TECHNICAL ADVANCE

Enrichment of Phosphoproteins and Phosphopeptide Derivatization Identify Universal Stress Proteins in Elicitor-Treated *Arabidopsis*

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Protein phosphorylation is a key biological process that regulates reactions involved in plant–microbe interactions. The phosphorylated form of a protein often represents only a small fraction of the total population and can be problematic to analyze in a mass spectrometer. We demonstrate how a titanium dioxide (TiO₂) resin can be employed for the enrichment of phosphoproteins, as well as a method to derivatize TiO₂-purified phosphopeptides to facilitate determination of the exact site of phosphorylation. The use of these methods was exemplified by the identification of two plant proteins that were shown to be phosphorylated after the elicitation of *Arabidopsis* cells with *Phytophthora infestans* zoospores and xylanase. Both of the proteins that were identified, At5g54430.1 and At4g27320.1, were found to contain a universal stress protein domain with conserved residues for ATP binding.

Additional keywords: 4-sulfophenyl isothiocyanate, SPITC.

Protein phosphorylation is a key biological process regulating a large number of essential reactions in living organisms. The phosphorylation of serine, threonine, and tyrosine can affect protein structure, enzymatic activity, and subcellular localization, as well as interaction with other proteins. Analysis of phosphoproteins, including determination of phosphorylation sites with mass spectrometry (MS) analysis of peptides, is thus highly important for gaining an understanding of interactions between plants and microbes. This knowledge can be used for development of new methods to reduce effects of pathogen attacks, such as phosphomimic mutation of signaling intermediates.

Plants have developed many different mechanisms to counteract pathogen attack. Some of these defense mechanisms are preformed, providing physical and chemical barriers to hinder pathogen infection, whereas others are first induced when the presence of a pathogen has been perceived. Recognition of a pathogen or an elicitor leads to a variety of local and systemic changes that serve to kill the pathogen or prevent its spreading through the plant. The different layers of induced defense include basal defense and resistance (*R*)-gene-mediated defenses. Basal defense relies on the plant recognizing general elicitors, which are structural components of microbes. One fungal

elicitor is xylanase, which can be a potent elicitor of defense responses in cultivars of the Solanaceae family. Ron and Avni (2004) were able to show that silencing of xylanase receptors prevents the binding of xylanase and, thus, inhibits the hypersensitive response in tomato. Interestingly, a kinase (AtMPK6) can be induced by different signaling pathways depending on whether a fungal or a bacterial elicitor is involved (Anthony et al. 2006).

Reversible protein phosphorylation is a key biological process for regulating pathogen-induced defense responses. Approximately 4% of all *Arabidopsis* proteins are predicted to be kinases, proteins that transfer a phosphate group to a substrate. In both basal and *R*-gene-mediated defenses, phosphorylation cascades can be activated within minutes. The specific and irreversible inactivation of mitogen-activated protein kinase (MAPK) pathways by bacterial effectors injected by the type III secretion system, and the fact that potato plants have been engineered to be resistant to *Phytophthora infestans* by superactivation of a MAPK pathway, both illustrate how important phosphorylation can be in plant–pathogen interactions (Shan et al. 2007; Yamamizo et al. 2006).

Different *Phytophthora* spp. are among the most devastating pathogens of dicot plants. *P. infestans* caused the Irish potato famine some 160 years ago and, since then, has been a major problem in potato production. *P. infestans* causes a premature wilting and tuber blight. This plant pathological problem is presently on the increase due to sexual reproduction of the oomycete and due to fungicides having become less efficient. The general public has also shown considerable concern regarding the use of fungicides. *Arabidopsis* exhibits a nonhost resistance to *P. infestans* and, therefore, is an attractive source of resistance to investigate.

Signaling proteins are often of low abundance and the phosphorylated form of the protein usually constitutes only a small fraction of the total population. Therefore, several different methods for phosphoprotein enrichment have been developed. Immunoprecipitation with antibodies specific to phosphorylated amino acid residues have been used but the technique suffers from the limited availability of effective anti-pSer/pThr antibodies. Selective enrichment of phosphoproteins by the use of immobilized metal-affinity chromatography (IMAC) and of metal hydroxide has also been described (Collins et al. 2005 [Ga³⁺]; Dubrovskaya and Souchelnyskiy 2005 [Fe³⁺]; Machida et al. 2007 [Ga³⁺, Fe³⁺, Zn²⁺, and Al³⁺]; Wolschin et al. 2005 [Al(OH)₃]).

Identification of phosphopeptides can be limited by their relatively low abundance and their ion suppression during

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analysis in a mass spectrometer. Several techniques for the enrichment of phosphopeptides, including IMAC (Witze et al. 2007) and strong cation exchange chromatography (Beausoleil et al. 2004), have been employed. Recently, titanium dioxide (TiO₂) purification of phosphopeptides has been successfully carried out (Bodenmiller et al. 2007; Caspersen et al. 2007; Larsen et al. 2005; Macek et al. 2007; Thingholm et al. 2006), but purification of intact phosphoproteins on TiO₂ has not yet been described.

The tendency of phosphopeptides to lose phosphoric acid can make it difficult to determine the exact site of phosphorylation. Methods for improving the assignment and sequence coverage of phosphopeptides include comparison of untreated with phosphatase-treated samples, as well as specific chemical modification of phosphopeptides that are important especially in analyses with time of flight (TOF) instruments. Modification of phosphorylated residues can be achieved by β -elimination using BaOH but is difficult to carry out successfully if the residue is followed by a proline (Adamczyk et al. 2001). This is a major drawback because S/TP sequences are common targets for large groups of kinases, such as cyclin-dependent kinases and MAPKs.

