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A Highly Sensitive Detection Method, Phos-tag™ Affinity SDS-PAGE, Used to Analyze a Possible Substrate of CDPK-Related Protein Kinase5 in *Arabidopsis*

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Abstract

Phosphorylation of proteins is an important post-translational modification. Methods to determine the phosphorylation state of proteins are very important to evaluate diverse biological processes. CRK5 is the CDPK-related protein kinase in *Arabidopsis*, WD-repeat protein (WDRP) might be CRK5-interact-protein based on Y2H results. Here, we used bimolecular fluorescence complementation (BiFC) further to study and visualize the interaction between CRK5 and WDRP in living cells. Then, we combined Phos-tag™ SDS-PAGE with western blot (WB) analysis, using WDRP antibody and the anti-6×His antibody, to detect phosphorylated WDRP. This approach confirmed that WDRP might be phosphorylated by CRK5 *in vitro*. Site mutation analysis suggested that serine-70 might be the amino acid phosphorylated by CRK5 in WDRP. Cell extracts isolated from WT, OERK5, and *crk5* used to analyze the kinase reaction using recombinant WDRP as substrate. These results demonstrated that WDRP was phosphorylated by cell extracts and that there may be additional kinases that phosphorylate WDRP in *Arabidopsis*. Phos-tag™ SDS-PAGE thus provides a suitable and convenient method for analysis of phosphorylation in plants.

Keywords

Calcium-Dependent Protein Kinase (CDPK), CDPK-Related Protein Kinase (CRK), WD-Repeat Protein (WDRP), Protein Phosphorylation, Phos-tag™

1. Introduction

Phosphorylation is an important post-translational modification of proteins that

regulates protein properties, resulting in dynamic control of enzymatic activity, localization, and complex formation with other proteins [1]. A complicated and varied network of kinases and phosphatases is involved in the rapid and accurate regulation of protein phosphorylation, which is required for several fundamental and vital functions of cellular functions. Methods to determine the phosphorylation state of proteins, also called phosphoproteomic methods, are thus very important in the evaluation of diverse biological processes.

In 2003, Prof. Koike's group first reported a selective phosphate-binding tag molecule, Phos-tag™ [2]. Since then, various methods for phosphoproteome research have been developed using Phos-tag™ derivatives. Phos-tag™ SDS-PAGE is an electrophoretic method that permits the separation of phosphorylated proteins and non-phosphorylated proteins based on the conventional SDS-PAGE technique. In conventional SDS-PAGE, the mobility of phosphorylated and non-phosphorylated proteins is usually the same; therefore, they do not separated efficiently. However, in Phos-tag™ SDS-PAGE, phosphorylated proteins trapped by immobilized Phos-tags in the separation gel, tend to migrate more slowly, than these proteins can be detected as gel-shifted bands. In addition, multiple phosphorylation states of the target protein individually can be detected as separate bands after Phos-tag™ SDS-PAGE. Furthermore, this system is compatible with the equipment and reagents used for conventional SDS-PAGE, and followed by general WB analysis with a specific antibody against a certain target, it is much more convenient for researchers to analyze the target proteins in cellular lysate [3]. The Phos-tag™ SDS-PAGE method is highly accurate and sensitive for the detection of the phosphorylation state of proteins [4]. Using this method, many researchers reported valuable findings related to protein phosphorylation modifications, not only in the fields of basic life science but also in clinical medicine field [5]-[13]. With the continuous improvement of technology, the Phos-tag™ SDS-PAGE method has gradually become a routine method for laboratory analysis of protein phosphorylation [14] [15] [16] [17].

Calcium acts as an important second messenger in plant cells. CDPKs are serine-threonine protein kinases for the transduction of calcium signals in plant cells [18] [19]. Another plant family with similar structure is the CDPK-related protein kinases (CRKs). CRKs characterized by a regulatory domain with high sequence similarity to CDPKs and degenerate EF-hand motifs [20]. Biochemical data confirmed that CRKs are serine-threonine protein kinases, and the kinase activity of some CRKs does not require calcium [21] [22]. However, there is evidence showing that some CRK isoforms could activated by exogenous Ca^{2+}/calmodulin [23] [24]. Recently, it has reported that CRKs have high tyrosine autophosphorylation activity, and they can phosphorylate tyrosine residue(s) on substrate proteins in *Arabidopsis* [25].

The conventional CDPKs are multifunctional kinases that are involved in the regulation of diverse aspects of cellular function [5] [26] [27] [28]. In contrast to CDPKs, there is relatively little information about the potential substrates and

functions of CRKs. In *Arabidopsis*, there are eight CRKs. Of these, AtCRK3 specifically interacts with and phosphorylates the cytosolic glutamine synthetase, AtGLN1, and AtCRK3 may play a role in the control of nitrogen remobilization during leaf senescence [29]. AtCRK5 is required for the proper polar localization of PIN2 in the transition zones of roots. Inactivation of CRK5 results in a root gravitropic defect and stimulates lateral root formation. CRK5 undergoes auto-activation and phosphorylates the hydrophilic T-loop of PIN2 *in vitro* [24].

To identify proteins capable of interacting with AtCRK5, the N-terminal region of AtCRK5 fusing with the GAL4 DNA-binding domain was used as bait to screen an activation domain-tagged cDNA library prepared from *Arabidopsis* [29]. Among 10^6 co-transformants, an AtWDRP construct lacking 30 amino acids at the C-terminus was identified under selective conditions and LacZ activity. These finding suggest that AtCRK5p could interact with a nearly full-length AtWDRP protein in yeast.

