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High-Throughput Phosphorylation Screening and Validation through Ti(IV)-Nanopolymer Functionalized Reverse Phase Phosphoprotein Array

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Abstract

Protein phosphorylation is one of the most important and widespread molecular regulatory mechanisms that controls almost all aspects of cellular functions in animals and plants. Here, we introduce a novel chemically functionalized reverse phase phosphoprotein array (RP3A) to capture and measure phosphoproteomes. RP3A uses polyamidoamine (PAMAM) dendrimer immobilized with Ti(IV) ions to functionalize nitrocellulose membrane, facilitating specific chelation of phosphoproteins from complex protein samples on the array. Globular, water-soluble Ti(IV)-dendrimer allows the RP3A surface to be highly accessible to phosphoproteins multidimensionally, and the captured phosphoproteins were subsequently detected using the same validated antibodies as in regular reverse-phase protein arrays. The novel chemical strategy demonstrated superior specificity (1:10 000), high sensitivity (fg level), and good quantitative nature ($R^2 = 0.99$) for measuring phosphoproteins. We further applied quantitative phosphoproteomics followed by RP3A to validate the phosphorylation status of a panel of phosphoproteins in response to environmental stresses in *Arabidopsis*.

Graphical Abstract

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Supporting Information

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Detection of different samples by RP3A (PDF)

The authors declare no competing financial interest.

Protein phosphorylation is the most prevalent post-translational modification (PTM), and the resulting activation/deactivation of proteins plays an essential regulatory role in a wide variety of biological processes.^{1,2} Western blotting, immunohistochemical staining, and enzyme-linked immunosorbent assay (ELISA) are indispensable tools for monitoring phosphorylation changes using phosphospecific antibodies. However, these approaches have been severely limited by the availability and quality of phospho-specific antibodies, constraining analyses only to well-characterized phosphorylation events.³ While applications of mass spectrometry (MS) lead to the discovery of numerous novel phosphorylation events every day, an effective antibody has to be made for every single phosphorylation site on individual proteins for validation, making it the bottleneck step in many discovery efforts.^{4,5} Researchers working on model organisms, such as *S. cerevisiae*, *Drosophila*, and *Arabidopsis thaliana*, do not have access to commercial phosphospecific antibodies and have to use undesirable methods such as ³²P radioactive labeling.

Reverse phase protein array (RPPA) is an emerging high-throughput approach that provides protein expression data across a large set of biological samples simultaneously.⁶⁻⁹ In a general RPPA workflow, denatured protein samples from cells, body fluids, tissues, and so on are directly spotted on a membrane. Subsequently, the membrane array is incubated with an antibody specific for the antigen of interest and visualized to estimate the protein concentration. Multiplexing is achieved by probing multiple arrays spotted with the same lysate with different antibodies simultaneously or probing different lysate with the same antibodies. The throughput, sensitivity, and requirement of one single antibody in contrast to antibody pairs in sandwich assays, together with its ability to deal with minuscule sample amounts, have propelled applications of the technology in basic, preclinical, and clinical research fields. For example, as the RPPA works with tiny amounts of proteins, the implementation of RPPA into clinical practice could help evaluate protein-based biomarkers.¹⁰ RPPA has also been used as a powerful tool for signaling pathway profiling.¹¹

Despite the success, RPPA, which relies heavily on the paucity of high-quality monospecific antibodies, is highly limited for the detection of proteins with post-translational modifications (PTMs) such as phosphorylated proteins, due to the relatively low availability and low quality of phosphospecific antibodies.¹² Moreover, an overwhelming number of nonphosphoproteins usually present in the sample and the high complexity with tens of thousands of proteins in cell lysates or tissue extracts make it extremely challenging to detect phosphoproteins which are usually of low abundance.¹³ With the daily discovery of novel phosphoproteins and phosphorylation sites involved in critical biological events, there

is an urgent need to fill this gap in phosphoprotein profiling and validation by RPPA in high throughput, which would greatly contribute to the comprehensive phosphorylation analysis and greatly expand the scope of RPPA applications. Accordingly, we report here a novel chemically functionalized RPPA, which we termed reverse phase phosphoprotein array (RP3A), to capture and measure phosphoproteins in complex biological samples in high throughput. We functionalized nitrocellulose membrane with polyamidoamine (PAMAM) dendrimer immobilized with Ti(IV) ions to enable specific chelation of phosphoproteins, and the captured phosphoproteins by RP3A were subsequently detected using the same validated antibodies as in regular RPPA. We demonstrated superior specificity, high sensitivity, and good quantitative nature of RP3A for phosphoprotein analyses. Finally, we integrated quantitative phosphoproteomics with RP3A to validate the phosphorylation status of a panel of phosphoproteins in response to environmental stresses in *Arabidopsis*.

