMV 35S promoter was replaced by an endogenous promoter, no stable expressing lines could be obtained. In contrast, expression of the entire genomic locus, including the promoter, exons, and introns and fused to a C-terminal PC2 cassette resulted in stable lines, in which the chimeric protein is supposed to be expressed as the endogenous protein. This construct was validated by complementation of the corresponding knockout T-DNA mutant. In conclusion, the full genomic locus seems to be of major importance for obtaining stable transgenic lines for critical genes of interest. Besides, the amount of expressed bait protein is a crucial factor in the success of the approach. From the literature, it can be stated, overall, that the

more a protein is expressed the higher is the amount of the purified bait protein. However, this rule, although often verified for the bait protein, does not apply to the purified protein partners in the complex(es) as plants usually do not adapt their expression and/or abundance to the level of the bait protein. In addition, if the physiological function of the bait protein necessitates a tight regulation, overexpression or underexpression will very probably lead to phenotypes different from the wild type. That is why the promoter for expression of the bait protein has to be chosen carefully. The locus promoter is probably the best choice. In addition, the genetic background needs to be considered, usually with two alternatives: a wild-type background or a bait protein mutant background. Working in a mutant background presents two major advantages in the TAP approach. First, the complementation of the mutant phenotype indicates with high confidence that the TAP-tagged protein is functional and that the protein complex(es) centered on it are functional as well. Second, it avoids the competition that would occur otherwise between the endogenous protein and the tagged protein for the formation of the complex(es).

To perform TAP purification, we use whole plantlets grown in liquid medium to obtain large amounts of material. Protein complexes are then purified from frozen material. Although cell cultures and transient in planta expression assays can theoretically be used, stable plant transformants are closer to physiological conditions. This is the reason why we chose this expression and culture system. Additionally, such strategy allows the use of mutant genotypes. Several biological replicates are performed for each TAP-tagged line, because some interaction partners are likely to be non-systematically identified by MS in a single purification experiment. Besides, even though the TAP provides rather clean samples, in which the number of proteins interacting nonspecifically with the affinity beads is limited, some contaminant proteins are still retrieved in the final sample. To distinguish between the true complex components and these contaminants, other transgenic lines expressing unrelated TAP-tagged proteins such as GFP (Green Fluorescent Protein) are used as controls. Purified proteins are digested by trypsin and the peptides analyzed by capillary liquid chromatography coupled to tandem mass spectrometry (nanoLC-MS/MS). Proteins repeatedly identified in samples of interest but not in controls are considered to be likely components of the TAP-tagged complexes.

The TAP-MS approach also allows analyzing PTMs occurring on protein complexes without making a priori assumptions on their nature or localization. This requires of course that sufficient material of the bait proteins and its interaction partners be obtained by TAP, to reach significant protein sequence coverage by the MS

analysis of the proteolyzed protein complexes. Searching for PTMs has become more and more possible with the sensitivity and mass measurement accuracy of current mass spectrometers and with the PTM-related options in database search software. Indeed, database search options, like the "error tolerant" search function in Mascot, allow screening for any possible covalent modification in proteins of interest. The high sensitivity and dynamic range of current mass spectrometers makes it possible to detect and identify modified sequences of proteins even though the modification affects below 1 % of the biomolecules of same sequence (the dynamic range typically covers about four orders of magnitude). The high mass accuracy of current mass spectrometry instruments (below 5 ppm) contributes to accepting with confidence the proposed modified peptide sequences or can result in their prompt rejection. Phosphorylation events are of particular interest as they are key elements in protein regulation. It is also possible to identify other kinds of PTMs as far as these modifications are stable enough to withstand the physicochemical conditions of the whole analytical process or do not prevent the ionization and detection of the respectively modified peptides during the LC-MS/MS analysis. Whereas PTM characterization without a priori assumption can be successful on purified proteins, there are limitations, because modified sequences present in substoichiometric amounts in the complex digests are globally more likely to escape identification by MS than major abundance species. The identification of these species present in low amounts would require their enrichment. In large-scale studies of phosphorylation events, for example, phosphoproteins and/or phosphopeptides are usually first enriched by IMAC or metal oxide affinity chromatography (MOAC) before MS analysis [11, 12]. Yet there exists no enrichment technique compatible with the simultaneous purification of peptides carrying a variety of PTMs. Nevertheless, the approach to identify PTMs without a priori assumptions is quite easy to perform and can provide important clues on the underlying regulatory mechanisms.

In a second step, complementary approaches, like yeast two-hybrid or in planta bimolecular fluorescence complementation (BiFC), need to be used to validate the identified protein partners of bait proteins. Upon confirmation of the specific protein–protein interactions, further genetic, phenotypic, and biochemical studies can be carried out to gain further insight into the roles of these proteins.

The following experimental procedures describe the different steps of a standard study of a protein of interest by TAP PC2 coupled to MS analysis. Cloning steps to obtain a protein fused to the TAP PC2 cassette and selection of *Arabidopsis* stable lines expressing the TAP-tagged protein are not exposed here as they are beyond the frame of this chapter.