Fragmented peptide matrix-assisted laser desorption ionization (MALDI) MS/MS spectra typically contain y and b and sometimes a, c, and z ions. General chemical derivatization methods that produce y-ion-dominated spectra have been used primarily in de novo sequencing. These methods include N-terminal derivatization by use of 3-sulphopropionic acid NHS-ester (Keough et al. 1999) or 4-sulfophenyl isothiocyanate (SPITC) (Chen et al. 2003; Gevaert et al. 2001), both of these resulting in a strong negative charge at the N-terminus and, thus, in an enhancement of the y-ion series. The effectiveness of these derivatization methods could be expected to be independent on the site of phosphorylation but the methods have not yet been applied to phosphopeptides from biological samples.

The importance of phosphorylation emphasizes the need for characterizing plant-microbe-mediated protein phosphorylation and identifying the phosphorylation sites in these proteins to obtain a better understanding of the biological role of the modification. This could serve as a basis for developing new methods of reducing the effects of pathogen attack. We describe two new signaling intermediates in the interaction between *P. infestans* and plants, as well as the development of methods for enrichment of phosphoproteins and the derivatization of phosphopeptides.

RESULTS

Phosphoprotein enrichment.

In efforts to discover phosphorylated signaling intermediates that take part in the response of plants to *Phytophthora* spp., we decided to develop a method for enrichment of phosphoproteins with TiO₂ Sachtopore NP beads that had been employed recently for phosphopeptide enrichment (Larsen et al. 2005; Witze et al. 2007). We first used a standard mix of six proteins, of which two are phosphorylated (PeppermintStick mix; Molecular Probes, Eugene, OR, U.S.A.). With this material, a determination of whether native or denaturing conditions should be used and quantitative analyses of the recovery of phosphoproteins could be done. The protein (2 μ g of each) was mixed with 200 μ l of the respective buffer and 20 mg of TiO₂ beads. This was incubated with shaking for 2 h and the beads were washed four times with 1 ml binding buffer and were subsequently eluted three times with 200 mM Na₂HPO₄ and 100 mM sodium pyrophosphate (NaPP with or without urea). After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation, the gels were first analyzed for phosphoproteins by staining them with Pro-Q Diamond (Molecular Probes) (Fig. 1A, upper panel), and then with Coomassie brilliant blue (CBB) for detection of proteins (Fig. 1A, lower panel). A specific detection of phosphoproteins was indicated by the lack of staining of the nonphosphorylated proteins whereas the two phosphoproteins at 45 and 23 kDa could be readily detected (Fig. 1A). Surprisingly, we found that, in order to enrich the two phosphoproteins, we needed to use both native and denaturing conditions because each method enriched only one of the proteins (Fig. 1A). Quantification of the Pro-Q-diamond signals from two independent experiments revealed that, under native conditions, 49% of the 45-kDa protein was recovered and, under denaturing conditions, 34% of the 23-kDa protein was recovered. Both the phosphoenrichment buffers efficiently removed unphosphorylated proteins from the mix, with the tendency for the denaturing conditions to contain even less nonphosphorylated proteins (Fig. 1A, lower panel). Analyses of the nonbound fractions showed that the 45-kDa protein did not bind in the denaturing buffer (data not shown). Boiling the beads with SDS buffer showed that, in native conditions, the 23-kDa phosphoprotein was bound to the beads and did not elute using the sodium phosphate- and sodium pyrophosphate-containing buffer (data not shown).

After the demonstration of the use of TiO₂ beads for enrichment of phosphoproteins from the mix with low complexity, we investigated whether the method was efficient on biologically relevant samples. Proteins from 4-day-old green cell cultures of *Arabidopsis thaliana* were extracted under native conditions and samples were diluted with 5 volumes of either extraction buffer or 8 M urea before addition of beads. After 2 h of binding, the beads were washed and proteins were eluted with vigorous shaking in elution buffer with or without 6 M urea. Again, the gels were first analyzed for phosphoproteins by staining them with Pro-Q Diamond (Fig. 1B, upper panel), and then with CBB for detection of proteins (Fig. 1B, lower panel). Phos-

Fig. 1. Phosphoprotein enrichment by titanium dioxide (TiO₂) resin under native and denaturing conditions. Upper panel, Pro-Q Diamond staining; lower panel, Coomassie brilliant blue (CBB) staining. **A**, PeppermintStick phosphoprotein mix; **B**, *Arabidopsis* cell culture extract; in, input; nat, eluate with native buffer; den, eluate with denaturing buffer.

Fig. 2. Enrichment of phosphoproteins in native conditions with different resins. **A**, Titanium dioxide (TiO₂) purification. **B**, Ni-NTA purification. Upper panel, Pro-Q Diamond staining; lower panel, Coomassie brilliant blue (CBB) staining. Ti, eluate from TiO₂ purification; Ni, eluate from Ni-NTA purification; 50/5 Im, binding in buffer containing 50 or 5 mM imidazole; 5/50 PO₄, binding in buffer containing 5 or 50 mM Na₂HPO₄.

phosphoprotein-enrichment was evident in both buffer systems compared with the total protein extract (Fig. 1B, lane “in”). The patterns of phosphoproteins after enrichment were only partially overlapping again, indicating that both native and denaturing conditions could be used in order to get a more comprehensive phosphoprotein analysis. Varying the pH of the extraction buffer for the TiO₂ enrichments from pH 6.5 to 8.0 did not affect the overall pattern (data not shown).

For comparative studies of the TiO₂ resin, we tested an IMAC resin (Ni-NTA beads) using 100 mM imidazole and 100 mM Na₂PO₄ for elution. Phosphoprotein enrichment under native conditions was evident by both resins (Fig. 2). The phosphoprotein patterns in the eluates from respective beads revealed clearly different profiles that suggest that the two resins are complementary to each other.