MATERIALS AND EXPERIMENTAL PROCEDURE

Materials.

All chemicals for the synthesis of Ti(IV) functionalized soluble nanopolymer, Amicon Ultra centrifugal filter units, the purified cJun, and the primary antibodies were obtained from Sigma-Aldrich. The secondary antibodies linked with IRDye 800 were purchased from LI-COR Biosciences. The sample printing pin was obtained from Arrayit. SnakeSkin pleated dialysis tubing (3500 MWCO, 22 mm dry diameter) and BCA Protein Assay Kit were purchased from Pierce.

Synthesis of Ti(IV) Functionalized Soluble Nanopolymer.

500 μ L of PAMAM (polyamidoamine) dendrimer generation 4 solution (provided as 10% (wt/vol) in methanol; Millipore-Sigma) was mixed with 5 mL of 250 mM MES buffer in water (2-(N-morpholino)ethanesulfonic acid; pH 5.5) in a 10 mL round-bottom flask with a magnetic stir bar. Then, 20 mg of 3-phosphonopropionic acid, 30 mg of *N*-hydroxysuccinimide (dissolved in 200 μ L of water), and 400 mg of EDC (1-ethyl-3-(3-(dimethylamino)propyl) carbodiimide hydrochloride) were added into the flask and stirred overnight to functionalize the dendrimer with phosphonic acid. The solution was dialyzed against water overnight using SnakeSkin pleated dialysis tubing (3500 MWCO, 22 mm dry diameter, Pierce) to remove any remaining unreacted reagents (replaced with fresh water 3 times, each time 6–8 h). Then, the reagent solution was dried down completely using a SpeedVac vacuum concentrator (LabConco). The reagent was resuspended in 5 mL of DMSO; then, 75 mg of DMAP (4-(dimethylamino)-pyridine) and 50 μ L of acetic anhydride were added, and the mixture was incubated for 2 h. The mixture was mixed with 5 mL of water and dialyzed overnight against water. The mixture was dried down to ~5 mL, and 50 μ L of titanium oxychloride stock solution was added; the mixture was incubated for 1 h with agitation at room temperature to chelate titanium ions with phosphonic acid groups on the dendrimer. The solution was finally dialyzed against 0.01% HCl overnight. The final product was diluted to 40 mL final volume with water and stored at 4 °C.

Preparation of Reverse Phase Phosphoprotein Array (RP3A).

Nitrocellulose membrane was incubated in a solution of Ti(IV) functionalized soluble nanopolymer (8 M) for 1 h, and then, the solution was removed. The membrane was immediately incubated with 1% BSA in TBST for 1 h to block the membrane. Then, the membrane was incubated with 1% TFA for 15 min to acidify the membrane. Finally, the membrane was dried completely (usually 30 min) and used for the following spotting of phosphoproteins.

Sample Spotting and Detection by RP3A.

Prepared protein lysates were spotted on the membrane using a pipet tip or microarray printing pin (Arrayit SMP15B). After the membrane was dried, it was washed with 0.05% SDS in TBST for three times, 5 min per wash. Then, the membrane was blocked with 1% BSA in TBST and incubated with a primary protein antibody overnight. After that, the membrane was washed with 1% BSA in TBST for three times, 5 min per wash. A corresponding secondary antibody linked with IRDye 800 (LI-COR Biosciences) was then used for direct fluorescence-based detection. The membrane was scanned using an infrared imaging system (LI-COR Odyssey), and the fluorescent signals were recorded and quantified.

Plant Sample Preparation for RP3A and Regular RPPA.