2 Materials

Remark 1: Use adapted protections when necessary (laboratory coat, nitrile gloves, eyeshields, respirator filter) as some chemicals used in the procedure are harmful.

Remark 2: Wash thoroughly reusable consumables and equipment.

2.1 Arabidopsis thaliana Plant Cultures

1. *Arabidopsis* culture medium (½ MS): 2.2 g/L MS including vitamins (e.g., Sigma), 10 g/L sucrose (e.g., Sigma), 0.5 g/L MES (e.g., Sigma), pH adjusted to 5.7 with KOH, autoclaved.
2. Small equipment and consumables: Petri dishes 145/20 mm, Parafilm, 50 mL Falcon tubes, liquid nitrogen.
3. Large equipment: sterile laminar flow hood, fridge, culture chamber [rotating platform, long day (16 h light–8 h dark), 24 °C].

2.2 Protein Extraction

1. Buffer 1: 50 mM Tris–HCl pH 7.5 with HCl, 150 mM NaCl, 10 % (v/v) glycerol, 0.1 % (v/v) NP40/IGEPAL CA-630. Prepare fresh Buffer 1 from sterilized or filtered reagent stock solutions.
2. Extraction Buffer: protease inhibitors (e.g., Complete EDTA-free, Roche, one tablet per 50 mL; and 1 mM PMSF), phosphatase inhibitors [1 mM NaF, 0.5 mM sodium orthovanadate (Na_3VO_4), 15 mM β-glycerophosphate, and 15 mM 4-nitrophenyl phosphate bis(tris) salt] in Buffer 1. Prepare fresh Extraction Buffer and let it on ice. Use stock solutions of protease/phosphatase inhibitors, stored according to the supplier recommendations.
3. Small equipment and consumables: mortars, pestles, 400 mL beakers, 200 mL glass bottles, spatulas, funnels, Miracloth, compatible centrifuge tubes, 50 mL Falcon tubes, liquid nitrogen.
4. Large equipment: centrifuge.

2.3 First Step of Purification

1. Ni Elution Buffer: 200 mM imidazole, protease inhibitors (Complete Mini EDTA-free, Roche, one tablet per 10 mL) and 1 mM PMSF, phosphatase inhibitors (1 mM NaF, 0.5 mM Na_3VO_4, 15 mM beta-glycerophosphate, and 15 mM 4-nitrophenyl phosphate bis(tris) salt) in Buffer 1. Prepare fresh Ni Elution Buffer and let it on ice. Use stock solutions of imidazole and protease/phosphatase inhibitors, stored according to the supplier recommendations.
2. Ni beads: Ni-NTA Agarose (e.g., Qiagen).

3. Small equipment and consumables: 15 mL Falcon tubes, 50 mL Falcon tubes, end-cut tips, rotating wheel for tubes, disposable plastic columns (e.g., Poly-Prep columns, Bio-Rad), retort stand tripod.
4. Large equipment: centrifuge.

2.4 Second Step of Purification

1. Strep Elution Buffer: 10 mM D-desthiobiotin in Buffer 1. Prepare fresh Strep Elution Buffer and let it on ice. Use a stock solution of D-desthiobiotin, stored according to the supplier recommendations.
2. Strep-Tactin beads: Strep-Tactin Sepharose (2-1201-010 IBA).
3. Small equipment and consumables: 15 mL Falcon tubes, 50 mL Falcon tubes, 1.5 mL Eppendorf tubes, end-cut tips, rotating wheel for tubes, 2 mL disposable plastic columns (e.g., Pierce), retort stand tripod.
4. Large equipment: centrifuge.

2.5 Protein Precipitation

1. Methanol Precipitation Solution: 0.1 M ammonium acetate, 1 % (v/v) β-mercaptoethanol, in absolute methanol. Prepare fresh Methanol Precipitation Solution under a fume hood and let it on ice. Add the 2-mercaptoethanol just before use.
2. 70 % Ethanol solution: 70 % (v/v) ethanol in water. Let at −20 °C or on ice.
3. Small equipment and consumables: 1.5 mL Eppendorf tubes, vortex.
4. Large equipment: −80 °C freezer, centrifuge.

2.6 Protein Digestion

1. Acetonitrile: HPLC grade.
2. Reducing agent: Tris-(2-carboxyethyl)phosphine (TCEP).
3. Alkylating agent: methyl methanethiosulfonate (MMTS).
4. Digestion enzyme: sequencing grade modified trypsin (e.g., Promega).

2.7 MS Analysis

1. HPLC solutions are prepared with HPLC grade solvents and Milli-Q water at 18.2 MΩ cm. Buffer A = H_2O–acetonitrile–formic acid, 98:2:0.1 (v/v/v); buffer B = H_2O–acetonitrile–formic acid, 20:80:0.1 (v/v/v).
2. Chromatographic system UltiMate 3000 (Dionex) equipped with a reversed-phase pre-column (Pepmap C18, 3 μm particle size, 100 Å porosity, 300 μm internal diameter, and 5 mm length) and a capillary column (Pepmap C18, same stationary phase characteristics as the pre-column, 75 μm internal diameter, and 15 cm length).