When proteins were enriched on TiO₂ beads, adding 5 mM sodium phosphate in the binding buffer clearly reduced the binding of proteins to the beads (Fig. 2A, lane labeled “5 PO₄”). Including imidazole in the binding buffer was not found to decrease the recovery of proteins or phosphoproteins (Fig. 2A). The presence of sodium pyrophosphate (10 mM) and glycerophosphate (10 mM) in the extraction and binding buffer resulted in the complete absence of proteins in the eluate (data not shown).

Omitting imidazole in the elution buffer led to there being no detectable level of proteins in the Ni-NTA eluate (data not shown). Binding to the Ni-NTA beads in the presence of 5 mM imidazole clearly reduced the recovery of proteins from the resin, whereas inclusion of 50 mM sodium phosphate in the binding buffer had little effect on the protein or phosphoprotein patterns (Fig. 2B). In contrast to titanium-based enrichment, the presence of sodium pyrophosphate and glycerophosphate in the extraction and binding buffer did not reduce the amount of phosphoproteins obtained from the Ni-NTA beads (data not shown). Thus, although both resins enriched phosphoproteins, they differed in their interaction with the proteins. The binding to the TiO₂ beads was found to be dependent upon the presence of phosphate and not of imidazole, whereas the opposite was the case for the Ni-NTA resin.

Table 1. Identified phosphoproteins after single TiO₂ enrichment of proteins from *Arabidopsis* cell culture under native (N) and denaturing (D) conditions^a

Accession number	Buffer	Peptide count	Total ion score	Best ion score	Protein name
AT1G76180.1	D	8	648	181	Dehydrin (ERD14)
AT1G48920.1	D+N	8 + 1	483 + 141	118 + 141	Nucleolin, putative
AT5G22650.1	D+N	6 + 1	438 + 57	108 + 57	Expressed protein
AT3G15730.1	N	7	394	125	Phospholipase D α 1 (PLD α 1)
AT1G56070.1	D+N	5 + 12	393 + 1,144	166 + 190	Elongation factor 2
AT3G52990.1	D+N	1 + 4	180 + 420	180 + 222	Pyruvate kinase, putative
AT4G31580.1	D+N	3 + 2	147 + 65	73 + 42	Splicing factor RSZp22 (RSZP22)
AT3G49430.1	D	3	120	55	Pre-mRNA splicing factor SF2
AT5G15450.1	N	1	93	93	Heat shock protein 100 (HSP100), putative
AT5G47210.1	D+N	1 + 1	43 + 83	43 + 83	Nuclear RNA-binding protein, putative
AT3G13920.1/					
AT1G54270.1	N	2	81	64	Eukaryotic translation initiation factor 4A-1/2
AT3G55460.1	D	1	80	82	SC35-like splicing factor, 30 kDa (SCL30)
AT5G43830.1	N	1	73	73	Expressed protein, similar to auxin downregulated protein ARG10 (<i>Vigna radiata</i>)
AT5G54430.1	N	1	60	60	Universal stress protein (USP) family protein
AT3G48050.1	N	1	54	54	Bromo-adjacent homology (BAH) dom /AT3G48060.1-containing protein
AT2G01210.1	D	1	45	45	Putative LRR transmembrane protein kinase
AT1G20630.1	N	1	44	44	Catalase 1, CAT1
AT5G61780.1	N	1	41	41	Tudor domain-containing protein/nuclease family protein, contains Pfam domain PF00567
AT1G24180.1	D	1	40	40	Pyruvate dehydrogenase E1 component α subunit, putative
AT5G52040.2	N	1	33	33	Arginine/serine-rich splicing factor RSP41

^a Dataset is generated from tandem mass spectrometry data of one sample from each buffer. The ion scores were generated by Mascot searches of the *Arabidopsis thaliana* TAIR database with carbamidomethyl as fixed modification and phosphorylated S, T, Y, and oxidized M as variable modifications. Only peptides with rank 1 are included.

To show the general applicability of TiO₂ beads for phosphoprotein-enrichment, we digested the TiO₂-enriched plant proteins shown in Figure 1 with trypsin and analyzed them by MS. Twenty phosphoproteins were identified from two single high-pressure liquid chromatography (HPLC) MS/MS samples enriched under native and denaturing conditions (Table 1). The spectra are collected in Supplementary Figure 1. Only a third of the phosphoproteins were overlapping between the two samples, again showing that a screen using both buffers could be useful. The assignments as phosphoproteins were done with comparison to the PhosPhAt 2.1 database, where only experimentally verified phosphopeptides were taken into account. In addition to this study on complex samples, several phosphoproteins have been identified from gel pieces such as nucleoside diphosphate kinase 1 NDK1 (At4g09320.1) and ribosomal protein S6 RPS6 (At4g31700.1).

Stress-induced phosphorylation.

To investigate the interaction between plants and *P. infestans* as well as to evaluate the use of the established phosphoprotein enrichment methods for the comparison of different samples, the *Arabidopsis* cell-suspension cultures were treated with xylanase or *P. infestans* zoospores. The proteins obtained were purified under native conditions on TiO₂ or Ni-NTA beads, separated on SDS-PAGE, and stained with Pro-Q Diamond and CBB (Fig. 3). Two bands with apparent molecular weight of approximately 30 kDa showed an increase in Pro-Q Diamond staining after 15 min of elicitation with xylanase and *Phytophthora* spp. Interestingly, the bands were hardly detected in the control sample whereas distinct bands were visible in the stressed samples after TiO₂ enrichment (Fig. 3A). In contrast, we could not detect any differences in protein staining of the two bands in control and treated samples from the Ni-beads (Fig. 3B). This indicates that the two resins bind these

proteins differently and that TiO₂ beads discriminated between the phosphorylated and unphosphorylated form of these proteins better than the Ni-beads. The results from the Ni-resin also show that no protein accumulation occurred during the 15 min of elicitation and that the difference in Pro-Q Diamond staining of the control and treated samples is only due to increased phosphorylation.

