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Phosphoproteins analysis in plants: A proteomic approach

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Abstract

The study of phosphoproteome on a global scale represents one of the challenges in the post-genomic era. Here, we propose an integrated procedure starting from the crude protein extract, that consists of sequential purification steps, and ending up in the identification of phosphorylation sites. This involves (i) an enrichment in phosphoproteins with a commercially available chromatography matrix, (ii) a 2-D gel analysis of the enriched fraction followed by the selective staining with the phosphospecific fluorescent dye Pro-Q Diamond, (iii) a phosphopeptide capture, from the tryptic lysate of 2-D spots, using IMAC micro-columns. In the end, the identification of the phosphoproteins and their corresponding phosphorylation sites were achieved by MALDI-TOF-TOF spectrometry. The method was applied to contrasting samples prepared from cell suspension cultures of *Arabidopsis thaliana* and roots of *Medicago truncatula*. The results obtained, demonstrated the robustness of the combination of two enrichment stages, sequentially at the protein and at the peptide levels, to analyse phosphoproteins in plants.

Keywords: *Medicago truncatula*; *Arabidopsis thaliana*; Phosphoproteins; IMAC; Mass spectrometry

1. Introduction

The reversible phosphorylation of proteins is a regulatory mechanism involved in a wide variety of cellular processes. From sequencing data, it has been inferred that 5% of the genome of the model plant *Arabidopsis thaliana* encodes kinases and phosphatases, representing more than 1000 kinases and phosphatases, controlling the phosphorylation status of thousands proteins (The Arabidopsis Genome Initiative, 2000; Gribskov et al., 2001; Kerk et al., 2002). Despite their tremendous importance, our knowledge of plant phosphoproteins remains incomplete because of several limitations. Phosphoproteins and particularly those involved in cellular signalling are present in low abundance. Furthermore, as a given protein may contain several phosphorylation sites, its phosphorylation status may depend upon the physiological conditions a cell has to face at a given time. Thus, phosphoproteomics aims at identifying the phosphorylated proteins as a whole but also at the peptide level to decipher the so-called phosphorylation code that governs cellular processes. The new generation of mass spectrometers dedicated to proteomics and the progress made in mass spectrometry analysis of phosphopeptides, open up new possibilities for tracking the phosphoproteome (Kalume et al., 2003; Yates, 2004; Reinders and Sickmann, 2005). One of the prerequisite is to enrich the starting biological material in phosphoproteins and/or phosphopeptides. In animal models, antibodies raised

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against tyrosine phosphorylated residues allow the specific immunoprecipitation of P-tyr containing proteins (Pandey et al., 2000). However, this class of phosphorylated proteins accounts for 0.05% of the overall phosphoproteome, only. Furthermore, this approach is not adapted to the study of plant phosphoproteins because only a few number of them are phosphorylated at Tyr residues (mainly the dual p-Thr/p-Tyr MAP kinases family) (Luan, 2003). Similar approaches using anti p-Ser or anti p-Thr antibodies are possible but only a limited number of positive results have been reported (Gronborg et al., 2002). Alternative approaches rely on the use of chromatography methods. A commercial kit was thus proposed for human and yeast cells but allowed only the identification of one phosphorylation site in the human cells sample after peptide sulfonation to enhance the signal of y ions in MALDI-TOF analysis (Metodiev et al., 2004). Similarly, an $Al(OH)_3$ based technique has also been reported to enrich phosphoproteins from plant samples (Wolschin et al., 2005) and further validated by the identification of different phosphorylation sites (Wolschin and Weckwerth, 2005). Very recently, another commercial kit was used in combination with phosphoprotein detection by the Pro-Q Diamond dye to detect a set of potentially phosphorylated proteins in *Arabidopsis* (Irar et al., 2006). However, no phosphorylation event was fully demonstrated by the identification of phosphorylation sites. By contrast, enrichment methods of phosphopeptides using affinity chromatography with immobilized metal ions (IMAC) were proved to allow the characterization of a high number of phosphorylation sites in *Arabidopsis* plasma membrane (Nühse et al., 2003). Therefore, despite an increasing number of reports, the capacity of recent tools to help the characterization of phosphorylation sites when starting from plant protein mixtures remains poorly validated, by contrast to strategies starting from peptide mixtures.