Here, we used bimolecular fluorescence complementation (BiFC) to further study the interaction between CRK5 and WDRP in living cells. We demonstrated a novel application to detect the phosphorylated WDRP *in vivo* and *in vitro* using Phos-tag™ SDS-PAGE and WBs. Our findings will provide new insight in the analysis of protein phosphorylation in the calcium signal system in plants.

2. Methods and Materials

2.1. Plant Materials and Growth Conditions

Seeds of wild-type *Arabidopsis thaliana* (Columbia-0) and mutant lines, were germinated after two days stratification at 4˚C on half-strength Murashige and Skoog (MS) medium [30] supplemented with 0.5% Sucrose in a growth chamber (22˚C with 120 μE m^{-2}·s^{-1} light, 16 h light/8 h dark). The 7-day seedlings were then transferred to soil under controlled greenhouse conditions (16 h light/8 h dark period; 22˚C to 24˚C day temperature and 18˚C night temperature, 80 to 120 μE·m^{-2}·s^{-1} light) as described [31].

The T-DNA insertion mutant *crk*5 was kindly provided by Dr. Ágnes Cséplő from the Institute of Plant Biology at the Biological Research Center, Hungary.

The pBS-T vector containing the complete ORF CRK5 was digested with *Bam* H I, and ligated into the binary expression vector pMD, which was digested with *Bam* H I, thus allowing the gene to be driven by the cauliflower mosaic virus (CaMV) 35S promoter. The construct was transformed into *Agrobacterium tumefaciens* (GV3101) by heat shock and used to transform *Arabidopsis thaliana* accession *Col*-0 plants by floral dipping [32] [33]. *Arabidopsis* transgenic lines were selected on a MS medium plus vitamins, supplemented with 50 μg/ml Kanamycin (Km). Primary transformants were checked by PCR using CaMV35S promoter specific primer (5'-ACGCACAATCCCACTATCCAAC-3') together with CRK5 reverse primer. Homozygous lines were selected by segregation analysis for Kanamycin-resistance. The expression levels of the transgene among the homologous transgenic lines were evaluated by RT-PCR.

2.2. Bimolecular Fluorescence Complementation (BiFC) Assay

To generate the BiFC constructs, the coding regions of *AtCRK*5 without stop codons were sub-cloned into 35S-SPYCE vector, and the coding regions of *AtWDRP* without stop codon were sub-cloned into 35S-SPYNE vector.

Transformation of onion epidermal cells was performed according to the following method. Each plate was shot twice. Each shot contains 270 μg gold particles (1.0 μm in diameter), and particles were coated with 2 μl of pSPYCE-35S-CRK5 and pSPYNE-35S-WDRP recombinant plasmid at 0.5 μg/μl. The gold-coated DNA particles were delivered into onion epidermal cells using the PDS-1000/He Biolistic Particle Delivery System (BioRad Laboratories, Hercules, CA, USA), and the bombarded onion epidermal peels were maintained at 25˚C for at least 12 h until they were examined by fluorescence microscopy (Nikon, Tokyo, Japan) according to Lee *et al.* [34].

2.3. Total Protein Extraction

Total protein was extracted from 4-day-old Col-0 or 8-day-old seedlings using extraction buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, protease inhibitor mixture and phosphatase inhibitor mixture). After centrifugation at 18,000 × g for 20 min at 4˚C, the supernatant was transferred to a new tube and the protein concentration was determined using the Bio-Rad protein assay kit.

2.4. Site-Directed Mutagenesis by PCR Amplification

The QuikChange® XL site-directed mutagenesis method was used to make point mutations. The complimentary oligonucleotides containing the desired mutation, flanked by unmodified nucleotide sequence were synthesized by Invitrogen company. The complimentary oligonucleotides for WDRP site mutations were shown in the following. $WDRP^{S70A}$ 5':
GCGACGTTCATGTTACTGCAGACAATGCC, $WDRP^{S70A}$ 3':
GGCATTGTCTGCAGTAACATGAACGTCGC, $WDRP^{S227A}$ 5':
GGTCATATTGCCAAGTCATTTAAAACGGATTGTTGC, $WDRP^{S227A}$ 3':
GCAACAATCCGTTTTAAATGACTTGGCAATATGACC, $WDRP^{S264A}$ 5':
GCAAAAGTGCTAGCGAAATTTAGAGCTCAC, $WDRP^{S264A}$ 3':
GTGAGCTCTAAATTTCGCTAGCACTTTTGC, $WDRP^{S289A}$ 5':
GTATGTTGACTTCTGCGGTCGATGGTACAATTCG, $WDRP^{S289A}$ 3':
CGAATTGTACCATCGACCGCAGAAGTCAACATAC.

2.5. Expression and Purification of CRK5, WDRP and WDRP Site Mutations

The full ORF sequence of CRK5 and WDRP were constructed into pET28a and pEA separately using *Bam*H I site, and then the recombinant plasmids were transformed into *E.coli* DE3 respectively. WDRP site mutations were constructed in to pET28a using *Bam*H I site. 1 mM IPTG was added to the culture, then CRK5-6×His or WDRP-6×His recombinant proteins was purified by Ni-nitrilotriacetic acid agarose affinity chromatography (QIAGEN) according to

the manufacturer's instructions. Fractions containing apparently homogeneous recombinant proteins were dialyzed with storage buffer (10 mM Tris-HCl, pH 7.5) at 4˚C, and stored at −80˚C.