3. NanoESI-LTQ-Orbitrap XL instrument (Thermo electron), piloted with the software Xcalibur.
4. Metallized tips (e.g., New Objective).
5. Identification of peptide sequences from MS/MS spectra is provided by the software Mascot (www.matrixscience.com).

3 Methods

Remark 1: For all the following steps, it is strongly recommended to plan the experiment including the tagged line of interest together with a control line.

Remark 2: It is useful to keep aliquots corresponding to the key steps of the TAP purification and use them for a western-blot analysis with an anti-Myc antibody. This allows for the evaluation of the yield of tagged-protein purification and the marking of the steps that can be potentially improved.

3.1 *Arabidopsis thaliana* Plant Cultures

1. Under a sterile laminar flow hood, pour 55 mL of *Arabidopsis* culture medium in a 15 cm petri dish. Prepare ten such dishes.
2. Sow sterilized *Arabidopsis* seeds in equal amounts in the ten petri dishes and seal the plates with Parafilm.
3. Keep the petri dishes at 4 °C for 3–4 days for stratification of the seeds.
4. After stratification, transfer the petri dishes in the culture chamber and allow plants to grow for 17–18 days. This should allow the production of 5 g of plant material per dish.
5. Harvest plants: quickly dry the samples with several layers of absorbent paper and transfer them in 50 mL Falcon tubes. Freeze the tubes in liquid nitrogen (*see* **Note 1**). Use one 50 mL Falcon tube for two petri dishes.

3.2 Protein Extraction

1. Grind all the frozen plants in prechilled mortar and pestle to obtain a homogenous fine plant powder (*see* **Note 2**). Regularly add liquid nitrogen to keep the samples frozen.
2. Weight the ground plant powder so as to use the same amount of material for the TAP between the tagged-line of interest and the control line.

Remarks: All steps from here to Subheading 3.6 should be conducted on ice or at 4 °C. Cool consumables and equipment on ice or at 4 °C well before use.

3. In a 400 mL beaker, pour two volumes of cold Extraction Buffer (e.g., 80 mL for 40 g of plants).
4. Add the plant powder progressively in the beaker and homogenize gently with a spatula (*see* **Note 3**).
5. Keep on ice for about 10 min.

6. Filter the crude solution through two layers of Miracloth into a 200 mL glass bottle using a funnel.
7. Repeat filtration with one layer of Miracloth.
8. Distribute the filtered solution in centrifuge-compatible tubes, equilibrate them, and centrifuge at 4 °C for 20 min at 16,000 × *g*.
9. Keep the supernatant in new 50 mL-Falcon tubes (*see* **Note 4**).

3.3 First Step of Purification

Remark: Prepare the Ni beads during the centrifugation of Subheading 3.2, **step 8**.

3.3.1 Preparation of Ni Beads

1. Disperse the Ni beads into homogeneous slurry and transfer 1 mL (500 µL beads) in a 15 mL Falcon tube using an end-cut tip.
2. Add 5 mL of Buffer 1 to the beads disperse gently and centrifuge at 4 °C for 2 min at 700 × *g* to pellet the beads. Discard supernatant.
3. Repeat the previous step twice.

3.3.2 Ni Purification

1. Distribute equally the prepared Ni beads in three 50 mL Falcon tubes.
2. Add equal amounts of the protein extract from Subheading 3.2, **step 9** in these tubes.
3. Gently rotate tubes on a rotating wheel for 2 h at 4 °C (*see* **Note 5**).
4. Centrifuge the tubes at 4 °C for 3 min at 700 × *g* to pellet the beads. Discard supernatant.
5. Add 2 mL of Buffer 1 to the beads homogenize gently and load them on a disposable plastic column using end-cut tips (*see* **Note 6**).
6. After the column has completely drained, add 2 mL of Buffer 1 to wash the beads.
7. Repeat the previous step.
8. Add 3 mL of Ni Elution Buffer to the column and collect the eluate in a new 15 mL Falcon tube.

3.4 Second Step of Purification

Remark: Prepare Strep-Tactin bead suspension during the incubation on Ni beads of Subheading 3.3.2, **step 3**.

3.4.1 Strep-Tactin Beads Preparation

1. Gently resuspend the Strep-Tactin solution and transfer 200 µL of resin slurry (100 µL beads) in a 15 mL Falcon tube with an end-cut tip.
2. Add 3 mL of Buffer 1 to the bead slurry, resuspend gently, and centrifuge at 4 °C for 2 min at 700 × *g* to pellet the beads. Discard supernatant.
3. Repeat the previous step.

