Phosphoproteomics as a tool to unravel plant regulatory mechanisms

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Received 1 September 2005; revised 12 September 2005

Reversible phosphorylation of proteins plays a key role in many regulatory processes that lie at the basis of life. With plants, much research has focused on protein kinases that are involved in the adaptation to different stress conditions, such as pathogen attack and cold. However, the substrates of these kinases are mostly unknown. With the recent advances in phosphoproteomic techniques, the large-scale identification of kinase substrates, including their phosphorylation sites, is finally possible. Studies in mainly non-plant systems have demonstrated the high potential of this method by uncovering numerous novel phosphorylation events. In this minireview, we focus on recent developments in the field of phosphoproteomics that are based on phosphopeptide isolation from complex mixtures by immobilized metal-affinity chromatography coupled to sequence identification by mass spectrometry. Combination of these methods with labelling techniques now allows quantitative analysis of phosphorylation between different samples. We discuss the potential of this technology to uncover entire phosphoproteomes and signalling pathways in plants in the future.

Introduction

Reversible phosphorylation is one of the most important and diverse post-translational modifications of protein function. It can influence multiple characteristics of proteins, including the enzymatic activity, subcellular localization, protein–protein interaction network and half-life. As much as 30% of all proteins may be phosphorylated at any time (Hubbard and Cohen 1993), indicating that the phosphoproteome of each multicellular organism is immense. Numerous cellular signalling pathways are based on the sequential phosphorylation of an array of proteins. Therefore, the analysis of signalling pathways in plants has often focused on protein kinases. Most of these studies, however, described the phosphorylation of single substrates by a particular kinase. Approximately 1000 genes in the Arabidopsis

Abbreviations – ABA, abscisic acid; CDPK, calcium-dependent protein kinase; CID, collision-induced dissociation; EGFR, epidermal growth factor receptor; IMAC, immobilized metal-affinity chromatography; LC-MS, liquid chromatography-mass spectrometry; MAPK, mitogen-activated protein kinase; MS, mass spectrometry; RLK, receptor-like kinase; SAX, strong anionic exchange; SCX, strong cationic exchange; SILAC, stable isotope labelling by amino acids in cell culture; SPS, sucrose–phosphate synthase.

110 Physiol. Plant. 126, 2006 Copyright © Physiologia Plantarum 2006, ISSN 0031-9317
Reversible phosphorylation in cold stress signalling

Calcium influx and the hormone abscisic acid (ABA) cause the activation of MAPKs in plants. The MAPKKs MKK1 and MKK2 are critical in this process, with MKK2 being activated by protein Ser/Thr. A. thaliana dehydrin ERD14 itself is also phosphorylated, and its binding affinity for calcium is increased upon cold treatment. The phosphorylation of rice calreticulin and maize ribosome somal protein S6 is increased upon cold treatment.

Reversible phosphorylation in plant defence signalling

The transcription of several protein kinases and phosphatases is upregulated during cold stress. Several families of protein kinases and phosphatases are induced by cold stress, and their genes are essential factors in cold signalling as well as other stresses. The involvement of phosphorylation in cold signalling has been clearly established by the role of AKIP1, a heterogenous nuclear RNA-binding protein. Phosphorylation of AKIP1 increases its affinity for dehydrin mRNA, and this binding stabilizes the mRNA, providing a mechanism for the regulation of protein level of dehydrin.

Moreover, the kinase complement of A. thaliana is more complex than that of humans, which consists of about 500 genes. Many plant genomes lack clearly recognizable tyrosine kinases, which is an intriguing difference with animal genomes. The absence of tyrosine kinases in plant genomes is consistent with the lack of tyrosine phosphorylation events in plant ontogeny, and among the early targets are two cytoskeleton destabilization and increased membrane rigidity and depends on calcium. Pathogen-derived elicitors induce many phosphorylation events, and among the early targets are two cyto- and plasma membrane syntaxin.

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A few successful studies have described the isolation of phosphopeptides from complex mixtures with strong enrichment strategies for mass spectrometry-based phosphoproteomic analysis.

Enrichment strategies for mass spectrometry-based phosphoproteomic analysis

Approaches to study phosphorylation events in plants

A. thaliana.

Enrichment strategies for mass spectrometry-based phosphoproteomic analysis

Approaches to study phosphorylation events in plants
Global identification of in vivo phosphorylation sites using IMAC coupled to mass spectrometric technology.

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phosphopeptides. Of these phosphopeptides, 79% was singly phosphorylated, 19% was doubly phosphorylated and 2% was triply phosphorylated. The functional classification of all the 151 phosphoproteins identified so far in our studies is shown in Fig. 3.

It has been stated that prefractionation (with for instance SAX) is required for the efficient recovery of monophosphorylated peptides from complex mixtures when using IMAC (Nühse et al. 2003a). However, we recover a high percentage of these phosphopeptides without any further prefractionation of the complex cytosolic mixture. Nonetheless, more elaborate prefractionation will be required for more complete coverage of phosphorylation sites and efficient identification of the lower abundant phosphopeptides. In our analysis of the intracellular phosphoproteome, we identified several described phosphosites of abundant metabolic enzymes and ribosomal proteins, which represent main groups among the phosphoproteins (Fig. 3). For instance, we determined a conserved phosphorylation site in sucrose–phosphate synthase (SPS) in two Arabidopsis thaliana isoforms that is analogous to the known pSer-158 (lower case p indicates phosphorylated residue) of spinach SPS (Fig. 4). In addition to this pSer, we identified several novel phosphorylation sites (Fig. 4). A major target of phosphorylation is represented by the mRNA splicing machinery (S. de la Fuente van Bentem, D. Anrather, E. Roitinger, D. Lecourieux and H. Hirt, manuscript in preparation). In addition to abundant phosphoproteins, also phosphorylation sites of low abundant signalling proteins were detected in our studies.