2.6. *In Vitro* Kinase Assays

In vitro phosphorylation assays were performed with 1 μg CRK5-6×His or 1 mg extractions isolated from seedlings (4 weeks) in 50 μl kinase buffer (25 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT, and 0.1 mM ATP), 2 μg WDRP-6×His was added as substrate and kept at 30˚C for 30 min. $CaCl_2$ was added, at a final concentration of 2 μM with or without 5 mM Ca^{2+}-chelator EGTA where indicated.

2.7. Mobility Shift Detection of Phospho-Proteins

Phos-tag Acrylamide (AAL-107) is commercially available from from Wako Pure Chemical Industries (Osaka, Japan). The kinase reaction mixtures were separated on 10% SDS-polyacrylamide gels which were prepared with 75 μM acrylamide pendant Phos-tag ligand and 150 μM $MgCl_2$ according to the instructions provided by the Phos-tag Consortium. Gels were electrophoresed. Prior to transfer, gels were first equilibrated in methanol-free transfer buffer containing 1 mm EDTA for 10 min and then in transfer buffer without EDTA for 10 min.

Transfer onto Immunoblot Membrane (Millipore) was performed at 75 V at 4˚C for 2 hours. The membrane was incubated for 1 h in TBST blocking buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, and 5% dry skimmed milk) and for 1 h with anti-6×His (1:2000, Roche) or anti-WDRP antibody (1:500 dilution, NewEast Bioscience) in blocking buffer. After washing with TBST three times, the membranes were incubated for 1 h with peroxidase-conjugated goat anti mouse or rabbit antibody (Pierce; dilution 1:2000), washed with TBST, and overlaid with Immunoblot Western Chemiluminescent horseradish peroxidase substrate to detect target proteins by autoradiography (Tanon 5200, Shanghai).

2.8. ABA, NaCl and PEG Stress

Seeds were sown on MS medium supplemented with 3% sucrose, and the plates were placed at 4˚C for 2 days in the dark prior to germination. To determine the effect of salt stress, 10-day-old seedlings germinated in normal medium were transferred to medium supplemented with 200 mM NaCl. To test the effect of ABA stress, the seeds were allowed to germinate under normal growth conditions and then 10-day-old seedlings were transferred to medium supplemented with 10 μM ABA. To determine the effect of drought, 10-day-old seedlings were transferred to medium supplemented with 20% PEG 6000.

3. Results

3.1. Identification of AtWDRP as an AtCRK5-Interacting Protein

AtCRK5 has been reported to be required for the proper polar localization of

PIN2 in the transition zones of roots. Inactivation of CRK5 results in a root gravitropic defect and stimulates lateral root formation. CRK5 undergoes auto-activation and phosphorylates the hydrophilic T-loop of PIN2 *in vitro* [24]. The conventional CDPKs are multifunctional kinases that are involved in the regulation of diverse aspects of cellular function. CRK5, as a multifunctional kinase, there might be other substrates *in vivo*, and CRK5 may take part in other physiological activities.

To identify proteins capable of interacting with AtCRK5, the N-terminal region of the kinase fusing to the GAL4 DNA-binding domain, and was used as a bait to screen an activation domain-tagged cDNA library prepared from *Arabidopsis* [29]. Among 10^6 co-transformants, an AtWDRP construct lacking 30 amino acids at the C-terminus was identified under selective conditions and LacZ activity. These findings suggest that AtCRK5p could interact with a nearly full-length AtWDRP protein in yeast. We directly visualized the interaction between CRK5 and WDRP in living plant cells. The cDNAs of WDRP and CRK5, without stop codons, cloned into pSPYNE-35S and pSPYCE-35S, respectively (**Figure 1(a)**). Whereas cells transfected with single plasmids and any combination with empty vectors produced no or only background fluorescence, a strong signal was observed throughout the membrane when WDRP-YFP^N was co-expressed with CRK5-YFP^C (**Figure 1(b)**). Importantly, the localization of the CRK5-WDRP complex corresponds to the subcellular distribution of CRK5, which reported by Rigó *et al.* [24].

3.2. AtWDRP with Potential Phosphorylated Amino Acid Sites

KinasePhos (http://kinasephos.mbc.nctu.edu.tw/) can computationally predict phosphorylation sites within given protein sequences [35]. This software used to predict the phosphorylation sites within WDRP. The deduced amino acid sequence of AtWDRP consists of 299 residues. The predicted result is shown in **Figure 2(a)**. There were four predicted phosphorylation sites in WDRP, all of which were serine and might be phosphorylated by CaMK II in animal cells, which demonstrated high similarity with CRKs in plants. CRKs, first cloned from maize and rice, are very similar to CaMK II based on sequence and structure studies, and were first named CaMK II-related protein kinases or CaM-binding protein kinases in plants [21] [36]. Further biochemical studies demonstrated that CRKs bind CaM, though binding to CaM is not necessary for activation of the kinase. Therefore, the biochemical characteristics of CRKs are different from CaMK II, whose activity is dependent on CaM strictly in animal cells [24] [37].