3.4.2 Strep-Tactin Purification

1. Add the 3 mL of Ni eluate from Subheading 3.3.2, **step 8** to the prepared Strep-Tactin beads.
2. Gently rotate the tube on a rotating wheel for 1.5 h at 4 °C (*see* **Note 5**).
3. Centrifuge the tube at 4 °C for 2 min at 700 × *g* to pellet the beads. Discard supernatant.
4. Add 300 µL of Buffer 1 to the beads, resuspend gently, and load them on a 2 mL-disposable plastic column using end-cut tips (*see* **Note 6**).
5. After the column has completely drained, add 300 µL of Buffer 1 to wash the beads.
6. Repeat the previous step.
7. Add 500 µL of Strep Elution Buffer to the column and collect the eluate in an Eppendorf tube.
8. After complete elution, reload the eluate to the column for a second elution cycle (*see* **Note 7**).

3.5 Protein Precipitation

3.5.1 Mix with Methanol Precipitation Solution

1. Split the Strep eluate in two Eppendorf tubes, adding ca. 250 µL per tube.
2. Add 5 volumes of cold Methanol Precipitation Buffer, ca. 1.25 mL per tube.
3. Vortex and let at –80 °C, overnight (*see* **Note 8**).

3.5.2 Recovery of Precipitated Proteins

1. Centrifuge the tubes at 4 °C for 30 min at 20,000 × *g* and discard supernatant under a fume hood (*see* **Note 9**).
2. Add 1 mL of cold Methanol Precipitation Solution per tube, vortex, and centrifuge at 4 °C for 15 min at 20,000 × *g*. Discard the supernatant under a fume hood.
3. Repeat the previous step.
4. Add 1 mL of cold 70 % (v/v) Ethanol solution per tube, vortex, and centrifuge at 4 °C for 15 min at 20,000 × *g*. Discard the supernatant.
5. Repeat the previous step.
6. Open the tubes and let the pellet to air-dry at room temperature (RT) (*see* **Note 10**).

3.6 Protein Digestion

1. Protein pellets are resuspended in ACN/50 mM tri-ethyl-ammonium bicarbonate (TEAB), 40/60 (v/v) (*see* **Note 11**). Complete solubilization of the pellets is achieved by 5–10 min sonication in an ultrasonic bath.
2. To facilitate trypsin digestion, proteins are heat-denatured at 95 °C for 5 min.
3. Disulfide bonds in proteins are reduced by addition of 5 mM TCEP and incubation at 60 °C for 1 h.

4. Resulting free sulfhydryl residues are alkylated by addition of 10 mM MMTS and incubation for 15 min on the bench.
5. Protein samples are finally digested by addition of 2 µL of trypsin at 0.4 µg/µL and overnight incubation at 37 °C (*see* **Note 12**).

3.7 MS Analysis

1. Peptides are first loaded onto the pre-column, with a 20 µL/min flow rate of buffer A. Salts are eliminated by a 5 min wash with buffer A.
2. After valve switching, pre-column and column are brought in series.
3. Chromatographic separation of peptides is performed at about 300 nL/min, by running a gradient from 100 % of buffer A to 70 % of buffer B. The gradient is developed in 50 min, followed by a 10 min flush of the system with 100 % buffer B to efficiently elute possible larger peptides.
4. Create an acquisition method using Xcalibur that automatically alternates between detection in MS mode in the Orbitrap cell of the peptides eluted from the chromatographic column, and fragmentation in MS/MS mode in the linear ion trap of the five species giving the most intense signals in the previous MS scan. Acquisitions are limited to the mass range [400; 1,600] Da in MS mode, and span [100; 2,000] Da in MS/MS mode.
5. To identify peptide sequences from MS/MS spectra, perform searches using the Mascot search engine, while considering the TAIR database (*see* **Note 13**), a maximum of two missed tryptic cleavages, full modification of cysteine residues by MMTS (modification called Methylthio(Cys)), possible methionine oxidation, 5 ppm error on precursor ions (*see* **Note 14**), 0.6 Da error on fragments, and specifying the use of an "ESI-trap" instrument (*see* **Note 15**). The "decoy search" option can be selected to get an estimate of the False Discovery Rate, i.e., an estimate of the fraction of wrong peptide identifications.
6. The previous search allows identification of proteins present in the TAP samples based on non-modified sequences. One can try to identify additional modified peptide sequences without a priori hypothesis on the nature of the covalent modification while using the "error tolerant search" option. All the protein entries identified in the previous search step will be grouped to form a limited database. In the second pass search, the selected enzyme (here trypsin) becomes semi-specific (i.e., only one end of the theoretical peptide matched to the experimental spectrum needs to result from a tryptic cleavage); the full list of modifications is tested; for each protein, single-amino-acid substitutions that can arise from single-base

DNA substitutions are assessed (see matrixscience.com for further detail). In particular, one can detect phosphorylated peptides, semi-tryptic peptides (possibly coming from protein degradation during cell lysis, or protein truncation in vivo) and $N=>D$ modifications that can occur spontaneously in vitro. This search can allow for increasing the sequence coverage of the bait protein and some of its partners.