In this paper, we made use of the chromatographic matrix provided by Qiagen, to obtain a phosphoprotein-enriched fraction from two model plants, *A. thaliana* and *Medicago truncatula*. In either case, the enriched fraction from cell suspension cultures (*A. thaliana*) or roots (*M. truncatula*), was analysed by 2-D electrophoresis. Subsequently, phosphoproteins were visualized by a phospho-specific protein dye and some of the dye reactive polypeptides were analysed by mass spectrometry to identify phosphorylation sites. To this end, in-gel digestion was performed with trypsin and the lysates were loaded onto IMAC micro-columns. After elution of the retained material, the phosphopeptides released were then analysed by MALDI-TOF and MALDI-TOF-TOF spectrometry. This integrated procedure allowed to identify phosphorylation sites in the two model plants.

2. Results and discussion

2.1. Affinity capture of phosphoproteins

Preliminary experiments showed that the use of the lysis buffer provided with the kit to extract the proteins, resulted in a low protein yield with both *A. thaliana* and *M. truncatula* samples. Therefore, the samples were extracted using procedures previously optimized for each plant material. After the extraction step, the protein samples were equilibrated in the lysis buffer using gel filtration prior to the enrichment step.

1-D gel analysis of the protein extract prepared under these conditions, before and after phosphoprotein enrichment, is reported in Fig. 1 for *M. truncatula*. The pattern of the Sypro Ruby-stained proteins in the crude extract (lane 1) and the flow-through (lane 2) were very similar. In contrast, the pattern of the retained fraction (lane 3),

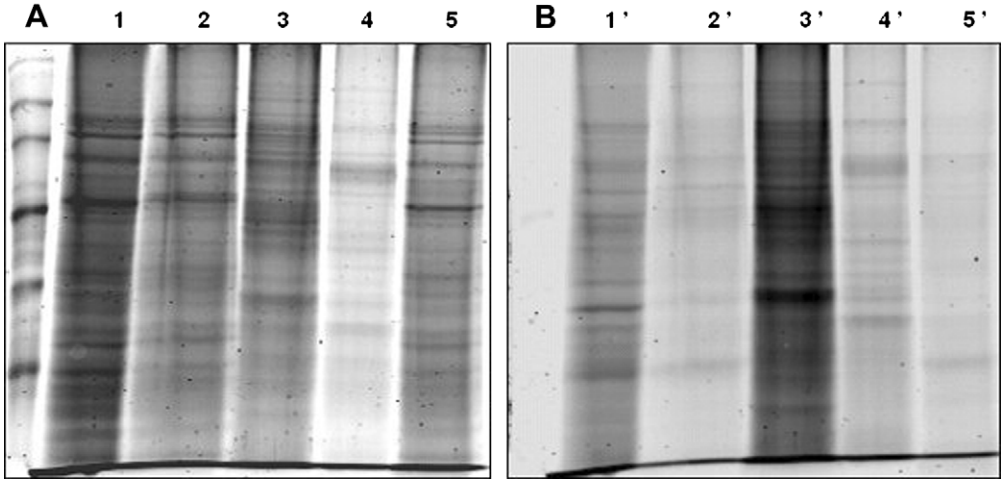

Fig. 1. SDS–PAGE analysis of *Medicago truncatula* roots proteins. 20 μg of crude extract (1), flow-through (2) and retained fraction (3) after phosphoprotein capture. Lanes 4 and 5 correspond to the retained fraction and the flow-through respectively, obtained after an incubation of the crude extract for 1 H at 30 °C. (A) The gel was stained with the general protein stain Sypro-Ruby. (B) Same lanes as A but the gel was stained with the phosphospecific dye Pro-Q Diamond.