The biotic stress induction that we detected prompted us to test how specific this increase in phosphorylation was. Abiotic stress such as salt stress with 0.1 M NaCl or osmotic shock by 0.2 M sorbitol did not alter the phosphorylation level of the two proteins (Fig. 3B). This might indicate a specific involvement in biotic stress of these proteins.

The differentially phosphorylated proteins were cut out from the gel, digested with trypsin, and analyzed by MALDI TOF MS (Fig. 4). The band of lower molecular weight was identified as being an *Arabidopsis* protein of unknown function (At5g54430.1) with similarity to bacterial universal stress proteins (USPs). The protein coverage was 54%. All the major peaks in the MS spectra originated from this protein (Fig. 4A and C). Peaks not matching the protein identified were used to search the *Arabidopsis* database but no additional protein could be identified. The higher molecular band was identified as At4g27320.1 (Fig. 4B and D). These two proteins belong to the same protein family and have a sequence identity with each other of more than 80% (Fig. 4B). The MALDI run of the At4g27320.1 and At5g54430.1 protein digests was then analyzed for the presence of phosphorylated peptides but none were found. This could be due either to low abundance of the phosphorylated peptides or to ion suppression of them during the MALDI analysis.

Phosphopeptide enrichment and analysis.

With the aim of purifying phosphopeptides from At5g54430.1 and At4g27320.1, conditions for TiO₂ purification were tested out using a mixture of trypsin-digested α - and β -casein. The protocol was based on Zhang and associates (2007), although TiO₂ beads from Sachtopore instead of from GL Science were employed. Briefly, the peptide mixture was diluted five times with a binding buffer (80% acetonitrile [ACN] and 5% trifluoro acetic acid [TFA]) and was then purified on a homemade column containing TiO₂ beads. Peptides were eluted with ammonia and were then purified on a homemade POROS OLIGO R3 reverse phase (R3) column. Use of 1.5% NH₄OH instead of 0.5% for elution of the TiO₂ column improved the recovery of phosphopeptides. Using 0.6% TFA for washing instead of a 0.1% solution (which is more often used) led to a higher recovery of phosphopeptides from the R3 column than the latter concentration yields (data not shown) (Larsen et al. 2005; Thingholm et al. 2006). Prepacked TiO₂ Nutip columns from Glygen were also tried but were found, in our hands at least, to be of no great benefit.

Applying the improved protocol, trypsin digests of At4g27320.1 and At5g54430.1 were purified on TiO₂ columns and desalted on R3 columns before the MALDI MS analyses. One peptide from each band was found at *m/z* 1,020 and 1,010, respectively (Fig. 5A) (data not shown). These correspond in mass to phosphorylated forms of the IHHPSPR (*m/z* 940.6, At4g27320.1) and the IHHPSSPR (*m/z* 930.6, At5g54430.1) peptides present in the original MALDI MS spectra (Fig. 4A) (data not shown). Neither of the putative phosphopeptides was present in the original spectra. The phosphopeptides were in the N-terminal part of At4g27320.1 and At5g54430.1.

To verify the identity of the peptides and to determine the phosphorylation sites, the peptides purified by TiO₂ were analyzed by MALDI MS/MS. MS/MS spectra of the 1,020- and

Fig. 3. *Phytophthora infestans* and xylanase-induced protein phosphorylation in *Arabidopsis* cells. **A**, Titanium dioxide (TiO₂) purification. **B**, Ni-NTA purification. Elicitation was done either with 3,000 zoospores per milliliter, xylanase at 100 μ g/ml, 0.1 M NaCl, or 0.2 M sorbitol for 15 min. These experiments have been repeated three times with similar results. M, PeppermintStick phosphoprotein molecular weight marker; -, control with no treatment; Pinf, *Phytophthora infestans* zoospores; Xyl, xylanase; Sorb, sorbitol; and CBB, Coomassie brilliant blue.

1,010-m/z peptides are shown in Figure 6. Both peptides clearly show a neutral loss of H_3PO_4 (-98, denoted as M^A) and a neutral loss of HPO_3 (-80, denoted as M^B), indicating that they are indeed phosphorylated. Both spectra contain b- as well as y-ions. The y-ion series of the 1,020-m/z peptide includes a phosphorylated y5 ion (m/z 633) and y3 ion (m/z 439), a y5 ion that has undergone neutral loss ($y5^A$, m/z 535), and an unphosphorylated y2 ion (m/z 272). This shows the phosphogroup to be located on the only serine residue that is present in the peptide (Fig. 6A). In the 1,010-m/z spectra, the presence of the m/z 623 ($y5$), 525 ($y5^A$), and 272 ($y2$) ions localizes the phosphogroup to the PSS fragment, although it is not possible to determine its exact position (Fig. 6B).

SPITC derivatization of phosphopeptides.

The tendency of phosphopeptides to lose phosphoric acid can make it difficult to resolve the site at which phosphorylation takes place. A chemical derivatization method that produces y-ion-dominated spectra is N-terminal derivatization by SPITC. The resulting peptide has a strong negative charge at the N-terminus, thereby enhancing the y-ion series. To our knowledge, SPITC derivatization has not previously been shown for phosphopeptides obtained from biological samples. We tested the possibility of applying this general and cheap modification method on TiO_2 -purified phosphopeptides because this derivatization method could be expected to be independent of the site of phosphorylation.