4 Notes

1. About 40 g of plant material are usually obtained with ten petri dishes of culture. Moreover, it is possible to store the harvested plants at −80 °C before continuing with post-processing.
2. It is easier to grind the plants "one tube after the other." Put back the ground plants to the tubes and keep them under liquid nitrogen until all material is ground and ready for subsequent step.
3. It can take some time to have a homogeneous solution because the frozen plant material decreases the temperature of the mix below 0 °C. It is possible to let the beaker at room temperature (RT) until complete homogenization, but control the temperature to avoid exceeding 4 °C.
4. The supernatant mainly corresponds to a cytosolic extract. However, some organelles, such as nuclei or chloroplasts, can be disrupted during the grinding step.
5. Do not rotate the tubes too fast. Use for example a tuning wheel at 10 rpm.
6. Use a retort stand tripod to fix the disposable plastic column and equilibrate the filter of the column with Buffer 1.
7. At the end, use a syringe or another kind of system to push air on top of the column so as to elute the maximum by "drying" the Strep-Tactin resin slurry.
8. Put several layers of Parafilm around the tubes as methanol is very "leaky."
9. Be careful not to discard the protein pellet which is sometimes difficult to see.
10. It is possible to store the precipitated proteins at −80 °C before continuing with the following step.
11. Proteolysis is performed in the presence of 40 % (v/v) acetonitrile, previously shown to accelerate the digestion of proteins by trypsin [13].
12. Digestion of protein samples by trypsin is usually advised to be performed with an enzyme to substrate mass ratio between 1/100 and 1/20. We did not estimate the amount of protein

material yielded by our TAP procedure applied to 40 g of plant material. However, given the signal detected in MS on digested TAP samples, we could estimate that 0.8 μg trypsin was sufficient.

13. We work with in-house installed Mascot software, allowing the downloading of any protein database of interest on site, and the updating of the database when relevant.
14. The LTQ-Orbitrap provides mass measurements with accuracy below 5 ppm when the Orbitrap analyzer is calibrated on a weekly basis. Better accuracy can be achieved when working with the "lock mass" option: during LC-M/MS analyses, the instrument takes as reference background ion(s) originating from ambient air to recalibrate all acquired MS spectra in real time [14].
15. The "Instrument" configuration specified for database search with Mascot determines the fragment ion types considered for interpreting MS/MS spectra. It is thus crucial to select the "Instrument" definition that best allows interpreting the experimental fragmentation spectra to obtain optimal identification results.

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Chapter 19

Quantitative Phosphoproteomic Analysis Using iTRAQ Method

Tomoya Asano and Takumi Nishiuchi

Abstract

The MAPK (mitogen-activated kinase) cascade plays important roles in plant perception of and reaction to developmental and environmental cues. Phosphoproteomics are useful to identify target proteins regulated by MAPK-dependent signaling pathway. Here, we introduce the quantitative phosphoproteomic analysis using a chemical labeling method. The isobaric tag for relative and absolute quantitation (iTRAQ) method is a MS-based technique to quantify protein expression among up to eight different samples in one experiment. In this technique, peptides were labeled by some stable isotope-coded covalent tags. We perform quantitative phosphoproteomics comparing *Arabidopsis* wild type and a stress-responsive *mapkk* mutant after phytotoxin treatment. To comprehensively identify the downstream phosphoproteins of MAPKK, total proteins were extracted from phytotoxin-treated wild-type and *mapkk* mutant plants. The phosphoproteins were purified by Pro-Q® Diamond Phosphoprotein Enrichment Kit and were digested with trypsin. Resulting peptides were labeled with iTRAQ reagents and were quantified and identified by MALDI TOF/TOF analyzer. We identified many phosphoproteins that were decreased in the mapkk mutant compared with wild type.

Key words Quantitative proteomics, iTRAQ, Phosphoproteome, Phytopathogen, Fusarium, Trichothecene, MAPK

1 Introduction

In all eukaryotes, mitogen activated protein kinase (MAPK) cascades play important roles of signal transduction in responses to various biotic and abiotic stresses, cell division, and cell differentiation [1–3]. MAPK cascades are composed of three sequentially activated kinases, MAPKKK, MAPKK, and MAPK. MAPKKKs activate MAPKKs by phosphorylation, and MAPKKs activate MAPKs by phosphorylation. Then, MAPKs directly phosphorylate downstream target proteins, such as transcription factor [4]. Therefore, the phosphoprotemics is a very important technology to identify target proteins phosphorylated by the MAPK-dependent signal transduction pathways [5–8]. As a first step, phosphorylated proteins were purified from total or fractionated proteins by use of

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some commercially available purification kit such as the immobilized metal ion affinity chromatography (IMAC) phosphopeptide enrichment kit and the Pro-Q Diamond Phosphoprotein enrichment kit (Invitrogen) [8, 9]. Fe^{3+} or Ga^{3+} is usually used as a metal ion for IMAC to trap the negatively charged phosphopeptide. Therefore, acidic non-phosphorylated proteins may affect the IMAC purification. Alternatively, after conventional gel electrophoresis, phosphoproteins can be selectively stained with fluorescent dye Pro-Q Diamond (Invitrogen) [9].