that was selectively eluted by the elution buffer provided with the kit, differed sharply from the two other fractions. Some polypeptides that appeared as faint bands in either the crude extract or the non-retained fraction were clearly enriched in the retained fraction. Conversely, major polypeptides of the crude extract or the non-retained fraction were absent in the retained fraction. Similar results were obtained for the *Arabidopsis* extract (data not shown). Quantitatively, 5% of the total protein sample was recovered in the enriched fraction from either *M. truncatula* or *A. thaliana* preparations. Such yield fell in the range of previous data obtained on yeast and human cells using the same kit and the exact procedure recommended by the manufacturer (Metodiev et al., 2004). In our hands, using the adapted extraction step described above, these yields were only obtained when loading a maximum of 1.3 mg protein on the column; important looses were obtained when higher amounts of proteins were loaded.

In order to check if the affinity column discriminated phosphoproteins, the crude extract was dephosphorylated by incubating the extract at 30 °C for 1 H prior to phosphoenrichment. The pattern of the Sypro Ruby-stained proteins corresponding to the retained fraction (lane 4) and the flow-through (lane 5) are reported in Fig. 1. The profile of the flow-through is unchanged when compared to the corresponding fraction obtained from the same crude extract kept at 0 °C (lanes 5 and 2, respectively). In contrast, that of the retained fraction is dramatically modified quantitatively (only 40% of the initial protein content was captured by the column) and qualitatively (lane 4 vs. lane 3). The staining of the same gel with Pro-Q Diamond, a fluorescent dye reported to be specific for phosphoproteins, confirmed that (i) the retained fraction (lane 3′) vs. the crude extract (lane 1′) is enriched in dye sensitive polypeptides (ii) the flow-through (lane 2′) contains only traces of dye sensitive polypeptides. Moreover, when the crude extract was incubated at 30 °C for 1 H before enrichment, the number and/or the intensity of polypeptides stained by the Pro-Q Diamond in the retained fraction was lowered (lane 4′). Thus the major Pro-Q sensitive protein band (lane 3′) was almost absent in the retained fraction (lane 4′) after dephosphorylation of the extract. Dephosphorylation conditions did not affect the pattern of the flow-through (lanes 5 and 5′ vs. lanes 2 and 2′) indicating the absence of phosphatase susceptible polypeptides in this fraction and showing also that proteolysis did not occurred. Together, these results show that the enrichment step on the Qiagen column is efficient to specifically capture potentially phosphorylated proteins.

Interestingly, the dephosphorylation step has been performed by using the endogenous phosphatases. The endogenous activity was estimated to 1500 Units/ml (based on *p*-nitrophenyl phosphate hydrolysis). Use of exogenous phosphatases such as λ or alkaline phosphatases added to dephosphorylate the sample did not modify the velocity or the efficiency of the dephosphorylation process (data not shown). Because the dephosphorylation process was inhibited by 25 mM NaF, it was probably due to endogenous acid phosphatases. However, the presence of NaF in the extract was incompatible with the use of the phosphoenrichment column because it prevented the phosphoprotein capture (data not shown). This suggests that NaF interacts with the matrix by the same mechanism as the phosphoprotein. For practical use, working at 4 °C, even in the absence of acid phosphatase inhibitors during the phosphoenrichment step, gave a good recovery of phosphoprotein.

