Optimized phos-tag mobility shift assay for the detection of protein phosphorylation in planta

Shah Hussain · Nhan Thi Nguyen · Xuan Canh Nguyen · Chae Oh Lim · Woo Sik Chung

Abstract  Post-translational modification of proteins regulates signaling cascades in eukaryotic system, including plants. Among these modifications, phosphorylation plays an important role in modulating the functional properties of proteins. Plants perceive environmental cues that directly affect the phosphorylation status of many target proteins. To determine the effect of environmentally induced phosphorylation in plants, in vivo methods must be developed. Various in vitro methods are available but, unlike in animals, there is no optimized methodology for detecting protein phosphorylation in planta. Therefore, in this study, a robust, and easy to handle Phos-Tag Mobility Shift Assay (PTMSA) is developed for the in vivo detection of protein phosphorylation in plants by empirical optimization of methods previously developed for animals. Initially, the detection of the phosphorylation status of target proteins using protocols directly adapted from animals failed. Therefore, we optimized the steps in the protocol, from protein migration to the transfer of proteins to PVDF membrane. Supplementing the electrophoresis running buffer with 5 mM NaHSO₃ solved most of the problems in protein migration and transfer. The optimization of a fast and robust protocol that efficiently detects the phosphorylation status of plant proteins was successful. This protocol will be a valuable tool for plant scientists interested in the study of protein phosphorylation.

Keywords  Electrophoresis mobility shift assay, Phos-tag, Plant, Protein kinases, Protein phosphorylation

Introduction

Most cellular regulatory events are affected by external stimuli that modulate protein activity by regulation protein localization and stability. Among these modulating events, post-translational modification plays an important role, by providing a dynamic means of regulating protein function (Jesper et al. 2006; Joseph 2000; Nishi et al. 2011). Among the different types of posttranslational modification, reversible phosphorylation modifies proteins involved in many cellular processes (Tony 2000). In a dynamic fashion phosphorylation can target a protein for degradation, stabilization, or direct it to become a part of a larger protein complex. Understanding protein phosphorylation is absolutely necessary for elucidating the molecular mechanisms of many diverse biological processes (Scott 2006).

Protein kinases are a major group of enzymes responsible for the specific phosphorylation of their target substrates. In Arabidopsis, about 1000 genes have been identified as protein kinases (Degeng et al. 2003), five times more than found in yeast and two times more than in other eukaryotic organisms (Mellisa and Shin-Han 2012). Most plants encode members of several specialized kinase classes, including mitogen-activated protein kinases, receptor like kinases, cytoplasmic protein kinases and calcium dependent protein kinases (Degeng et al. 2003). Generally, it is believed that these kinases use the γ-phosphate of adenosine triphosphate to phosphorylate serine, threonine, or tyrosine residues in the target protein (Steven and Tony 1995). In most biological systems, phosphorylated proteins can be antagonistically converted to a non-phosphorylated form via protein phosphatases to switch a particular cellular response on or off (Sophie and Nigel 2001).
Several methods are currently available for determining whether a kinase is active or inactive and to check the phosphorylation status of the target protein both in vitro and in vivo. These include the use of radiolabeled isotopes, phospho-specific peptide antibodies, and mass spectrometry based analysis (Bruno and Ruedi 2006; James et al. 2006; Jesper and Matthias 2004; Marie 2002; Mark et al. 2003; Scott 2006; Toshiyoshi et al. 2007; Xiang et al. 2007). Proteins phosphorylated at serine, threonine, or tyrosine residues can be efficiently identified through the use of radiolabeled ATP. However, concerns about the disposal and usage of radioisotopes have led to the development of non-radioactive based methods for determining the phosphorylation status of target proteins (Young-Whan et al. 1999).