Fig. 4. Matrix-assisted laser desorption ionization dual mass spectrometry (MALDI MS) analysis of proteins that are phosphorylated after stress. **A**, MALDI MS spectra of trypsin-cleaved band from the lower band. Asterisks denote peaks that belong to At5g54430.1. **B**, Protein alignment of At5g54430.1 and At4g27320.1. Peptides identified by MS are shaded. The universal stress protein domain is underlined. The asterisk denotes identified phosphorylated residues that were phosphorylated after *Phytophthora* elicitation. **C**, Identified At5g54430.1 peptides. It was not possible to assign two peptides (shown in italics) from At5g54430.1 due to a minor difference in size. **D**, Identified peptides from At4g27320.1.

The derivatization method was developed using the At5g54430.1 phosphopeptide of m/z 1,020 purified on TiO_2 . TiO_2 eluate (20 μl) in ammonia (pH 11) was mixed with 10 μl of SPITC at 20 mg/ml in 100 mM NaHCO_3 and 10 μl of 3.3% formic acid (in order to adjust the pH to 8.5). The mixture was incubated at 55°C for 15 min. An additional 5 μl of SPITC was added and the sample was incubated again for 15 min at 55°C. After acidification with 2.2 μl of concentrated formic acid, the derivatized phosphopeptide was purified on R3 resin and analyzed by MALDI MS (Fig. 5B). An underivatized sample was analyzed as a control (Fig. 5A). Comparison of the two MS spectra showed the method developed here to be highly effective in derivatizing the m/z 1,020 phosphopeptide into m/z 1,235 (+215 Da).

MS/MS analysis of the underivatized m/z 1,010 phosphopeptide from At4g27320.1 showed there to be two possible sites of phosphorylation, each of them labeled by a question mark in Figure 7A. Accordingly, a TiO_2 -enriched sample was SPITC derivatized and MS/MS spectra were collected from the derivatized phosphopeptide of m/z 1,225 (Fig. 7B). The most abundant fragment peak corresponds to the mass of the underivatized phosphorylated peptide, resulting from loss of the 215-Da SPITC group. The smaller peak at m/z 1,052 corresponds to a 173-Da loss of sulphanic acid ($\text{HO}_3\text{S}-\text{C}_6\text{H}_4-\text{NH}_2$). Peaks matching the loss of phosphoric acid (−98) and phosphate (−80) from both the SPITC-derivatized and underivatized peptide are present as minor peaks in the spectra. No N-terminal ions were to be found in the spectra. Instead, a prominent y-ion series was observed. The presence of the m/z 439 phosphorylated y3 ion and the m/z 272 unphosphorylated y2 ion show that the phosphogroup is located to the second serine of the IHHSPR phosphopeptide.

Alignment of the N-terminal part of At4g27320.1 and At5g54430.1 with related proteins from other plant species is

Fig. 5. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) spectra of 4-sulfophenyl isothiocyanate (SPITC) labeling of titanium dioxide (TiO_2)-purified phosphopeptide from At5g54430.1. **A**, TiO_2 eluate before derivatization. The major peak is the m/z 1,020 phosphopeptide. **B**, SPITC-derivatized TiO_2 eluate. The major peak is the derivatized m/z 1,020 phosphopeptide ($1,020 + 215 = 1,235$).

shown in Figure 8. The aligned part does not exist in bacterial USPs. Both proteins that were identified were phosphorylated in this part and, in monocots and dicots, the phosphoserine is conserved.

DISCUSSION

We have demonstrated how a TiO_2 resin can be used for the enrichment of phosphoproteins as well as a method to derivatize TiO_2 -purified phosphopeptides for facilitating determination of the exact site of phosphorylation. Use of these methods was exemplified by the identification of two plant proteins that could be shown to be phosphorylated after elicitation of *Arabidopsis* cells with *P. infestans* zoospores and xylanase but not after abiotic stress like salt and sorbitol. This is the first time TiO_2 was used to enrich intact phosphoproteins in any biological system.

TiO_2 and Ni-NTA enrichments of phosphoproteins showed different phosphoprotein patterns, suggesting that they could be used for the enrichment of partially differing phosphoproteome subpopulations. The Ni-NTA purification was, as expected, found to be dependent on imidazole but not phosphate, with the opposite being the case for TiO_2 purification. The phosphate-dependent binding of proteins to the TiO_2 resin indicates that the interaction occurred by means of the phosphorylation itself. Indeed, phosphate anions are known to bind strongly to TiO_2 in a bidentate bridging configuration (Connor and McQuillan 1999). This is in agreement with the finding that, from the Ni-beads, no differences in protein staining of

Fig. 6. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS/MS) spectra of phosphopeptides from At5g54430.1 and At4g27320.1. $y^A/b^A/M^A$ labels ions or fragments that have undergone a neutral loss of phosphoric acid; $M^\#$ labels a fragment that has undergone a neutral loss of phosphate; and $[M+H]^+$ labels the unfragmented parent ion. **A**, MS/MS spectra of the m/z 1,020 phosphopeptide. Insert shows enlargement of m/z 250 to 800. **B**, MS/MS spectra of the m/z 1,010 phosphopeptide. Insert shows enlargement of m/z 250 to 800.

the At4g27320.1 and At5g54430.1 proteins were detected between control and elicited samples. In contrast, hardly any At4g27320.1 or At5g54430.1 protein could be detected in the TiO₂-control sample, whereas distinct bands were present in the treated TiO₂-bead-enriched samples. It might be the histidine residues on the identified phosphopeptides that are involved in the less phospho-dependent binding to the Ni-beads. This is also in agreement with our detected influence by imidazole on protein-Ni binding in contrast to the protein-TiO₂ interaction.