In order to have a better resolution of the selected phosphoproteome, the retained fraction was resolved by 2-D gel electrophoresis and the polypeptides were first stained with Pro-Q Diamond, and subsequently with Sypro Ruby. Because preliminary experiments showed that most of the proteins of the retained fraction focused in a range of rather acidic pH, the first dimension was performed on 4–7 immobiline strips to get the best resolution. The images of the gels stained with Pro-Q Diamond and Sypro Ruby protein stain are reported respectively in Fig. 2A and B for *M. truncatula* and Fig. 2C and D for *A. thaliana*. In both cases, more than 100 spots stained with the Pro-Q Diamond dye were detected using Melanie 3 image analysis software. The fluorescent dye did not stain the molecular weight standards, except the phosphorylated ovalbumin (45 kDa) used as a positive control, in agreement with the specificity assumed for this dye. The Pro-Q stained spots were also detected with Sypro Ruby dye. However, the staining intensity of some spots was stronger with Pro-Q Diamond than with Sypro Ruby stain. Considering the reported linearity ranges of responses for the two dyes, this could correspond to the presence of proteins of low abundance with a high number of phosphorylation sites. Furthermore, a minor proportion of proteins, not detected with the Pro-Q Diamond, was stained with Sypro Ruby suggesting that non-phosphorylated proteins were present in the retained fraction. Based on these observations and the recovery yield obtained, it can be assumed that the retained fraction is enriched in potentially phosphorylated proteins with a up to 15–20-fold enrichment factor. Moreover, after enrichment, the use of 2-D gels associated with a double staining procedure provides a more accurate visualization of the phosphoproteins pattern in comparison to previous studies using a 1-D gel analysis combined either to Coomassie blue or Pro-Q Diamond staining (Wolschin et al., 2005; Wolschin and Weckwerth, 2005). This procedure described in the present paper was reproducible since the analysis of more than 10 samples corresponding to independent experiments exhibited the same protein pattern.

2.2. Protein identification and phosphopeptides analysis

Spots stained both by the Pro-Q Diamond and the Sypro Ruby dyes were analysed by MALDI-TOF MS. Fifty proteins out of 108 were identified in the enriched fraction from *M. truncatula*. For *A. thaliana*, 19 proteins out of 28 analysed spots were identified. Most of the identified proteins, coexisting as multiple isoforms (visualized by spots sharing the same MW but differing in their p*I*),

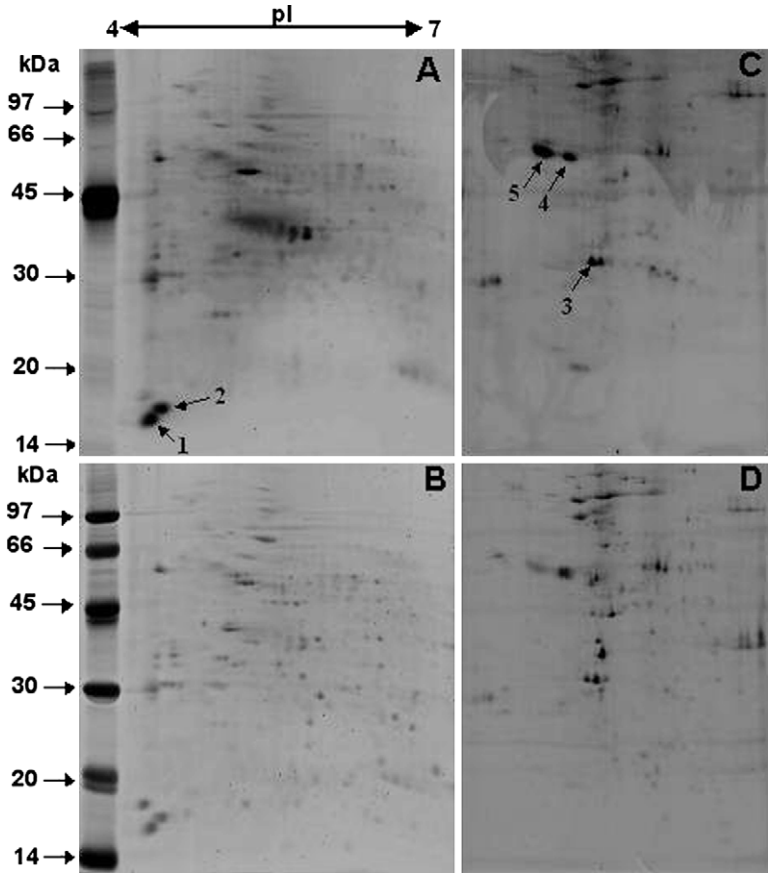

Fig. 2. 2-D gel analysis of *Medicago truncatula* (left panels) and *Arabidopsis thaliana* (right panels) proteins after selective enrichment. Proteins (50 μg) were applied to a 7 cm pH 4–7 IPG Strip for the first-dimension, followed by SDS–PAGE analysis. The gels were successively stained with Pro-Q Diamond (A, C) and Sypro Ruby (B, D).