Plant samples from *Arabidopsis thaliana* were used in this study (*Genus: Arabidopsis; Species: Arabidopsis thaliana; Ecotype: Col-0*). For cold treatment, the 10-day-old seedlings were placed in a chamber at 4 °C, and the seedlings before and after cold treatment were collected. For ABA, mannitol, and flg22 treatment, the 10-day-old seedlings were transferred to 4 mL of liquid MS media, and then ABA, mannitol, and flg22 were added to the media with a final concentration of 50 μ M, 800 mM, and 100 nM. At the indicated time point, the seedlings were collected for protein extraction. The total proteins were extracted by using the following extraction buffer: 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM PMSF, 10 μ M antipain, 10 μ M aprotinin, 10 μ M leupeptin, and phosphatase inhibitor cocktail set II (Sigma-Aldrich). The concentration of total proteins was measured by using Quick Start Bradford Dye Reagent (BioRad), and finally, the concentration of each protein sample was adjusted to the same level. Samples then were diluted with 2% SDS and boiled for spotting.

Arabidopsis thaliana Sample Preparation for Phosphopeptides Enrichment and LC-MS/MS Analysis.

The sample preparation procedure is based on a published paper.¹⁴ Briefly, the extracted proteins were digested with Lys C for 3 h and then digested with trypsin for another 12 h to obtain peptides mixture. Phosphopeptides were enriched using a PolyMAC-Ti kit (Tymora Analytical, West Lafayette, IN).¹⁵ Briefly, the digested peptides were resuspended in 200 μ L of Loading buffer, and the sample was vortexed. Next, 50 μ L of the PolyMAC/Magnetic Capture beads were added to the sample and incubated for 20 min. The solvent was removed using a magnetic separator rack, and the beads were washed with 200 μ L of Washing buffer 1 (50 mM glycolic acid in 0.5% TFA and 95% ACN) for 5 min. The beads were then incubated with 200 μ L of Washing buffer 2 (80% ACN in ddH₂O) for 5 min, and the solvent

was removed as before. Finally, the beads were incubated twice with 100 μ L of Elution buffer for 5 min each. The eluents were combined and completely dried in a SpeedVac.

LC-MS/MS Analysis.

The resulting peptides were resuspended in 0.1% formic acid and injected into a Thermo Scientific Easy-nLC-1000 system. A 45 cm long C18 column with 75 μ m i.d. was packed in-house with 2.2 μ m C18 resin. The mobile phase buffer A constituted 0.1% formic acid in ultrapure water, and buffer B constituted 0.1% formic acid in 80% acetonitrile. The following gradient was used in the LC-MS/MS experiment: the gradient changed from 6% to 30% buffer B within 60 min and then from 30% to 50% buffer B within 10 min; it was increased to 95% buffer B within 5 min, followed by washing the column for 5 min. At the end, the gradient was reduced back to 6% buffer B and the column was equilibrated for 5 min. The flow rate of the UPLC was 250 nL/min. The electrospray ionization emitter tip was generated on the prepacked column with a laser puller (Model P-2000, Sutter Instrument Co.).

The Easy-nLC-1000 UPLC system was coupled online with a high resolution hybrid linear ion trap orbitrap mass spectrometer (LTQ-Orbitrap Velos Pro, Thermo Fisher). The mass spectrometer was operated in the data-dependent mode in which a full-scan MS was followed by 10 MS/MS scans of the most abundant ions. High-resolution MS scans were acquired in the Orbitrap (30 000 fwhm at m/z 400) to monitor peptide ions in the mass range of 350–1500 m/z , followed by collision-induced dissociation MS/MS scans in the ion trap (isolation width of 3 m/z , normalized collision energy of 30%) of the ten most intense precursor ions. Ions with unassigned charge state as well as singly charged species were excluded. The maximum ion injection times of the survey scans and the MS/MS scans were 250 and 25 ms, respectively, and the ion target values were set to 1×10^6 and 5×10^3 , respectively. Dynamic exclusion duration was set to 60 s. Data were acquired using Xcalibur software.

MS Data Analysis and Label-Free Quantification.

The raw files were analyzed using MaxQuant software (version 1.5.5.1) with the Andromeda search engine. The main search peptide mass tolerance for precursor ions was set to 10 ppm, and ITMS MS/MS mass tolerance was set to 0.6 Da. Enzyme specificity was set to trypsin, and a maximum of two missed cleavages were allowed. Carbamidomethyl cysteine was set as a fixed modification. Methionine oxidation, protein N-terminal acetylation, and phosphorylation on serine, threonine, or tyrosine residues were chosen as variable modifications. The spectra were searched against the Uniprot database (*Arabidopsis thaliana*, version 2015_01) with common contaminants and concatenated with the reversed versions of all sequences. Protein identification required at least one unique or razor peptide per protein group. The XIC peak intensity was extracted with a match time window of 1.0 min for “match between runs”. The required false discovery rate was set to 1% at the peptide and the protein level, and the minimum required peptide length was set to 6 amino acids. Contaminants, reverse identification, and proteins only identified by site were excluded from further data analysis using Perseus software (version 1.5.5.0). The peak intensities were $\log_2$ transformed and normalized by subtracting the medians. Missing values were imputed by