The efficient recovery of known phosphoproteins indicates that even fairly small stoichiometric changes in phosphorylation might be detected by the use of TiO₂ beads with complementing buffers. The detection of over 10 phosphoproteins from single HPLC MS/MS runs of *Arabidopsis* samples enriched by TiO₂ beads under native and denaturing conditions also show the general applicability of the method. More than half of the

phosphoproteins were unique for each buffer, again showing the advantage of using the two different buffers. The MS/MS data of over 20 phosphoproteins taken together with the efficient recovery of phosphoproteins, the clear enrichment shown by the phosphoprotein-stained gels, as well as the inhibition of binding of proteins to the TiO₂ beads by addition of phosphate demonstrate that this method could be of general use.

Two proteins were found to be more phosphorylated after challenge of *Arabidopsis* cells with fungal xylanase and zoospores of *P. infestans*. These two proteins, At5g54430.1 and At4g27320.1, were identified as belonging to a superfamily of USPs (PFAM: PF00582). The USP-domain-containing proteins were first identified in prokaryotic organisms, in which they are known to accumulate in response to a plethora of stimuli, including nutrient starvation, H₂O₂, heavy metal, and heat stress (Kvint et al. 2003, Nachin et al. 2005). In plants, the

Fig. 7. Matrix-assisted laser desorption ionization dual mass spectrometry (MALDI MS/MS) spectra of the At4g27320.1 phosphopeptide before and after 4-sulphophenyl isothiocyanate (SPITC) derivatization. [M+H]⁺ denotes the parent ion; MS denotes the SPITC-derivatized parent ion; y^Δ labels a y-ion that has undergone a neutral loss of phosphoric acid; M[#] denotes a parent fragment that has undergone a neutral loss of phosphate; and M^Δ labels a parent fragment that has undergone a neutral loss of phosphoric acid. **A**, MS/MS spectrum of the 1,010 phosphopeptide before derivatization. Question marks denote on which residue the phosphogroup might be situated. **B**, MS/MS spectrum of the SPITC-derivatized phosphopeptide (m/z 1,225).

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At5g54430		LPNIKIHHPPSPRHSHHHSSSTPSSAATPTPTAGARRKIGVAVD
At4g27320		LPNIKIHHPPSPR-HSHHHSSSTP-SAATPTPTAGARRKIGVAVD
125934794 (Bn)		LPTIKIHHPPSP---HHHHSSSTPSSAATPTPTAGARRKIGVAID
125936449 (Bn)		LPNIKIHHPPSPR-HSHHHSSSTP-SAATPTPTAGARRKIGVAVD
TC228830 (Gm)		LPQIKIHHPPSPR---HHHPSSAGA---ATPTPTAGARRKIGVAVD
TC45490 (Pop)		LPTIKIHHPPSPR-HSHHHS-----HLHAATPTAGARRKIGVAVD
TC2771 (Pv)		LPQIKIHHPPSPR-----HPSPAG---ATPTPTAGARRKIGVAVD
TC177617 (Sl)		LANIKIKC-SSPR----FPPPTTP--SATDTPTAGAQRKIGIAVD
BF616994 (Hv)		AGQAATIQQSPR---YFFS-----SLAGKDASSHRRRIAIAVD
TC250617 (Ta)		AAVAATIQQSPR---YFFS-----SLA--ASSPHRRRIAIAVD

Fig. 8. Alignment of the N-terminal part of At5g54430.1 and At4g27320.1 with apparent plant homologues. The asterisk denotes the phosphorylated serine residue. Bn, canola; Gm, soybean; Pop, poplar; Pv, common bean; Sl, tomato; Hv, barley; and Ta, wheat.

USP domain is found both in small proteins and together with other functional domains such as protein kinase domains. The biochemical function of the 150-amino-acid residue USP domain is still not clear (Kerk et al. 2003). The structure of the *Methanococcus jannaschii* protein MJ0577 has been resolved and was found to contain a bound molecule of ATP (Zarembinski et al. 1998). Residues known to be involved in ATP binding were found to be conserved in both of the *Arabidopsis* proteins identified in this study (Kerk et al. 2003), indicating that these proteins also bind ATP and could function as molecular switches in biotic stress-related cellular processes. This is supported by the fact that these proteins were not phosphorylated upon abiotic stress like salt or sorbitol treatments.

The determination of phosphorylation sites in the proteins that were identified was facilitated by our method for derivatization of TiO_2 -purified phosphopeptides with SPITC. A prominent y-ion series with no N-terminal ions were present in the spectra. Interestingly, the neutral loss detected in the derivat-

ized samples was less pronounced than in the underivatized samples. This could also help in determining the location of phosphorylation sites. Both proteins were phosphorylated at the N-terminal region (Fig. 4B), a part that does not exist in the USPs from bacteria. The phosphorylation site was found to be conserved in USP-domain-containing proteins from many plant species, including both monocot and dicot plants (Fig. 8). This conservation indicates that phosphorylation of these proteins may have been conserved for more than 100 million years. The phosphorylation of a serine-proline motif means that it is possible that they are substrates of MAPKs known to be activated during responses to xylanase and *Phytophthora* elicitors (Benschop et al. 2007; Zhang et al. 1998). These proteins show an example where derivatization of the phosphogroup by β -elimination with BaOH would be difficult (Adamczyk et al. 2001), which is an important drawback with that method because the SP motif is a target that large groups of kinases have in common.