Though WDRP was predicted to be phosphorylated by CRK5, it also remains to be determined whether endogenous WDRP is phosphorylated in plant cells. We used the Mn^{2+}–Phos-tag^{TM} SDS-PAGE method to analyze the phosphorylation state of WDRP in plant cells. Four-week seedlings were used to analyze the phosphorylation state of WDRP in plant cells. First, the full protein was extracted from four-week wild-type seedlings and then separated in Mn^{2+}–Phos-tag^{TM}

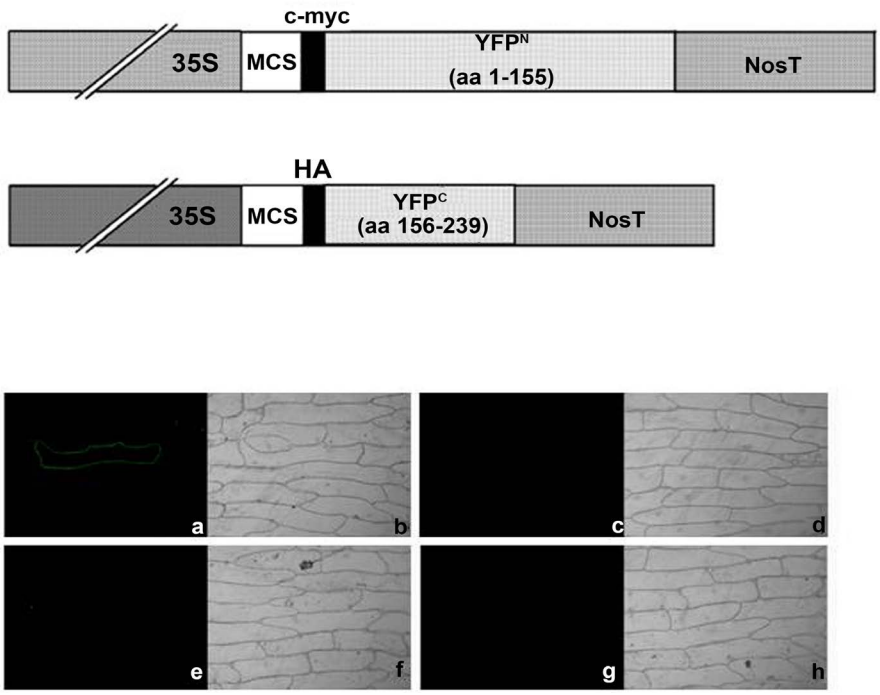

Figure 1. BiFC visualization of the interaction of CRK5 and WDRP. 1) Schematic representation of plant-compatible BiFC vectors. 2) BiFC provides for visualization of the interaction between AtCRK5 and AtWDRP in transiently transfected onion epidermal cells. Epifluorescence (a) and bright field images (b) of onion epidermal cells co-transfected with CRK5-YFPC and WDRP-YFPN. Epifluorescence (c) and bright field images (d) of onion epidermal cells co-transfected with YFPC and YFPN. Epifluorescence (e) and bright field images (f) of onion epidermal cells co-transfected with CRK5-YFPC and YFPN. Epifluorescence (g) and bright field images (h) of onion epidermal cells co-transfected with YFPC and WDRP-YFPN.

gel. The specific protein was detected by WB analysis with anti-WDRP. The bottom and top gels are normal SDS-PAGE and 75 μM Mn^{2+}–Phos-tag™ SDS-PAGE in **Figure 2(b)**, respectively. Immunoblots of seedling lysates analyzed by Phos-tag™ SDS-PAGE reveal that a fraction of the endogenous WDRP migrates at the characteristic molecular weight (marked with non-P) but that a majority of the protein migrate more slowly, in two major bands (marked with 1 and 3) and one weak band (marked with 2) (**Figure 2(b)**, top gel). These results demonstrate that there were at least three phosphorylated WDRP variants in cells, except endogenous WDRP. On the contrary, there was only one band at the characteristic molecular weight in the normal SDS-PAGE result (**Figure 2(b)**, bottom gel).

We also detected the phosphorylation state of WDRP in the OECRK5 transgene line and the *crk*5 line. In the OECRK5 transgene line, the *CRK*5 gene was over-expressed, while in the *crk*5 line, the *CRK*5 gene was knocked out. No differences were found in the phosphorylation state of WDRP among WT, OECRK5, and *crk*5 (**Figure 2(b)**).

These results implied that WDRP could be phosphorylated and CRK5 might not be the only kinase that can phosphorylate WDRP in plant cells.