To profile protein expression pattern, two-dimensional gel electrophoresis is often used. After electrophoresis, gels were stained with colloidal CBB (Coomassie Brilliant Blue) or fluorescent dye such as SYPRO Ruby (Invitrogen). Alternatively, multiple protein samples labeled with different fluorescent dyes (CyDyes; Cy2, Cy3, or Cy5) are separated by the same gel electrophoresis (2D-DIGE) [9]. In these gel-based methods, comparative analysis among different samples is performed by calculated signal intensities of protein spots, then cutting spots are identified by Matrix Assisted Laser Desorption Ionization-time-of-flight mass spectrometry (MALDI-TOF MS) or LC-MS (Liquid chromatography-mass spectrometry (LC-MS) [9]). Recently, non-gel-based quantitative proteomics by mass spectrometry has been developed. In these techniques, peptides are labeled with isotope-coded affinity tags (iCAT) or isobaric tags for relative and absolute quantification (iTRAQ) [5, 8]. In addition, in vivo incorporation with isotope labeled amino acids can be performed by SILAC (stable isotope labeling by amino acids in cell culture) [8]. These MS-based techniques are very useful for quantitative proteomics [5, 8]. Among them, the advantage of iTRAQ method is that up to eight samples can be analyzed simultaneously.

Some *Arabidopsis* MAPKKs regulate the innate immune responses to the bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000 (*Pst*DC3000) [10–15]. These MAPKKs were also involved in the disease resistance against a fungal phytopathogen, *Fusarium sporotrichioides* producing trichothecene phytotoxin (T-2 toxin) ([16]; unpublished results). T-2 toxin activated novel MAPK cascades including these MAPKKs ([16]; unpublished results). Therefore, to screen the target proteins regulated by the MAPKK in response to T-2 toxin, the phosphoproteins were purified from T-2 toxin-treated wild-type and mapkk mutant plants by the Pro-Q® Diamond Phosphoprotein Enrichment Kit. Purified phosphorylated proteins were digested by the trypsin, and resulting peptides were labeled with iTRAQ methods. Then, these peptides were quantified and identified by MALDI TOF/TOF analyzer. Thus, in this chapter, we introduce the quantitative phosphoproteome technique using iTRAQ to identify downstream target proteins of the MAPK cascades in plants.

2 Materials

2.1 Plant Growth and Trichothecene Phytotoxin Treatment

1. Murashige and Skoog agar medium: Murashige and Skoog Plant Salt Mixture (Wako, Osaka, Japan), vitamine mixture (100 mg/L Myoinositol, 0.5 mg/L Nicotinic acid, 0.5 mg/L Pyridoxyn, 0.1 mg/L Thiamine, 2 mg/L L-Glycine), 2 % (w/v) sucrose, 0.2 % (w/v) gellan gum, and adjusted to pH 6.0 by 0.1 N KOH.
2. T-2 toxin (10 mg/mL) is dissolved in 99.5 % (v/v) ethanol (*see* **Note 1**).

2.2 SDS-Polyacrylamide Gel for 2D Electrophoresis

2.2.1 Stock Solution

1. 30 % (w/v) acrylamide–*N*,*N*′-Methylenebisacrylamide solution (29.2:0.8). Store at 4 °C in the dark.
2. 1 M Tris–HCl, pH 8.8. Store at room temperature.
3. 10 % (w/v) SDS solution. Store at room temperature.
4. 25 % (w/v) Ammonium persulfate is dissolved in water, and stored at −20 °C.
5. 10× Running buffer; 2.5 M Tris base, 19.2 M Glycine, and 10 % (w/v) SDS.

2.2.2 10 % SDS-Polyacrylamide Gel

1. Mix gently 10 mL of 30 % (w/v) acrylamide/*N*,*N*′-Methylenebisacrylamide solution, 8.4 mL of deionized water, 11.2 mL of 1 M Tris–HCl, pH 8.8, 300 μL of 10 % SDS solution, 24 μL of *N*,*N*,*N*,*N*′-Tetramethyl-ethylenediamine (TEMED), and 100 μL of 25 % (w/v) ammonium persulfate (APS). Pour the mixture to gel plate and then add *N*-butyl alcohol to the top of gel.

3 Methods

3.1 Preparation of Samples

1. The Columbia (Col-0) ecotype of *Arabidopsis thaliana* (L.) Heynh. was used as wild type in this study. A T-DNA insertion mutant of *MKK* was obtained from the *Arabidopsis* Biological Resource Center, Ohio state University, Columbus, OH.
2. Surface-sterilized seeds were sown on Murashige and Skoog agar medium in disposable plastic petri dishes.
3. The seeds were placed at 4 °C under dark condition for 2 days and then were grown at 22 °C under a 16–8-h light–dark cycles.
4. For T-2 toxin treatment, 10-day-old *Arabidopsis* plants grown on MS agar medium without trichothecenes were transferred to 0.5 μM T-2 toxin-containing medium.
5. At 3 h after T-2 toxin treatment, the roots of *Arabidopsis* plants were cut by a scissors, and roots were quickly collected into tubes in liquid nitrogen.