Among the non radioactive based approaches, PTMSA is a method of choice for determining the phosphorylation status of target proteins. The discovery of the alkoxide-bridged dinuclear zinc (II) complex by Kinoshita and his group (2004) marked a central breakthrough for this technique, as it binds the phosphate monoester dianion. This complex was therefore named “phos-tag”. Similarly, the manganese (II) homologue has the ability to bind phospho-serine and phospho-tyrosine residues (Eiji et al. 2009a). The phos-tag bound to polyacrylamide enables it to alter the mobility of a phosphorylated protein relative to its non-phosphorylated version during polyacrylamide gel electrophoresis, thereby producing double bands when visualized with protein specific antibodies (Eiji et al. 2008). High molecular weight proteins, even those larger than 200 kDa, can be easily analyzed with this novel technique by using 3% acrylamide gel supplemented 0.5% w/v agarose than 200 kDa, can be easily analyzed with this novel technique by using 3% acrylamide gel supplemented 0.5% w/v agarose. Proteins phosphorylated at serine, threonine, or tyrosine residues can be efficiently identified through the use of radiolabeled ATP. However, concerns about the disposal and usage of radioisotopes have led to the development of non-radioactive based methods for determining the phosphorylation status of target proteins (Young-Whan et al. 1999).

As varying concentrations of the component materials provide different results, optimum concentrations must be empirically determined for each protein. Unfortunately, most plant scientists have experienced problems during gel formation, running proteins in the gel, transferring proteins to the membrane, and during development of the membrane. Based on our observations, we found that PTMSA running buffers using native conditions are very important for protein migration and subsequent detection of phosphorylated protein residues. We suspect that partial hydrolysis in 1 X Tris-Glycine might be the reason of the disorganization in the phos-tag gel. Through empirical experimentation, we have optimized a protocol for the detection of plant protein phosphorylation that minimizes potential problems.

**Materials and Methods**

**Plant material**

*Arabidopsis thaliana* Columbia-0 (Col-0) seedlings were grown under long day conditions with a 16-h light / 8-h dark cycle for 10 days. A transgenic line over expressing 3 x Flag-tagged-protein (hereafter referred to as protein X) was used for the detection of phosphorylation status in planta. Ten days old seedlings were treated with 200 mM mannitol for 1 and 3 hours to induce phosphorylation.

**Protein Extraction**

The samples were ground in liquid nitrogen and protein was extracted using plant protein extraction buffer. The samples were kept on ice for 5 min and vortexed vigorously 2 ~ 4 times. The sample was centrifuged at 13,000 rpm for 10 min. The supernatant was transferred to a new, pre-chilled tube and centrifuged again for 5 min to remove tissue debris.

**Gel Preparation**

The glass, spacer, and tubes were washed with clean water to remove dust. 10 ~ 12% gel solution containing 25 ~ 100 µM phos - tag and 50 ~ 200 µM ZnCl₂ or MnCl₂ (1:2 ratio) were
made and TEMED (5 µL/5 mL) was added but 10% APS was not. The gel solution was degassed at 40 ~ 50 cm Hg for 3 ~ 5 min using a vacuum to remove oxygen and other dissolved gasses. Then 10% APS was added and mixed gently to prevent the formation of bubbles. The gel solution was poured gently between the glass slabs. Isopropanol was added on the top of the gel solution to remove bubbles and to prevent the gel from drying during polymerization. After polymerization, the isopropanol was gently removed. The top of the gel was once washed with water and dried with filter paper. Finally the stacking gel was prepared according to standard procedure.

Gel electrophoresis

After polymerization of the gel solution, the comb was removed and the electrophoresis system was assembled. The chamber was filled with running buffer (1 X Tris-Glycine buffer) supplemented with NaHSO₃ or NaHCO₃ (to 500 mL 1 X Tris-Glycine buffer add 5 mL 0.5 M NaHSO₃ or NaHCO₃ (for a final concentration of 5 mM). The protein samples were prepared by denaturing for 3 ~ 5 min and placing on ice for 5 min. 70 ~ 100 µg total proteins were loaded. Electrophoresis was started with an initial voltage of 50 V. Voltage was increased to a maximum of 80 V after 1 hr.

Removing ZnCl₂

After electrophoresis, the gel was washed in 1 X transfer buffer supplemented with 1 mM EDTA for 10 min to remove excess Zn²⁺ ions. Next, we washed the gel with 1 X transfer buffer without EDTA twice for 10 min to remove EDTA.