Many newly developed gel-free quantitative proteomics methods rely on extended access to mass spectrometers with isotopic resolution (Peck and Thelen 2007). For gel-based analyses, less time is required for MS analysis and treatment of data because uninteresting proteins are deselected at an early step. Two-dimensional (2D) gel separation is often used mainly because of its capacity for separating the phosphorylated from the unphosphorylated forms of proteins. However, a limitation of 2D gels is that they are very poor at separating basic proteins, and many proteins fail to dissolve in the first dimensional loading buffer. The methods for purification of intact phosphoproteins that we describe were found, together with Pro-Q Diamond staining of samples separated by one-dimensional gel samples, to be a robust way of finding differentially phosphorylated proteins (Fig. 9). Identification of phosphopeptides from gel-separated intact proteins can be more straightforward than from gel-free systems, because it is based on information of the size of the proteins and the identification of several peptides from a given protein. Therefore, such methods can be expected to be suitable for many biologists. However, it should be pointed out that a prefractionation step is most probably required to find many interesting differentially phosphorylated proteins in such complex organisms as plants.

One way to identify differentially phosphorylated proteins and to determine the sites of phosphorylation is to excise the gel bands and digest them by trypsin and first analyze a fraction directly by HPLC/MSMS (Fig. 9). If the complexity of the sample is sufficiently low, the protein and phosphopeptide identification as well as determination of the phosphosite can be successful. If no phosphopeptides are identified, however, they need to be enriched prior to HPLC/MSMS (Figs. 4A, 5A, and 9). Increased concentration of ammonia in the elution of peptides from TiO_2 and increased TFA concentration in the wash of the R3 column made a significant improvement compared with protocols that were published earlier (Larsen et al. 2005; Zhang et al. 2007). If the sites still cannot be determined, we recommend, especially for TOF users, derivatization of the phosphopeptides by the use of SPITC to get an unambiguous assignment (Figs. 5B, 7, and 9). In addition to phosphosite determination, the SPITC derivatization of peptides is also valid for organisms without a well-annotated proteome in which de novo sequencing is often necessary in order to identify the protein. This is often the case for molecular studies of plant-microbe interactions.

The USPs described here are the first plant proteins to be shown to be phosphorylated upon treatment with *Phytophthora* spores. With the definite determination of phosphorylation sites, they are interesting candidates for phosphomimic mutation in order to circumvent the pathogen-driven downregulation of

Fig. 9. Summary of methods used in this article and how they relate to each other. Pro-Q Diamond staining visualizes phosphorylated proteins and colloidal Coomassie stains proteins. TiO_2 , titanium dioxide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; R3, POROS OLIGO R3 reverse phase chromatography; SPITC, 4-sulphophenyl isothiocyanate; HPLC, high-pressure liquid chromatography.

kinase activity seen at least in bacteria–plant interactions (Shan et al. 2007).

MATERIALS AND METHODS

Cell culture and elicitor treatment.

Suspension cell cultures of *A. thaliana* ecotype Landsberg (La-e) were used 4 days after subculture of 5 ml of culture into 100 ml (weekly). Cells were treated for 15 min with a final concentration of xylanase at 100 µg/ml, 0.1 M NaCl, 0.2 M sorbitol, or 300,000 *P. infestans* zoospores prepared according to Eucablight standards (Swedish isolate 03046 provided by Björn Andersson, Swedish University of Agricultural Sciences). Cells (50 ml) were centrifuged at 4,000 rpm for 2 min at room temperature. The pellet was immediately frozen in liquid nitrogen and stored at –80°C.

Enrichment of phosphorylated proteins.

Extraction buffer (16 ml; 100 mM morpholineethanesulfonic acid [pH 6.1], 200 mM NaCl, 2% glycerol, 25 mM sodium fluoride, 1 mM sodium molybdate, 0.2% Tween 20, 0.2% triton X-100, 10 µM leupeptin, 1 nM calyculin A, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate) was added to a frozen pellet of plant cells and the mix was sonicated until thawed. Cells were refrozen in liquid nitrogen and sonicated again. Cells were centrifuged at 4°C for 5 min at 4,000 rpm in a swing-out rotor and the supernatant was transferred to a new tube and centrifuged again for 5 min. Binding was done in the extraction buffer or under denaturing conditions according to Wolschin and associates (2005) but with a final urea concentration of 6.4 M urea. Extracts were incubated end over end with 50 µl of 50% Ni-NTA slurry (Qiagen) or 20 mg of 300 Å Sachtophore NP TiO₂ beads (Sachtleben Chemie, Duisburg, Germany) at 4°C for 2 h. Beads were washed two times for 20 min with 5 ml of binding buffer. Proteins were eluted from Ni-beads three times in 35 µl of 100 mM imidazole, 25 mM NaF, and 1 mM sodium molybdate with vigorous shaking for 10 min followed by incubation for 3 min at 40°C, unless otherwise stated. The TiO₂ beads were eluted the same way with elution buffer (200 mM Na₂HPO₄, 75 to 100 mM NaPP, 0.2% Tween 20, and 0.2% triton X-100 with or without 6 M urea). The eluates were pooled before gel separation.

SDS-PAGE.

Samples were loaded on SDS-PAGE gels alongside 0.4 µl of PeppermintStick phosphoprotein molecular weight standards (0.5 µg/µl per protein, Molecular Probes). Gels were stained with Pro-Q Diamond Phosphoprotein Gel Stain according to the protocol provided by Molecular Probes. The gels were analyzed using a Typhoon Scanner (GE Healthcare, Little Chalfont, U.K.) with the use of a 532-nm laser for excitation and an LP 560-nm emission filter. The gels were subsequently stained with colloidal Coomassie. Protein bands of interest were excised from the gel using a scalpel, and stored at –20°C in 1.5-ml maximum recovery tubes (Axygene, Union City, CA, U.S.A.).