3.2 Protein Extraction and Purification of Phosphorylated Protein

1. *Arabidopsis* roots were ground in a pestle under liquid nitrogen to obtain a fine powder. For protein extraction, about 1 g of these fine powders were mixed with 5 mL of HEPES buffer containing 1 % (v/v) Triton-X 100, 1 mM PMSF, and 1/1,000 protease inhibitor cocktail (e.g., Sigma) (*see* **Note 2**), then were homogenized with vortex mixer.
2. These extraction solutions were centrifuged (14,000 × *g*, 4 °C, 15 min), and the supernatant was collected. Protein contents in these solutions were measured by RC DC Protein Assay Kit (e.g., Bio-Rad) (*see* **Note 3**).

3.3 Purification of Phosphorylated Protein

1. Dilute the protein extractions to a final protein concentration of 0.1–0.2 mg/mL with wash buffer. Equilibrate the Pro-Q® Diamond Phosphoprotein Enrichment column (Invitrogen) with 1 mL of wash buffer twice.
2. Apply the appropriate volume (maximum input 1 mg) of diluted protein solutions to the column and then wash the column with 1 mL of wash buffer three times.
3. Elute the column with 250 μL of elution buffer five times, collect the elute fractions, and concentrate up to approximately 50 μL by the Vivaspin filtration concentrator. When 1 mg of proteins extracted from *Arabidopsis* roots is loaded onto the column, approximately 50 μg of phosphoproteins is recovered.
4. Add six volumes of cold acetone to purified phosphoproteins and then mix gently.
5. Incubate for 4 h at −20 °C. Then, carefully decant the acetone.
6. The precipitates are dissolved into dilution buffer (AB Sciex). Protein concentrations in these solutions are also measured by RC DC Protein Assay Kit (Bio-Rad) (*see* **Note 3**).
7. A small amount of the purified phosphoproteins are checked by SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE).
8. Each gel is stained by Pro-Q® Diamond Phosphoprotein Gel Stain (Invitrogen) according to the manufacture's recommended protocol.
9. After staining, gels are visualized by scanning using a Typhoon™ 9400 imager (GE Healthcare) using at a 532 nm laser and an emission filter of 560 nm band pass (LP) with 100 μm resolution. The photomultiplier tube (PMT) is set 600 V by using normal sensitivity. Gel images show Fig. 1.
10. The scanned images are analyzed by the ImageQuant V5.2 software package (GE Healthcare).

3.4 Reducing the Proteins and Alkylation of Cysteine

1. Purified phosphoproteins from wild-type and *mkk1* mutant proteins (100 μg each) are labeled with the iTRAQ™ Reagents.
2. Add 1 μL Denaturant (2 % w/v SDS) in the kit (AB Sciex), and mix gently.

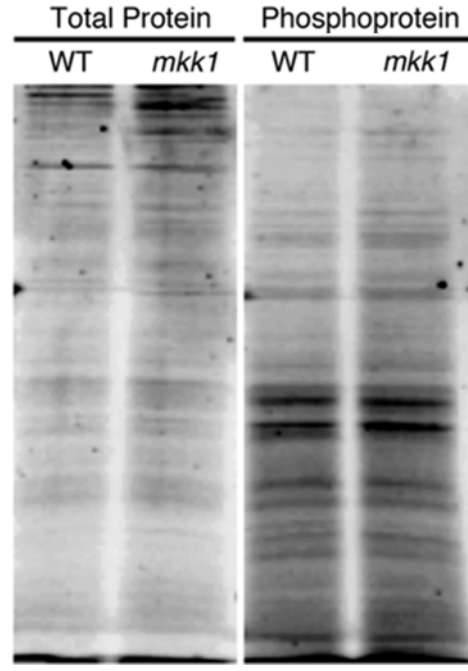

Fig. 1 Gel images of total protein and phosphoprotein. Total protein or phosphoprotein was prepared from T-2 toxin-treated root of *Arabidopsis thaliana*. The wild-type and *mkk1* mutant plants were treated by T-2 toxin for 3 h. Then 5 μg of each protein was loaded into SDS-PAGE. The SDS-PAGE was carried out at 10 mA constant voltage for 1 h

3. Add 2 μL Reducing Reagent (AB Sciex) and mix gently.
4. Incubate the tubes at 60 °C for 1 h, and then centrifuge briefly.
5. Add 1 μL Cysteine Blocking Reagent and mix gently.
6. Incubate the tubes at room temperature for 10 min.

3.5 In-Gel Digestion by Trypsin

1. Add 10 μL of the trypsin solution and mix by pipetting.
2. Incubate the tubes at 37 °C overnight.
3. Briefly centrifuge the tubes.