were previously reported as phosphoproteins. In order to assess the capacity of the procedure to enable the characterization of phosphorylation sites, different spots were selected for further analysis. These included (i) well known phosphorylated proteins such as 60S acidic ribosomal proteins (spots 1–3) involved in the regulation of translation during various physiological processes (Freeman et al., 2002; Montoya-Garcia et al., 2002), (ii) a possibly phosphorylated protein such as a HSP associated protein like (spot 4) or a protein of unknown function (spot 5). In order to select phosphopeptides, the corresponding digests were analysed by IMAC using home-made micro-columns. IMAC resulted in phosphopeptide enrichment, although not all IMAC-retained peptides were phosphopeptides, largely because the well-known affinity of acidic peptides for this chromatographic media (not shown). Fig. 3 shows the results obtained for *M. truncatula* (MtC00735_1_AA) and *A. thaliana* (At5g39570/MIJ24_40) corresponding to a 60S acidic ribosomal protein P2 and a protein of unknown function, respectively. The upper panels show the MALDI-TOF MS spectra of the IMAC non-retained fraction. The peptide mass fingerprints of these fractions confirmed the identification of these proteins assessed from whole tryptic digests. Analyses of the IMAC retained fractions (middle panels) revealed the presence of ions, not detected in the non-retained fraction, and corresponding to predicted phosphopeptides. Moreover, the metastable loss of H_3PO_4 clearly detected for these ions, confirmed their phosphorylated status. Finally, the fragmentation spectra reported in the lower panels unambiguously identified the phosphorylation site on the phosphopeptides. The phosphorylation sites of the different analysed proteins are reported in Table 1. The two 60S acidic ribosomal proteins P2 of *M. truncatula*, MtC00735_1_AA and MtC0078_1_AA, contained the same phosphopeptide with or without 1 and 2 missed cleavages. The same phosphorylation site was located at the C-terminal end of a dodecapeptide highly conserved in eukaryotes. For the 60S acidic ribosomal protein P0-B of *A. thaliana* (At3g09200), the identified phosphorylation site also concerns the C-terminal end of the protein and, as suggested by Wolschin and Weckwerth (2005), could reflect a better accessibility of this region to digestion or enrichment. However, this is not a general rule because the phosphorylation site identified for the HSP associated protein like (At4g22670) is located in the N-terminal part of the protein. The analysis of At5g39570/MIJ24_40, a protein of unknown function, revealed also the presence of one phosphorylation site.