Protein transfer and membrane blocking

The protein was transferred the PVDF membrane using Bio-Rad, Trans-Blot Turbo Transfer System. After transfer, the membrane was washed in 1 X TBS - T for 5 min. The membrane was incubated in 5% skimmed milk at 4°C for 5 h or overnight. Following day the membrane was washed 3 times in 1 X TBS - T for 10 min.

Western blot analysis

Total proteins were extracted from 10 days old seedlings according to the method of Kim et al. (2017). 80 µg proteins were separated by 10% SDS - PAGE. After transfer to PVDF membrane proteins were detected by mouse anti-flag (1:5000, Sigma, USA) as primary antibody and HRP conjugated secondary antibody (1:5000) and visualized using ECL kit (Bio-Rad Laboratories, USA).

Results and discussion

Previous methods showed irregular protein migration in 1 X Tris-Glycine buffer

During our initial experiment, we failed to observe plant protein phosphorylation using 10% SDS gel containing phos-tag, even using low voltage (50 V) in 1 X Tris-Glycine buffer (Tris, SDS, Glycine). The protein migration rate was severely affected by the presence of metal ions. The lanes were highly disorganized and, in some cases, the separation of higher molecular weight protein (above 60 kDa) was impaired. Furthermore, transferring proteins from the gel to the PVDF was extremely inefficient, with most proteins failing to transfer using the wet-transfer method even after removal of metal ions through multiple washes with EDTA and 1 X transfer buffer. By comparing the phos-tag gel with a normal SDS gel we suspected that the failure in protein migration and gel disorganization might be due to the concentration of the phos-tag and metallic ions. Therefore, we checked different concentrations of the phos-tag and ZnCl₂ (MnCl₂) in 8-12% gel (Table 1).

Change in concentration of the phos-tag and ZnCl₂ has same effect on protein migration in 1 X Tris-Glycine buffer

Eiji et al. (2009a) found that the ratio between the phos-tag and ZnCl₂ must be 1: 2. To check that change in phos-tag and ZnCl₂ has any effect on acryl-amide concentration in the gel. We tested different concentrations of the component materials. It has been found that the problem associated with running and blotting was mostly independent of the concentration of phos-tag, ZnCl₂ and acryl-amide in 1X tris-glycine buffer (Table 1).

Running buffer is an important factor in PTMSA

Our next target in the optimization of PTMSA was to check the running buffer. As protein migration and transfer were extremely inefficient in 1 X Tris-Glycine buffer, we were unable to determine the phosphorylation status of the target protein (Fig. 2, Middle blot). Therefore, we replaced 1 X Tris-Glycine with 1 X MOPS. This led to a marked improvement in the gel condition and the lane organization (Fig. 1B and Table 1). However, higher molecular weight proteins above 60 kDa were not separated. These findings led us to think
about other possible factors that might be responsible for the abnormal migration and transfer of proteins in PTMSA.

Reduction of free radicals by addition of sodium bisulfite improves PTMSA

We suspected that the Tris-Glycine buffers might undergo partial hydrolysis in the presence of the electric current during the run. This could ultimately lead to the disorganization by changing the porosity and integrity of the acryl-amide gel. Previously, Kinoshita and his group (2009) tried to overcome this problem by using a gel system containing dizinc (II) acrylamide-phos-tag in Bis-Tris buffer at a neutral pH. However, we failed to obtain satisfactory results using the same system. We suspect that due to hydrolysis, free radicals are generated that might be the source of these problems. We chose sodium bisulfite (NaHSO₃) as a mild reducing agent to quench the free radicals produced during hydrolysis in 1 X Tris-Glycine buffer. Similarly, NaHSO₃ can also neutralize the oxidation effect of ZnCl₂ in the gel. We found that supplementing 1 X Tris-Glycine buffer with 5 mM NaHSO₃ produced good result (Fig. 1C). With the addition of NaHSO₃, we observed normal migration of proteins in the gel. We next tested different concentrations of phos-tag: ZnCl₂ (25: 50 µM and 50: 100 µM) and acrylamide (10 and 12%) and obtained similar results. Protein transfer efficiency from the gel to the PVDF membrane was tested by using both the dry and semi-dry protein transfer method using a Bio-Rad transblot transfer system (USA) for a maximum 14 minutes. After