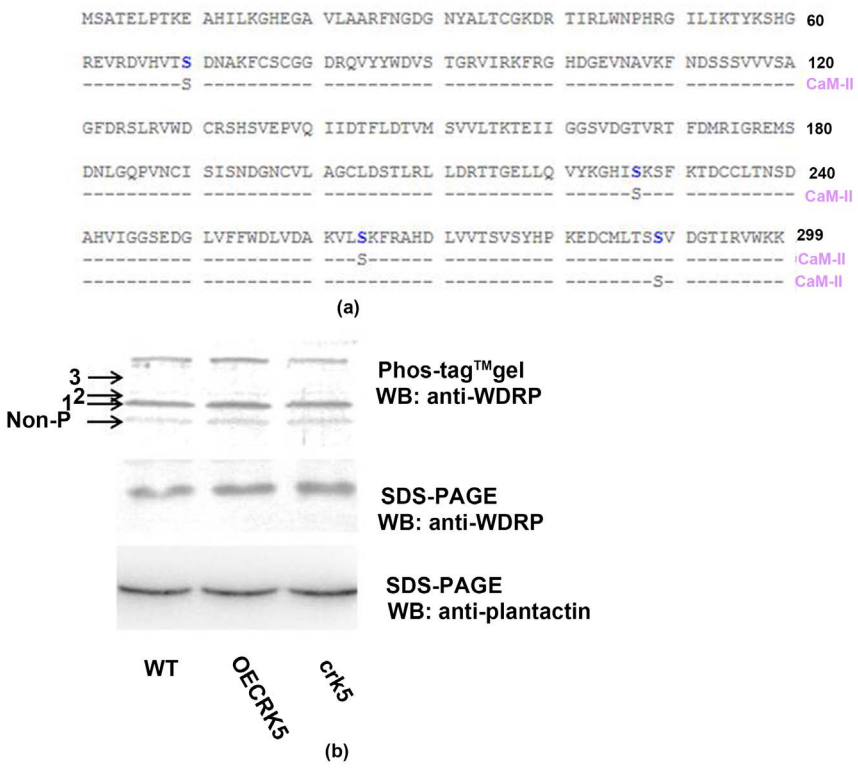

Figure 2. AtWDRP can be phosphorylated in plant cells. (a) AtWDRP has putative phosphorylated sites predicted by KinasePhos; (b) Phos-tag™ gel was used to detect the phosphorylation state of WDRP in plant cell. Anti-WDRP (1:500) was used to detect the different state of WDRP. Normal SDS-PAGE and WB was used as a control; anti-WDRP (1:500) and anti-plant actin (1:2000, Abbkine) were used to detect the WDRP and actin, respectively.

3.3. AtWDRP Can Be Phosphorylated by CRK5 in the *in Vitro* Kinase Analysis

In order to further confirm the phosphorylation of AtWDRP by AtCRK5, we completed an *in vitro* kinase assay with purified recombinant AtCRK5 and AtWDRP proteins. AtCRK5 and AtWDRP were cloned into pET28a and pEA, respectively, and then the recombinant protein, AtCRK5-6×His or AtWDRP-6×His, was purified from *Escherichia coli* by Ni-NTA resin and then assayed by SDS-PAGE as a single polypeptide, consistent with the predicted molecular mass of the fusion protein (**Figure 3(a)**). Purified AtCRK5-6×His and AtWDRP-6×His were used for kinase activity assays.

Phosphorylation by AtCRK5 was performed in a 50 μl reaction mixture containing 25 mM Tris-HCl, pH 7.5, 0.5 mM DTT, 5 mM $MgCl_2$, 100 μM ATP, 1 mg/ml AtWDRP-6×His and either 1 μM CaM and 2 μM $CaCl_2$ or 5 mM EGTA at 30˚C for 30 min. The reaction was initiated by the addition of purified AtCRK5-6×His, terminated by adding a one-fifth volume of 5×SDS sample buffer, and analyzed by Mn^{2+}–Phos-tag™ SDS-PAGE as described above.

Mn^{2+}–Phos-tag™ SDS-PAGE results demonstrated that after the *in vitro* kinase assay, there was a protein band with slow mobility, in addition to the