random sampling from a generated narrow normal distribution around the detection limit before statistical analyses were performed. We also filtered phosphorylation sites unable to be localized ($p < 0.75$). Principal component analysis (PCA) was performed with a Benjamin-Hochberg FDR cutoff of 0.05. The significantly enriched phosphorylation sites were selected by the two sample t test with a permutation-based FDR cutoff of 0.05 and $S0 = 0.2$. All the localized and significantly changed phosphorylation sites were submitted to Motif-X to determine the enriched phosphorylation motifs with the *Arabidopsis* Uniprot database as background. The significance was set at 0.000001; the width was set at 13, and the minimum number of occurrences was set at 20.

All mass spectrometry proteomics data were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD010208.

RESULTS AND DISCUSSION

The new phosphoprotein-specific RPPA platform, reverse phase phosphoprotein array (RP3A), is illustrated in Figure 1 for specific phosphoproteome capture. We devised the chemical strategy based on a soluble nanopolymer (i.e., dendrimer) which was functionalized with Ti(IV) ions for the specific chelation of phosphoproteins. This design takes advantage of the water-soluble, globular nature of the dendrimer, allowing for the access by low abundance phosphoproteins in a complex sample for optimum efficiency and maximum yield. We first modified the polyamidoamine (PAMAM) dendrimer generation 4.0 with phosphonate groups. Subsequently, titanium(IV) was immobilized on the surface through the chelation to phosphonate groups. The synthesized Ti(IV)-dendrimer was then incubated with nitrocellulose membrane to generate multidimensionally functionalized RPPA. In a RP3A experiment, denatured whole cell lysates or tissue extracts are spotted on the RP3A membrane for effective capture of phosphoproteins through the chelation of high density of Ti(IV) ions on dendrimer with phosphoproteins in three dimensions. The captured phosphoproteins are detected using the same validated antibodies as in regular RPPA, where any changes in signal can be attributed to changes in phosphorylation or in the expression of the phosphoprotein.

The novel RP3A platform has the following unique features: First, it utilizes the features of Ti(IV) chelation which has high specificity toward phosphoproteins and has widely been used in phosphoprotein and peptide enrichment and capture,^{16–18} and the chelating step between Ti(IV) and phosphate groups on phosphoproteins occurs immediately during sample printing and can tolerate denaturing conditions and salts in the sample. Second, due to its high density in terminal groups, dendrimer can be easily chemically modified and functionalized with abundant Ti(IV) ions for highly efficient phosphoprotein capture. Third, globular and water-soluble dendrimer makes the RP3A surface highly accessible by phosphoproteins multidimensionally.¹⁹ Overall, the RP3A platform overcomes the limitation of RPPA in detecting proteins with phosphorylation modifications, not only by eliminating the requirement of phospho-specific antibodies but also by significantly reducing the complexity of the crude sample to facilitate the detection of low-abundance phosphoproteins.