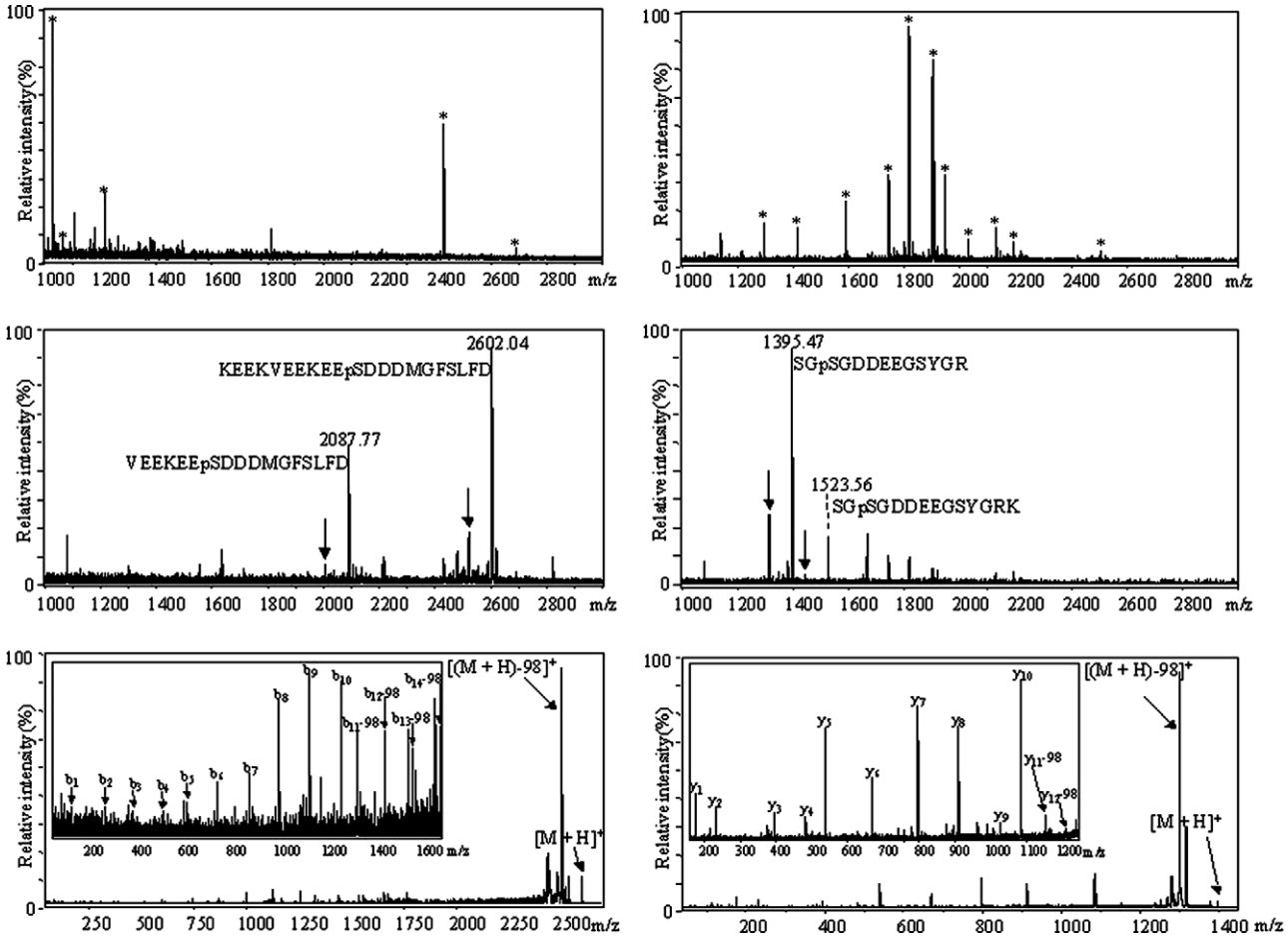

Fig. 3. Identification of phosphoproteins and phosphorylation sites. Top, peptide mass fingerprint from peptides (*) non-retained on IMAC microcolumns. Middle, IMAC-selected phosphopeptides and corresponding forms dephosphorylated upon metastable decomposition (arrow). Bottom, fragmentation spectra showing b and y ion series (insets). Left, *M. trunculata* 60S acidic ribosomal P2 protein (MtC00735_1_AA); right, *A. thaliana* expressed protein of unknown function (At5g39570/MIJ24_40).