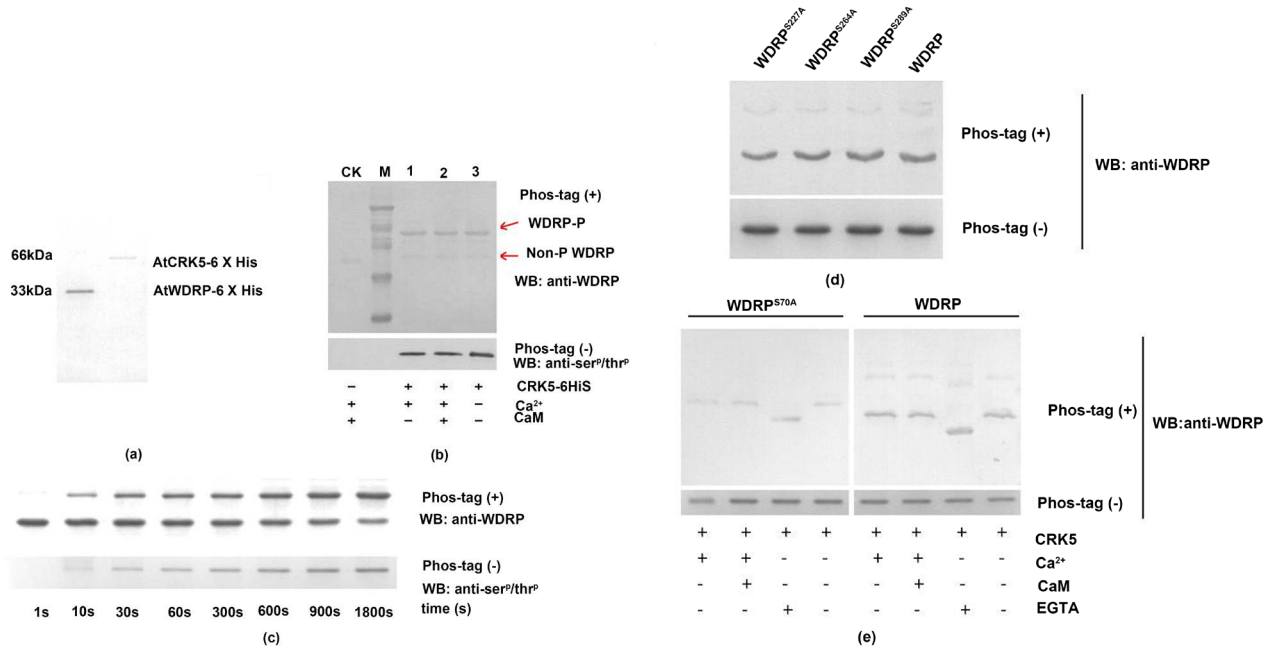

Figure 3. AtWDRP can be phosphorylated by CRK5 in the *in vitro* kinase assay. (a) Coomassie blue-stained SDS gel of purified recombinant WDRP and CRK5. WDRP and CRK5 were expressed in *E. coli* and then purified by Ni-NTA resin; (b) Mn^{2+}–Phos-tag^{TM} SDS-PAGE and WB results demonstrate that AtWDRP can be phosphorylated by CRK5 in a calcium/calmodulin-independent manner in the *in vitro* kinase assay. Normal SDS-PAGE and WB were used to analyze the phosphorylated WDRP using the anti-phosphorylated serine/threonine (1:1000) antibody. In the CK reaction system, only WDRP protein was added to the reaction buffer; there was no CRK5 added; (c) The time course analysis of the kinase reaction using Mn^{2+}–Phos-tag^{TM} SDS-PAGE and normal PAGE. In Mn^{2+}–Phos-tag^{TM} SDS-PAGE and WB, WDRP was detected by the anti-WDRP antibody. In normal SDS-PAGE and WB, the phosphorylated WDRP was detected by the anti-phosphorylated serine/threonine (1:1000) antibody. Purified, recombinant WDRP was incubated with CRK5. The reaction was started by the addition of CRK5, and samples of reaction mixtures were withdrawn at the indicated times, and then 5× SDS gel sample buffer were added to stopped the reaction; (d) Site-directed mutagenesis of WDRP (WDRP^{S227A}, WDRP^{S264A}, and WDRP^{S28A}) were phosphorylated by recombinant CRK5 in the *in vitro* kinase reactions. In kinase reactions, Ca^{2+} and CaM were added. Site-directed mutagenesis were detected by the anti-WDRP antibody after Mn^{2+}–Phos-tag^{TM} SDS-PAGE or normal SDS-PAGE and WB; (e) WDRP^{S70A} could not be phosphorylated by recombinant CRK5. Site-directed mutagenesis were detected by the anti-WDRP antibody after Mn^{2+}–Phos-tag^{TM} SDS-PAGE or normal SDFS-PAGE and WB.

WDRP protein band. Based on the principles of Mn^{2+}–Phos-tag^{TM} SDS-PAGE, the band with slow mobility was phosphorylated WDRP. These results implied that WDRP can be phosphorylated by CRK5 and that phosphorylation does not dependent on calcium and calmodulin (Figure 3(b)).

There was only one phosphorylated protein band in Figure 3(b) when AtCRK5 was added to the kinase reaction buffer, but in Figure 2, there are at least three phosphorylated protein bands in the extraction buffer isolated from seedling. As for the assay in Figure 2, there was much more kinase present in the plant cells and the phosphorylation of AtWDRP was complete; therefore, the phosphorylation state of WDRP was complex. WDRP might be phosphorylated by the other kinases in seedling extraction in addition to CRK5.

Temporal changes in the ratios of non-phosphorylated to phosphorylated WDRP have been analyzed using Mn^{2+}–Phos-tag^{TM} SDS-PAGE and WB. Con-

sistent with this observation, normal SDS-PAGE and WB were also used to show the time course of kinase reaction using the anti-phosphorylated serine/threonine (1:1000) antibody. Time-course analyses demonstrated that CRK5 rapidly phosphorylated WDRP in the presence of Ca^{2+}/CaM, soon after CRK5 was added to the reaction mixture at 30˚C, and the phosphorylated WDRP accumulated, as shown in normal SDS-PAGE and Mn^{2+}–Phos-tag™ SDS-PAGE (**Figure 3(c)**).

Site mutation analysis was used to examine the amino acids susceptible to phosphorylation in WDRP. There were four predicted phosphorylation sites in WDRP, all of which were serine, which might be phosphorylated by CaMK II, based on the KinasePhos results. We changed the four serines to alanine respectively, and then the four mutants (named $WDRP^{S70A}$, $WDRP^{S227A}$, $WDRP^{S264A}$, and $WDRP^{S289A}$) were expressed in *E. coli*. After purification, recombinant CRK5-6×His was added to the kinase reaction buffer which contained different mutants in the kinase reaction buffer. We found that only $WDRP^{S70A}$ could not be phosphorylated by CRK5 in kinase reaction buffer (**Figure 3(d)** and **Figure 3(e)**). This result implied that serine 70 might be the phosphorylated amino acid in WDRP when CRK5 was added to the reaction buffer.

3.4. AtWDRP Can Be Phosphorylated by Seedling Lysate

To further determine if WDRP, the possible substrate of protein kinase CRK5, was phosphorylated by plant cell extraction, we performed an *in vitro* kinase reaction, Mn^{2+}–Phos-tag™ SDS-PAGE, and WB analyses.