### Table 1 Troubleshooting conditions for optimization of the phos-tag gel in different running buffers

<table>
<thead>
<tr>
<th>Phos–tag (µM)</th>
<th>ZnCl₂ / MnCl₂ (µM)</th>
<th>Gel %</th>
<th>Running buffer</th>
<th>NaHSO₃</th>
<th>Gel Electrophoresis</th>
<th>After transfer to PVDF membrane</th>
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<tr>
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Notes: "MnCl₂ was used instead of ZnCl₂, "-" indicates the absence of NaHSO₃ in running buffer during gel electrophoresis, '+' indicates the presence of NaHSO₃ in running buffer during gel electrophoresis.

![Fig. 1](image) 10% gel containing 100 µM phos-tag were subjected to run in (A) 1 X Tris-Glycine buffer, (B) 1 X MOPS buffer, (C) 1 X Tris-Glycine buffer supplemented with 5 mM NaHSO₃. Total proteins extracted from plants were stained using coomassie brilliant blue
transfer, we checked the protein transfer efficiency by incubating the membrane in Coomassie Brilliant Blue (CBB) for 10 min and found that transfer from the gel to the PVDF membrane was successful. These observations suggest that an optimal running buffer is critically important in PTMSA for normal protein migration as well as for transfer to the PVDF membrane.

The optimized PTMSA protocol can determine plant protein phosphorylation status

Our final goal for optimizing PTMSA was to determine the phosphorylation status of target proteins in planta. Based on preliminary results, we hypothesized that our protein of interest is a putative substrate of a plant MAP kinase cascade (unpublished data). We therefore used our modified PTMSA protocol to determine the phosphorylation status of protein X in planta. Arabidopsis seedlings over-expressing an epitope tagged (3 x Flag) version of protein X were grown on 1 / 2 X MS media for 10 days. Seedlings were treated with 200 mM mannitol for 1 and 3 hours to activate the MAP kinase cascade suspected of phosphorylating protein X. Before PTMSA, a standard Western blot was performed to determine the expression level of the proteins X (Fig. 2, Upper blot). PTMSA was performed by mixing the gel solution, degassing the solution for 3 ~ 5 minutes under vacuum to remove oxygen, and subjecting the solution to polymerization for 10 ~ 20 minutes. 80 µg of total protein were loaded onto a 1 mm thick 10% acrylamide gel and subjected to 60 ~ 80 V. After transfer to PVDF membrane and consecutive treatment with the primary anti-Flag 1: 5000 and secondary anti-mouse IgG-HRP 1: 5000 antibodies, the membrane were developed with ECL solution. We were indeed able to detect retarded bands representing the phosphorylated protein using 100 µM phos-tag and 200 µM ZnCl₂ (Fig. 2, Lower blot). Based on these results, we conclude that our optimized protocol is a simplified and robust version of the phos-tag mobility shift assay that will be a useful tool for plant scientists to determine the phosphorylation status of proteins in planta.

Important considerations

The phos-tag mobility shift assay has proven to be a difficult assay, as many researchers have faced problems in optimizing the gel. To overcome the difficulties cleaned glasses and working equipment should be used, as dust might reduce the binding ability of the phos-tag. All the solutions should be properly handled and prepared. Zn⁺² ions should be removed by completely washing in transfer buffer with EDTA. Next completely remove EDTA by washing in 1 X transfer buffer. Traces of Zn⁺² or EDTA can cause problems in protein transfer. Smiley bands are often obtained after detection of signals that may be due to the attraction of the phosphorylated proteins to the phos-tag.

Limitations

The current protocol is convenient for determining the phosphorylation status of proteins in planta. However, we found that the migration of the protein size marker does not match the expected location of our protein in the phos-tag gel. This might be due to the presence of 20 mM Tris-phosphate in the protein size marker (PM2510, SMOBIO) which can interact with the phos-tag. Therefore, it is recommended to test the expression of the protein on a normal SDS-acrylamide gel without phos-tag to determine the size of the target proteins prior to performing PTMSA.

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