We first systematically evaluated the specificity, sensitivity, and quantitative capabilities of this new RP3A platform using a transcription factor component, cJun, and its phosphorylated counterpart. The purified recombinant GST-cJun, in either phosphorylated or nonphosphorylated form, in different concentrations ranging from 0 to 250 pg/ μ L was spiked into B cell lymphoma cell lysate at 1:200 ratio and spotted on the RP3A and regular RPPA membranes, and the GST-cJun signal was detected using anti-GST primary and fluorophore-linked secondary antibodies. As shown in Figure 2A, RP3A displayed over 99% specificity in selecting the phosphorylated cJun over the control nonphosphorylated cJun, whereas the regular RPPA membrane detected both phosphorylated cJun and nonphosphorylated cJun. The results also demonstrated a good quantitative capability of the RP3A approach (Figure 2A; $R^2 = 0.9886$) and outstanding sensitivity (Figure 2B). We then evaluated the technology by detecting the phosphorylated protein in a more complex sample. To mimic a complex sample, we spiked GST-cJun in phosphorylated and non-phosphorylated forms into undiluted plasma sample (one of the most complex biological mixtures available) at a 1:10 000 (5 ng/50 μ g) ratio. The capture of phospho-cJun from human plasma was efficient, leading to high signal. On the other hand, the spotted sample with spiked in control cJun did not produce any detectable signal (Figure S1). Interestingly, the results of spike-in experiment also demonstrated that Ti(IV)-dendrimer functionalized nitrocellulose membrane has extremely high binding capacity, likely due to the globular, nanosize of Ti(IV)-dendrimer that significantly increases the surface binding area for phosphoproteins in three dimensions. Because of the high binding capacity and the ability to simplify the sample significantly by enriching phosphorylated proteins only, we can use a much higher amount of starting material than in a regular RPPA analysis. A typical starting sample limit for regular RPPAs is 0.5 μ g/ μ L due to platform binding capacity, while with our Ti(IV)-dendrimer membrane array, we can start with a much higher protein concentration (50 μ g/ μ L), thus ensuring improved detection of low-abundant phosphoproteins. Overall, the RP3A shows remarkable analytical features through its ability to simplify the sample significantly and specifically enrich phosphoproteins.

We then coupled the RP3A platform with an MS-based quantitative phosphoproteome strategy to validate phosphorylation changes in biological samples. MS-based phosphoproteomics is an untargeted screening technique to identify and/or quantify a list of phosphoproteins, known and unknown. The RP3A platform can be primarily used as a validation technique because it allows for the quantification of phosphoproteins of interest from multiple samples in parallel. Combining these two techniques could provide a streamlined protocol to screen and validate phosphoproteins of interest in high throughput. *Arabidopsis thaliana* frequently serves as a convenient model for addressing biological processes in plants. Studying phosphorylation changes and signaling regulation in *Arabidopsis* allows us to understand molecular mechanisms underlying the responses to environment stresses in plants.^{20,21} However, compared with other organisms such as human and mouse, there are relatively few commercial antibodies specifically against phosphorylated proteins in *Arabidopsis*, making the validation of phosphoproteins from plants extremely difficult. In this study, we first applied quantitative phosphoproteomics to profile phosphoproteome changes in *Arabidopsis* with different treatment conditions and then used RP3A to validate some of the important phosphoproteins. *Arabidopsis* were

extracted with 30 min of cold stress (4 °C) and room temperature as the control. Proteins were extracted and digested, and phosphopeptides were enriched and followed by single-run mass spectrometric analysis for each sample. We performed three biological replicates for each plant sample. Among over ten thousand phosphorylation sites we identified in this study, 6029 were unique class I phosphorylation sites (localization probability >0.75). Among them, a total of 1657 phosphorylation sites representing 1075 phosphoproteins were significantly up-regulated in cold-treated samples (FDR < 0.05, S0 = 0.2) (Figure 3A). Principle component analysis (PCA) clearly distinguished overall phosphorylation and signaling after the cold treatment from the basal phosphorylation level in three samples at room temperature (Figure 3B). Motif analysis shows three main phosphorylation motifs [-pS-P-], [-pS-D-X-E-], and [-R-X-X-pS-], and [-pS-P-] was in particular enriched upon cold stress, which indicated that kinases recognizing the [-pS-P-] motif are likely crucial in the regulation of cold defense mechanisms in *Arabidopsis* (Figure 3C). Previous studies also reported that MAPK cascades, particularly the MKK4/MKK5-MPK3/MPK6 cascade, are critical for plants to respond to both biotic and abiotic stresses.^{22,23} Therefore, we then chose MPK3, MPK4, and MPK6 as the model proteins for the following RP3A analyses. Luckily, for all three phosphoproteins, there are antibodies against total protein and against specific phosphorylation sites, respectively. We first verified these phosphorylation changes by using an immunoblotting assay with phosphospecific antibodies. The immunoblotting assay showed that phosphorylation of all three proteins, MPK3, MPK4, and MPK6, increased (2.1–2.4-fold) after treatment for 30 min under cold condition (Figures 3D and S2), which is in agreement with the quantitative proteomics results (1.5–2-fold). We analyzed the phosphorylation changes with our RP3A approach in parallel, in which antibodies against individual proteins were employed for detection (Figure S3). The RP3A approach was able to measure similar phosphorylation changes of MPK3, MPK4, and MPK6 without the use of phosphospecific antibodies (Figure 3E), and the ratio of phosphorylation changes measured by RP3A (1.3–1.7-fold) is overall in good agreement with both the proteomics results and the immunoblotting assay. However, since RP3A is not site-specific and measures changes in total phosphoprotein amount, it is conceivable that the ratio of RP3A measurements might be smaller than that of the mass spectrometry and immunoblotting assay.