AtWDRP-6×His was expressed in *E. coli* and then purified by Ni-NTA resin. Plant cell extracts were isolated from different four-week seedlings of WT, OECRK5, and *crk*5. Anti-6×His antibody was replaced by anti-WDRP to identify the endogenic WDRP. Immunoblots of kinase reaction mixtures revealed that WDRP-6×His migrates at its characteristic molecular weight (marked non-P in **Figure 4**), but that a majority of the protein migrates more slowly in three major bands (marked 1, 2, and 3) when seedling extracts were added to the reaction system, regardless of whether the extract was from WT or OE-CRK5 (**Figure 4**). As for *crk*5, the major band (marked with 1) slightly above the non-phosphorylated WDRP-6×His was fainter than in WT and OE-CRK5. This result implied that CRK5 might be related to this phosphorylated WDRP species. When CRK5 was knocked out, the phosphorylation level of WDRP was decreased. In addition, this result demonstrated that WDRP could be phosphorylated by other kinases except CRK5. CRK5 belongs to a large protein kinase family, which includes CDPKs and other CRKs, some of which might similarly phosphorylate WDRP. We have not identified which additional member(s) of the protein kinase family phosphorylate WDRP. On the contrary, only one band, marked with non-P, was shown when no extract was added to the reaction system (**Figure 4**).

When EGTA (an aminopolycarboxylic acid, a chelating agent, which compared to EDTA, has a lower affinity for magnesium, making it more selective for

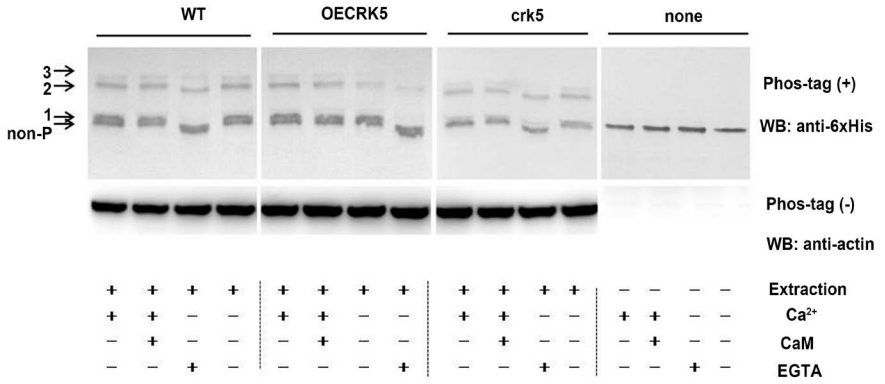

Figure 4. AtWDRP can be phosphorylated by cell extraction detected by Mn^{2+}–Phos-tag™ SDS-PAGE and WB. WDRP was expressed in *E. coli* and then purified by Ni-NTA resin. Extractions were isolated from different four-week seedlings of WT, OECRK5, and *crk5*. WDRP was added to different extraction buffer, the reaction was stopped by adding 5 × SDS gel sample buffer. Anti-6×His antibody was used to detect WDRP. None: no extraction was added to the reaction buffer.

calcium ions) was added to the kinase reaction system, the phosphorylation state of WDRP was different from the other reaction conditions. In WT, OECRK5, and *crk*5 extraction systems, the phosphorylated WDRP-6×His band (marked 3) was almost missing, which result implied that this phosphorylation state of WDRP was dependent on calcium (**Figure 4**). In addition, separation of the phosphorylated forms by Phos-tag™ SDS-PAGE requires the presence of Mn^{2+} in the running gel [3]. As shown in **Figure 4**, if $MnCl_2$ was omitted from the running gel (divalent cations were somewhat chelated with EGTA), all WDRP species migrated more quickly than the other conditions.

3.5. ABA, NaCl, and PEG Did Not Affect the Phosphorylation State

Using the Phos-tag™ SDS-PAGE method, we analyzed the phosphorylation state of WDRP in four-week seedlings (**Figure 2(b)**). These results implied that WDRP was phosphorylated in plant cells and there were at least three main phosphorylation states for WDRP. Protein phosphorylation is part of signal transduction or the stress response. To analyze whether the phosphorylation of WDRP was involved in the stress response, ABA, NaCl, and PEG were added to the culture medium separately, and then the 10-day seedling extracts were used to analyze the phosphorylation state of WDRP (**Figure 5**). Regardless of whether ABA, NaCl, or PEG was added to the medium, WDRP was phosphorylated, and two different phosphorylation states were present. These results implied that the phosphorylation of WDRP might not be related to ABA, NaCl, or PEG.

4. Discussion

Phos-tag™ SDS-PAGE is an electrophoretic method that permits the separation of phosphorylated and non-phosphorylated proteins and is based on the conventional SDS-PAGE technique.