Table 1
Characteristics of the phosphoproteins and of their phosphorylation sites isolated from *M. truncatula* and *A. thaliana*

Accession number and spot number	Protein identification	Theoretical		Observed		Phosphopeptide sequence	Position
		p*I*	M_r (kDa)	p*I*	M_r (kDa)		
MtC00078_1_AA(1)	60S Acidic ribosomal protein, P2	4.4	11.4	4.4	16	KVEEKEEpSDDDMGFSLFD	96–113
MtC00735_1_AA(2)	60S Acidic ribosomal protein, P2	4.7	15.7	4.5	18	VEEKEEpSDDDMGFSLFD-	133–149
						KEEKVEEKEEpSDDDMGFSLFD	129–149
At3g09200(3)	60S Acidic ribosomal protein, P0-B	4.7	34.1	5.1	32	VEEKEEpSDEEDYGGDFGLFDEE	299–320
At4g22670(4)	HSP associated protein like	4.6	46.6	4.9	58	VEEEEEEDEIVEpSDVELEGDTVEPDNDPPQK	77–107
At5g39570(5)	*A. thaliana* genomic DNA, chromosome 5, P1 clone:MIJ24	4.7	43.5	4.6	61	SGpSGDDEEGSYGR-	337–349
						SGpSGDDEEGSYGRK	337–350

3. Concluding remarks

In strategies where separation of proteins is necessary prior to phosphopeptide analysis, the proposed combination of phosphoprotein and phosphopeptide enrichment, together with the use of a phosphospecific protein dye, appears to constitute a robust approach to fish out phosphorylated proteins and identify their phosphorylation sites. The identification of a phosphopeptide in the protein of unknown function isolated from *A. thaliana* demonstrates the reliability of this approach. Furthermore, MALDI-TOF-TOF MS/MS enables to identify, in a fast and single step, the phosphoprotein (based on the peptide mass fingerprint of the IMAC non-retained fraction) and also the different phosphopeptides and their phosphorylation sites (based on the mass spectra of the IMAC retained fraction and subsequent ion fragmentation). Thus, this integrated procedure allows rapid characterization of soluble phosphoproteins in various plant materials. Despite the limits of 2-D gel analysis it could be useful to characterize

changes in phosphoproteome in response to an array of stimuli.

4. Experimental

4.1. Plant material and growth conditions

Seedlings of *M. truncatula* Gaertn. cv. Jemalong A17, were obtained as described by Sauviac et al. (2005). After a 4-day growth, the roots were harvested, transferred into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and kept at −80 °C until use. Cell suspension cultures of *A. thaliana*, were obtained as described by Chevalier et al. (2004). After a 7-day growth, the cells were filtered and frozen in liquid nitrogen and kept at −80 °C until use.

4.2. Protein extraction

4.2.1. Root extracts fractionation

Approximately 1 g of frozen *M. truncatula* roots was ground with a pestle in 1.5 ml Eppendorf tubes in the presence of liquid nitrogen. After thawing the resulting powder, proteins were extracted with 50 mM Tris–HCl buffer (pH 7) containing 0.1 mM EDTA, 5 mM DTT, 10 mM $MgCl_2$, 0.25% CHAPS, 2 μM leupeptine, 0.1 mM PMSF, 2 μM microcystine, and 250 units of benzonase (Qiagen). After centrifugation at 20,000*g* for 20 min at 4 °C, the collected supernatant corresponding to the soluble protein extract was used for further purification/enrichment steps.

4.2.2. Cell suspension fractionation

Approximately 1 g of *A. thaliana* frozen cells was ground in a mortar in the presence of liquid nitrogen. The fine powder was extracted with 50 mM HEPES pH 7.5, 1 mM *O*-vanadate, 10 mM NaF, 2 mM DTT, 12 μM Leupeptine, 1 mM PMSF and 0.1% phosphatase inhibitor cocktail 1 (Sigma) under stirring for 15 min. After centrifugation at 20,000*g* for 30 min at 4 °C, the supernatant containing the soluble proteins was immediately used for further purification/enrichment steps.