Finally, we demonstrated the unique utility of the RP3A platform with high-throughput screening of phosphorylation changes in a panel of six proteins under four different treatment conditions (Figure S4). We examined three abiotic treatments including cold, abscisic acid (ABA), and mannitol, while the bacterial pathogen-associated molecular pattern flagellin (flg-22) treatment mimics the biotic stress in *Arabidopsis*. Previous studies have shown that the functions of many proteins, such as kinases, receptors, and transcription factors, are regulated by phosphorylation in *Arabidopsis* under environmental stresses. For example, abiotic treatment with ABA and mannitol induces the phosphorylation of SnRK2.6, which subsequently phosphorylates and activates downstream proteins,^{24,25} while biotic stress treatment flg-22 induces phosphorylation of MAP kinases.²⁶ Some of the phosphorylation changes are known, while the rest are unknown due to the lack of appropriate validation methods. In this experiment, submicroliter amounts of protein samples were spotted onto the RP3A membranes, and the signals were detected using the

primary antibodies and fluorophore-functionalized antirabbit or antimouse secondary antibodies. For the detection of phosphorylation changes of MPK3, MPK4, and MPK6, we used the available antibodies against three individual proteins; for ICE1, SnRK2.6, and PYL5, since there is no protein or phosphoprotein antibody available, we used the transgenic plants expressing individual GFP-tagged proteins, GFP-ICE1, GFP-SnRK2.6, or GFP-PYL5. Thus, phosphorylation changes of these proteins can be measured using a high quality anti-GFP antibody on the RP3A platform. As a comparison, the identical spotting, incubation, and detection procedure was carried out with the unmodified nitrocellulose membrane as regular RPPA analyses.

One example of detection of protein SnRK2.6 by RP3A and regular RPPA is shown in Figure 4A. We can clearly observe increased phosphorylation in SnRK2.6 after treatment with ABA, while the expression of SnRK2.6 apparently remained constant with or without ABA treatment (Figure 4B). To compare the actual phosphorylation changes, the intensity obtained on RP3A was normalized with the regular RPPA to obtain the fold change of the phosphorylation on each protein. The comparative results are shown in Figure 4C, which illustrated the phosphorylation changes in 6 proteins under 4 treatments with three replicates. Overall, phosphorylation changes measured by the RP3A approach fit the known literature very well. The phosphorylation changes measured by the RP3A approach showed that the phosphorylation levels of MPK3, MPK4, and MPK6 were increased after cold and flg22 treatment, which is consistent with previous studies showing that both cold and flg22 can activate MPK cascades.^{24,25} On the other hand, the MPK3, MPK4, and MPK6 could not be activated by ABA treatment,²⁷ which was confirmed by our RP3A analyses indicating that the phosphorylation levels of these three MAP kinases were not or only mildly induced after the ABA treatment. The ABA- and mannitol-induced phosphorylation of SnRK2.6 detected by RP3A was in agreement with our previous study.²⁸ These results suggest that RP3A is a reliable method to measure the phosphorylation changes. Using this technology, we also examined some phosphorylation events that have not been studied yet. For example, we found that mannitol treatment induced the phosphorylation of MPK3 and MPK4 but not MPK6, which suggests that MPK3 and MPK4 are probably involved in the response to osmotic stress induced by mannitol treatment. Our RP3A data also showed that ABA treatment induced the phosphorylation of PYL5. This new discovery indicated that the phosphorylation may promote the protein stability of PYL5 under stress conditions and shows clues for investigating the significance of PYL5 phosphorylation in plant signaling in response to environmental stress in our future study. The data also highlighted the unique feature of this new analytical method which allowed us to detect the phosphorylation of PYL5 and SnRK2.6 as well as ICE1 even if there is no available antibody specific for the phosphorylation forms of these proteins. It is difficult for the traditional immunoblotting assay to validate their phosphorylation change. Through the introduction of a GFP-tag on these proteins, our new RP3A platform enables the efficient detection of the phosphorylated forms of these proteins and estimates their phosphorylation changes. These results further demonstrated that the RP3A approach is a powerful tool to identify and validate phosphorylation changes of specific proteins in complex biological samples in high throughput, which will be extremely valuable to screen or validate important phosphorylation events without resorting to expensive and time-consuming development of