In-gel digestion.

The gel pieces were destained in 100 µl of 50 mM NH₄HCO₃ in 50% ethanol three times for 30 min each time. The liquid was removed and 100 µl of ethanol was added for 10 min. The gel pieces were rehydrated in 10 µl of 10 mM dithiothreitol (DTT) in 50 mM NH₄HCO₃ at 37°C for 30 min. The liquid was removed and 100 µl of ethanol was added for 10 min. The gel pieces were rehydrated in 10 µl of 55 mM iodoacetamide in 50 mM NH₄HCO₃ and incubated for 30 min

at room temperature in darkness. The liquid was removed and 100 µl of 50 mM NH₄HCO₃ in 50% ethanol was added for 5 min. The liquid was removed and 100 µl of ethanol was added for 10 min. The gel pieces were rehydrated in 2.8 µl of sequencing-grade trypsin at 50 ng/µl (Promega Corp., Madison, WI, U.S.A.) on ice for 45 min. Excess trypsin was removed and 20 µl of 50 mM NH₄HCO₃ was added to cover the gel pieces. The samples were incubated at 37°C overnight and the digests were frozen until further use.

In-solution digestion.

Proteins were reduced with 10 mM DTT at 37°C for 1 h. Iodoacetamide was added to a final concentration of 20 mM and the sample incubated at room temperature in darkness for 1 h. Trypsin (0.1 to 1 µg) was added and the samples were incubated at 37°C overnight.

Enrichment of phosphorylated peptides.

Peptides from gel-excised protein bands were purified on handmade pipette tip-columns (epT.I.P.S 0.1 to 10 µl, no. 0300 073.002; Eppendorf AG, Hamburg, Germany) containing a C8 disk (3M Bioanalytical Technologies, Neuss, Germany) and 2 µl of Sachtophore NP beads. Trypsin digests were mixed with 6 volumes of binding buffer (5% TFA in 80% ACN). Columns were washed with 25 µl of binding buffer and the peptides then loaded onto the column. The column was washed twice with 25 µl of binding solution each time, followed by three washes with 0.5% TFA in 80% ACN. The peptides were eluted using 20 µl of ammonia solution, made with 60 µl of 25% NH₄OH and 940 µl of water. TiO₂ eluates were either SPITC derivatized (see below) or acidified with 1 µl of concentrated formic acid and purified on a handmade column containing a C8 disk and 2 µl of R3 chromatography beads in a 10-µl pipette tip. TFA (0.6%) was used to wash the column and 20 µl of 0.1% TFA in 80% ACN was used for elution. Desalted samples were evaporated in a speedvac and reconstituted in 0.5% TFA before HPLC separation.

SPITC derivatization and purification.

TiO₂-purified phosphopeptides (20 µl) in ammonia were mixed with 10 µl of 3.3% formic acid and 10 µl of SPITC at 20 mg/ml in 100 mM NaHCO₃ and incubated at 55°C for 15 min. SPITC at 20 mg/ml (5 µl) was added and again incubated for 15 min at 55°C. Concentrated formic acid (2.2 µl) was added and the derivatized phosphopeptides purified on R3 with four washing steps with 20 µl of 0.5% TFA and 1% ACN. SPITC-labeled peptides (+215) were manually selected for MS/MS analysis.

Liquid chromatography.

Samples were separated by reversed-phase nanoLC using an 1100 Series Nanoflow LC system (Agilent Technologies, Waldbronn, Germany). The mobile phases used for separation were (A) 0.05% (vol/vol) TFA and (B) 90% (vol/vol) ACN and 0.05% (vol/vol) TFA. Digested peptide extract (8 µl) was loaded on the column from glass vials. The samples were loaded onto a precolumn (Zorbax 300SBC18, 5 by 0.3 mm) at a flow rate for buffer A of 0.050 ml/min. After 14 min, the separation column (Zorbax 300 SB C18, 150 by 0.075 mm) was connected with the precolumn. The separation was carried out at a flow rate of 300 nl/min with the following elution profile: 10 to 16 min, 0 to 5% B; 16 to 62 min, 5 to 60% B; 62 to 67 min, 60 to 80% B; 68 to 72 min, 80 to 100% B; 73 to 78 min 100% B; 79 to 80 min, 100% B. Fractions were collected on MALDI targets from time 16 to 67 min with 0.5- to 1-min intervals using the 1100 Series LC micro collection/spotting system.

MALDI-TOF MS and MS/MS.

MS spectra were recorded automatically using a 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA, U.S.A.) mass spectrometer equipped with an Nd:YAG laser (355 nm) operating at a frequency of 200 Hz. All spectra were collected in positive reflector mode. The instrument was externally calibrated with a mixture of angiotensin (1,042.542 Da) and neurotensin (1,672.917 Da). If present, the obtained MS spectra were internally calibrated using two trypsin autolysis peptides with the *m/z* values 842.51 and 2,211.097 Da. Proteins were identified using the GPS Explorer software, with an in-house Mascot search engine (Matrix Science, London). MS data were first searched with oxidized methionines as a variable modification. Phospho (S/T/Y), Deamidation (N) were then added as variable modifications and carbamidomethylation of cysteine residues as static modification. Parameters specified in the search were as follows: missed cleavages, 1; peptide mass tolerance, ± 40 ppm; and fragment ion mass tolerance, ± 0.2 to 0.5 Da. MS/MS spectra were interpreted manually. The standard matrix was 5mg/ml α -cyano-4-hydroxy cinnamic acid (Sigma-Aldrich, St. Louis), in 50% ACN, 0.1% TFA, and 25 mM sodium citrate.

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