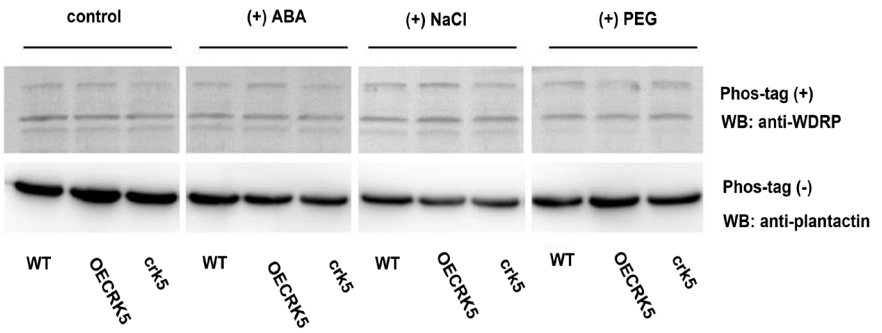

Figure 5. ABA, NaCl, and PEG stress did not change the phosphorylation state of WDRP in plant cells. Ten-day seedlings germinated in normal medium were transferred to medium supplemented with 200 mM NaCl, 10 μM ABA or 20% PEG 6000. After treatment, total protein was extracted from seedlings using extraction buffer (25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, protease inhibitor mixture, and phosphatase inhibitor mixture). After centrifugation, the supernatant was transferred to a new tube and 5X SDS gel sample buffer was added to the extraction, then Mn^{2+}–Phos-tag™ SDS-PAGE was used to separate the protein, the target protein WDRP was checked by WB using anti-WDRP antibody. Anti-plant actin was used as control.

There are many advantages to the Phos-tag™ SDS-PAGE method. First, radioactive and chemical labels are avoided. Radioactive and chemical labels are traditional methods to analyze protein phosphorylation. As radioactive labels are harmful to health and environment, special protective measures should be used in lab. However, the preparation of protein samples, operating procedure, and reagents used for Phos-tag™ SDS-PAGE are almost identical to those for conventional SDS-PAGE. Downstream procedures, such as WB analysis, are applicable. If a protein has multiple phosphorylation sites and exist in multiple phosphorylation state, the resulting differences in the electrophoretic mobility of the various phosphorylated forms of the protein in a lysate sample result in the formation of several bands that can be individually detected. Temporal changes in ratios of phosphorylated to non-phosphorylated proteins can also be analyzed quantitatively [4].

Thus, the Phos-tag™ SDS-PAGE method facilitates the resolution and separation of proteins with different phosphorylation states and the analysis of the corresponding individual bands on the gel. This is beneficial for investigations of the characteristic functions of various proteins and the relevance of phosphorylation state in cellular processes. This will allow researchers to identify in greater detail the phosphorylation target molecules that are implicated in particular physiological functions or dysfunctions in the cell and their roles in pathogenic mechanisms of diseases. Krauß *et al.* used this method to analyze the phosphorylation state of GLI3, a transcription factor, protein phosphatase 2A (PP2A), and the ubiquitin ligase, MID1, which regulate the nuclear localization and transcriptional activity of GLI3 [38]. On gels containing increasing concentrations of the acrylamide-dependent Phos-tag™ ligand, only one GLI3 band was detected, suggesting that GLI3 is a mono-phosphorylated protein in the cellular

system examined, where PKA was not stimulated. Furthermore, the separation of the WT and mutant GFP-GLI3 constructs on Phos-tag™ gels demonstrated no differences between WT and mutant. Taken together, these data strongly argue against the PP2A-dependent regulation of GLI3 localization by its own phosphorylation pattern, thereby suggesting the involvement of phospho-modified co-factors. Sutherland *et al.* exploited Phos-tag™ SDS-PAGE to separate multiple phosphorylated MYPT1 species and quantify MYPT1 phosphorylation stoichiometry [12]. Tau is a microtubule-associated protein which is hyperphosphorylation with more than 40 phosphorylation sites. Kimura et al applied the Phos-Tag technique to the analysis of tau phosphorylation *in vitro* and *in vivo* [13]. This method separates tau into many bands during SDS-PAGE depending on its phosphorylation states, creating a bar code appearance. Phos-Tag technique could provide other types of valuable information such as disease-specific phosphorylation [13]. A Phos-tag-based micropipette-tip method was developed for rapid and selective enrichment of phosphopeptides [39].

In this paper, *in vitro* phosphorylation assays demonstrated that WDRP can be phosphorylated by CRK5 in a kinase reaction system where only one band moved slower than non-phosphorylated WDRP on Phos-tag gel. This result implied that WDRP was phosphorylated by CRK in the *in vitro* kinase reaction system with sufficient kinase and reaction time. On the contrary, in plant cells, the phosphorylation of WDRP was regulated and demonstrated different phosphorylated states; there were at least three bands, which represented three phosphorylated forms of WDRP (**Figure 4**). These results demonstrate that WDRP can be phosphorylated by other kinases in plant cells, and it has different phosphorylated states that might be related to the complex regulation. To find out which signal or signals regulate the phosphorylation state of WDRP, ABA, NaCl, or PEG was used separately to treat the seedling; no differences were found on the Phos-tag™ gel compared with control. This result implied that the phosphorylation of WDRP might not be related to ABA, NaCl, or PEG. The function of the phosphorylation activity between WDRP and CRK5 need to be further analyzed.

In the future, detection by Phos-tag™ SDS-PAGE followed by immunoblotting is expected to increase the sensitivity of visualization of multiple phosphorylated variants of a given protein. Phos-tag™ gel-based approaches combined with the MS-based phosphoproteomic method, site-directed mutagenesis analysis, and the *in vitro* kinase reaction, have shed new light on the phosphorylation dynamics of a typical intracellular signaling molecule in biological and medical fields.

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Author Contributions

Di Xi performed the genetic and biochemical studies. Le-Ping He prepared all of the expression constructs and protein purification. Sequence analyses were performed by Lei Zhang. Lei Zhang designed the research and prepared the article.

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