phospho-specific antibodies. When RP3A is used as a screening technique, the phosphoprotein expression difference information obtained by RP3A can be further verified by MS that can provide phosphorylation site-specific information; RP3A can also be used as a validation technique to validate the phosphorylation changes obtained by mass spectrometric analysis because it can provide the information on phosphorylation changes without the need of a phosphospecific antibody. RP3A is useful in either situation because of its unique high-throughput feature. When RP3A is used a validation approach, compared with other biochemical techniques such as standard phosphoprotein enrichment in solution followed by protein-level antibody detection with Western Blotting, RP3A allows for the detection of phosphorylation changes in a large number of samples, e.g., clinical samples, on the same membrane in parallel.

CONCLUSION

In summary, we present a novel reverse phase phosphoprotein array for high-throughput profiling of protein phosphorylation changes in complex biological samples. The RP3A approach is appealing because a majority of signaling molecules do not have commercial antibodies to measure specific phosphorylation status, particularly in biological models and plants. Compared to regular RPPA, nitrocellulose membrane was chemically modified by water soluble PAMAM dendrimer immobilized with multiple Ti(IV) ions. The Ti(IV) ions on the nanopolymer recognize phosphate groups on proteins with high specificity, enabling the capture of phosphoproteins from complex mixtures such as serum, lysates, and tissue extracts. The novel RP3A platform shows extremely high selectivity (1:10 000) and high sensitivity (fg level). This approach also shows great universality and adaptability in profiling phosphorylation changes in complex samples for large scale applications, as demonstrated by profiling important phosphoproteins in multiple plant samples using the RP3A platform. In model systems such as in plants, the detection of interesting proteins can be achieved by introducing an epitope tag fused to target proteins. For PTMs such as phosphorylation, the introduction of epitope tags alone cannot measure protein PTMs. The use of both recombinant proteins (antibodies against standard epitope tags are usually high quality) and RP3A can efficiently facilitate the screening or validation of phosphorylation changes on important target proteins, not limited by the availability of high quality phosphoproteins. However, the RP3A measures changes in total phosphoprotein amount and may not reflect changes on individual sites when phosphoproteins have multiple phosphorylation sites. In these cases, the RP3A quantitation might be less sensitive and results may not exactly correlate with the MS results. Nevertheless, we foresee that this strategy could become a useful technique for sensitive profiling of phosphoproteins in a large number of biological samples for validation. Importantly, the novel RP3A platform can greatly expand RPPA applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Design and experimental workflow of RP3A. Ti(IV)-dendrimer is immobilized on the nitrocellulose membrane first, and the sample lysates are then spotted on the membrane. After washing away the nonphosphoproteins, the phosphoproteins were probed by antibodies against the protein of interest.

Figure 2.

(A) Quantification of fluorescent signals from cJun by RP3A. (B) Detection of subpg level of cJun by RP3A.

Figure 3.

(A) Volcano plot represents the phosphorylation sites on three MPKs were highly induced upon cold stimulation. (B) Principal component analysis (PCA) of triplicate samples under treatment or control. (C) Motif analysis of the up-regulated phosphorylation sites using Motif-X algorithm to analyze. (D) Western Blotting analyses of phosphorylated MPK3, MPK4, and MPK6 against their phosphorylated antibodies. (E) Quantification of MPK3, MPK4, and MPK6 under different conditions using RP3A; quantification ratio of phosphorylation changes is normalized by signals from total protein amount detected using regular RPPA.

Figure 4.

(A) Detection of GFP-tagged SnRK2.6 by RP3A and regular RPPA. (B) Comparison of the phosphorylation fold change of a GFP-tagged protein SnRK2.6 before and after treatment with stress measured by RP3A. (C) Heatmap of phosphoproteins (MPK3, MPK4, MPK6, ICE1, SnRK2.6, PYL5) before and after four different treatments and phosphoproteins detected by RP3A and regular RPPA in parallel. Fold change of the phosphorylation on each protein is obtained by normalizing the intensity obtained on RP3A with the intensity obtained on regular RPPA.