4.3. Phosphoprotein enrichment

Prior to phosphoprotein enrichment, the protein extracts were desalted on a PD-10 column (GE Healthcare) equilibrated in the lysis buffer provided with the PhosphoProtein Purification kit (Qiagen). Phosphoprotein enrichment was carried out according to the manufacturer's instructions with minor modifications. Approximately 1 mg of the equilibrated protein extract (300 μg per ml) was loaded on the Qiagen column. After washing with the equilibration buffer, the bound phosphoproteins were eluted with 2 ml elution buffer in four fractions of 0.5 ml each. Protein content of the fractions was quantified by the Bradford assay and the fractions containing proteins were pooled. The proteins were phenol extracted and precipitated with 10% ammonium acetate in 80% methanol according to Peck et al. (2001). After centrifugation (15 min, 20,000*g*), the pellet was washed with ice-cold 80% methanol and dried under reduced pressure.

4.4. 1-D and 2-D Gel electrophoresis

The dried pellet was re-suspended in 130 μl re-hydration solution as described by Chevalier et al. (2004) for *A. thaliana*, or consisting of 2% IPG buffer with 9 M urea, 1% CHAPS, 1% Triton X100 and 0.002% bromophenol blue for *M. truncatula*. The first dimension was run on 7 cm Immobiline DryStrips pH 4–7 (GE Healthcare). The focused proteins were then reduced with DTT, alkylated with iodoacetamide, and run on a 10% polyacrylamide SDS–PAGE as described by Chevalier et al. (2004). The same running conditions were used for 1-D gel electrophoresis. Phosphoproteins were stained with Pro-Q Diamond (Molecular Probes) according to the manufacturer procedure; total proteins were subsequently stained with Sypro Ruby dye (Molecular Probes). Images were acquired using fluorescence imagers (Typhoon 9400, GE Healthcare and FLA 5000, Fujifilm).

4.5. Mass spectrometry analysis

Spots of interest were excised from the gels and digested in-gel with trypsin according to Borderies et al. (2003). Peptides were desalted and concentrated through C18 ZipTipT (Millipore, Saint-Quentin en Yvelines, France) according to the manufacturer's instructions. Peptide mass mapping was performed on a MALDI TOF mass spectrometer (Voyager-DE STR, PerSeptive Biosystems, Framingham, MA, USA; Biflex III, Bruker, Germany) as previously described (Chevalier et al., 2004; Borderies et al., 2003). Protein identification was performed by mining a non-redundant protein sequence database (NCBI) or a local database (http://sequence.toulouse.inra.fr/Mtruncatula.html) using either MS-FIT (Protein Prospector, http://prospector.ucsf.edu) or Mascot (http://www.matrixscience.com) programs. The following criteria were used for database search: monoisotopic mass accuracy 20 ppm, carbamidomethylation of cysteine, maximum one missed cleavage allowed as fixed conditions, and oxidation of methionine, pyroglutamic acid as variable modifications. Peptide phosphorylation sites were characterized by MALDI-TOF-TOF MS/MS (Ultraflex 2, Bruker). Databases were queried using 0.5 Da mass accuracy in MS/MS and the same parameters as above but allowing Ser/Thr/Tyr-phosphorylation as variable modification and eventually a higher number of missed cleavages.

4.6. IMAC enrichment

Phosphopeptides were purified from tryptic peptide mixtures by nanoscale Fe^{3+} affinity chromatography according to Stensballe et al. (2001). Briefly, slurry of IMAC resin

was prepared using Ni^{2+}-NTA silica resin (Qiagen). The resin was washed successively, with 0.1 M EDTA and 0.2 M acetic acid, and subsequently charged with 0.1 M $FeCl_3$ in 0.1 M acetic acid. Excess Fe^{3+} was washed with 0.2 M acetic acid. Acidified tryptic digests of the phosphoproteins were loaded on customized nanoscale columns packed with the resin in the end of a GELoader tip (Eppendorf-Netheler-Hinz, Hamburg, Germany). The column was washed with 0.1 M acetic acid/acetonitrile (4/1, v/v). The tryptic phosphopeptides were eluted from the column with 2 μl DHB matrix [20 μg/μl matrix in 50/44/6 (v/v/v) acetonitrile/water/phosphoric acid] directly on the MALDI target.

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