3.6 Labeling the Peptide with iTRAQ™ Reagents

1. Add 70 μL of ethanol to each iTRAQ™ reagent vial.
2. Dissolve each iTRAQ™ reagent by vortex, and add one iTRAQ™ reagent vial to one sample tubes. Peptides derived from wild-type and *mkk1* mutant plants were labeled with tags 115 and 117, respectively.
3. Vortex each tube to mix.
4. Incubate each tube at room temperature for 1 h.
5. Combine the each iTRAQ™-labeled sample, and vortex to mix.

4 Purification of iTRAQ™ Reagents-Labeled Peptides

1. Add at least tenfold volumes of Cation Exchange Buffer-Load (AB Sciex), and vortex to mix.
2. Adjust the pH of samples is between 2.5 and 3.3 by Cation Exchange Buffer-Load (AB Sciex) using pH indicator papers.
3. Prepare Cation-Exchange Cartridge according to the manufacturer's standard protocol.
4. Inject 1 mL of the Cation Exchange Buffer-Clean into Cation-Exchange Cartridge.
5. Inject 2 mL of the Cation Exchange Buffer-Load into Cation-Exchange Cartridge.
6. Inject the peptide mixture into Cation-Exchange Cartridge (1 drop/s).
7. Inject 1 mL of the Cation Exchange Buffer-Load into Cation-Exchange Cartridge.
8. To elute the iTRAQ™-labeled peptides, inject 500 μL of the Cation Exchange Buffer-Load into Cation-Exchange Cartridge.
9. Dry the iTRAQ™-labeled peptides by the freeze dryer.
10. Add 30–50 μL of 0.1 % (w/v) trifluoroacetate (TFA) and vortex to mix.

4.1 Nano-liquid Chromatography

1. The iTRAQ™-labeled peptides are injected to nanoLC/MALDI fraction system (Dina-MaP) (KYA technologies).
2. The iTRAQ™-labeled peptides are fractionated to 384 spots using HiQ sil C18W-3 column (15 mm i.d. × 50 mm, 120 Å pore size, 3 mm particle size) at a flow rate of 300 nL/min in 0.1 % (w/v) TFA and 2 % (v/v) acetonitrile (buffer A). The gradient of Dina-MaP is carried out by buffer A and 70 % (v/v) acetonitrile with 0.1 % (w/v) TFA (buffer B).
3. After equilibration in buffer A, a multiple gradient started 10 min after the injection signal as follows: 5 % (buffer B) at 24 min, 50 % (buffer B) at 159 min, 100 % (buffer B) at 174 min, 100 % (buffer B) at 199 min, 0 % (buffer B) at 200 min, and 0 % (buffer B) at 210 min. Fraction collection is started 10 min after the injection and was finished 200 min. Fractions are collected every 30 s for a total of 384 spots per fraction. The eluates and α-Cyano-4-hydroxycinnamic acid matrix solution are mixed on the plate.

4.2 Identification and Quantification of Proteins by MALDI TOF/TOF Analyzer

The labeled peptides are analyzed using a 4800 Plus MALDI TOF/TOF™ Analyzer (AB Sciex) [9]. MS/MS data are evaluated by considering amino acid substitution and modification against the NCBI database using the Paragon algorithm [17] of ProteinPilot™ v2.0 software (AB Sciex). Gained results are shown in Table 1.

Table 1
Comparative analysis between T-2 toxin-treated wild-type and *mkk1* mutant plants using iTRAQ

Protein	Accession no.	ProtScore	% Cov	*mkk1*/WT
Unknown protein	gi|30697677	2.0	15.3	0.37
AT hook motif DNA-binding family protein	gi|15237481	1.5	13.0	0.47
Heat shock protein 89.1	gi|6466963	10.1	19.3	0.49
Cysteine-rich receptor-like protein kinase 8	gi|7682800	1.8	3.9	0.50
Pre-rRNA-processing protein	gi|15241108	1.7	20.7	0.61
O-Methyltransferase 1	gi|2781394	2.0	9.9	0.61
General regulatory factor 8	gi|30698124	7.3	48.0	0.63
Translation initiation factor-related	gi|30693092	2.0	6.7	0.64
Eukaryotic translation initiation factor 5A-1	gi|9295717	2.0	7.4	0.64
Receptor lectin kinase	gi|15224347	1.5	4.7	0.64
Unknown protein	gi|21554893	2.0	14.1	0.64
Galactose oxidase/kelch repeat superfamily protein	gi|21593163	1.2	15.3	0.65
VHS domain-containing protein/GAT domain-containing protein	gi|30686081	2.0	13.1	0.66

5 Notes

1. T-2 toxin should be stored at −20 °C.
2. Do not use phosphate buffer for purification of phosphoproteins.
3. RC DC Protein Assay Kit is compatible with the following reagents and buffers; 2 % (w/v) CHAPS, 350 mM DTT, 0.1 M EDTA, 0.5 M imidazole, Laemmli sample buffer with 5 % (v/v) β-mercaptoethanol, 2.5 M sodium hydrate 2 mM TBP, 0.5 M Tris, 2 % (v/v) Triton, and 2 % (v/v) Tween 20.

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