**IMAC-based quantitative phosphoproteomics as a tool to elucidate plant signalling pathways**


A recently developed technique to measure differences between proteins in two distinct samples by MS is Stable Isotope Labelling by Amino acids in Cell culture (SILAC; Ong et al. 2002). This in vivo method is based on the feeding of separate cultures with distinct isotopically labelled amino acids, which can later be differentiated during MS analysis. SILAC has been used as an effective tool to measure relative phosphorylation differences of proteins extracted from cell cultures grown under different conditions. SILAC followed by IMAC and tandem MS has been used for differential phosphorylation profiling of human and yeast-signalling pathways (Blagoev et al. 2004, Gruhler et al. 2005a, Kratchmarova et al. 2005). SILAC has been also successfully applied to Arabidopsis thaliana for quantitative proteomics and may therefore be used for quantitative analysis of the phosphoproteome during plant signalling (Gruhler et al. 2005b). A drawback of SILAC is that only phosphopeptides containing the labelled amino acid can be considered in the analysis. Fortunately, Arabidopsis thaliana, labelling was most efficient with 13C6-Arg (Gruhler et al. 2005b), which can be used conveniently in combination with trypsin digestion since this protease cleaves after basic amino acids.

An alternative approach to determine relative differences between different samples is the in vitro labelling of peptides before or after isolation from a complex mixture. Peptides can be labelled during esterification with deuterated methanol before IMAC. However, deuterated peptides appear to behave differently in the LC

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**Fig. 1.** Schematic representation of an experimental setup of phosphoproteomic studies on Arabidopsis thaliana. A. thaliana root cell suspensions were isolated by centrifugation and ground in liquid nitrogen to yield total extracts. Several intermediate steps from the protocol are omitted for simplicity. IMAC, immobilized metal-affinity chromatography; LC-MS, liquid chromatography-mass spectrometry.
run than the corresponding non-deuterated derivative (Chen and White 2004). The development of a multiplex set of reagents allows the incorporation of mass labels at the N-termini and lysine side chains of peptides in a digest mixture (Ross et al. 2004). Using four isoforms of this so-called iTRAQ reagent, Zhang et al. (2005) gave the first insights into the dynamic changes of phosphorylation profiles during EGFR tyrosine kinase signalling. Regarding in vitro peptide labelling methods, the success largely depends on the incorporation efficiency, and mass spectrometers with high mass accuracy are required for the relative quantification of phosphopeptide differences. However, compared with in vivo labelling methods, this requires additional handling time.

Conclusions and future prospects

The current MS-based phosphoproteomic technology has established itself as an invaluable tool in the

![Fig. 2. Specificity of phosphopeptide isolation by Fe$^{3+}$-immobilized metal-affinity chromatography (IMAC). Base peak chromatograms of full mass spectrometry scans of different IMAC fractions. IMAC load, flow-through and eluate fractions were separated by nano-reversed phase (C18) high-performance liquid chromatography applying a gradient of 2.5–40% acetonitrile in 0.1% formic acid coupled to a Finnigan LTQ quadrupole linear ion trap mass spectrometer. Three hundred micrograms of an Arabidopsis thaliana cytosolic protein extract was used for phosphopeptide isolation. Upper panel: chromatogram of the complex peptide mixture (0.1 μg) after trypsin digestion, showing a range of abundant peptides. Middle panel: flow-through of the IMAC material (0.1 μg), which contains virtually all abundant peptides. Lower panel: bound phosphopeptide fraction (from 300 μg of the starting mixture) that was eluted from the IMAC resin by phosphate buffer. The amounts that were used for analysis are indicated in each panel. Estimated from the relative abundance, isolated phosphopeptides represent only about 1/1000 of the total peptide fraction. NIU, normalized intensity units.](image)

![Fig. 3. Functional classification of intracellular phosphoproteins identified in Arabidopsis thaliana. Phosphopeptides belonging to 151 phosphoproteins were determined in our analyses of both nuclear and cytosolic extracts of A. thaliana cells (Fig. 1). Assignment of phosphoproteins to different classes is based on both their functional domains as predicted by the SMART database and database hits.](image)
Identification at novel phosphorylation sites. Future improvements of this technique will be used to decipher the global phosphoproteome and ultimately the dynamic behaviour of the complete phosphoproteome in plants. The pre-purification of single organelles or prefractionation of complex mixtures by, for instance, SAX/SCX will increase the coverage of the phosphoproteome in each study. The use of a protease panel to extend the sequence coverage of the phosphoproteome is a simple and efficient method (Rush et al. 2005). Automated and sensitive phosphorylation site mapping by the esterification-IMAC procedure combined with LC-MS will enhance the identification of complete phosphoproteomes (Ficarro et al. 2005). The recent development of specific labelling techniques greatly aids the quantification of phosphorylation profiles and their stress-induced changes in time. Especially iTRAQ labelling and SILAC have shown to be successful in combination with IMAC and MS. These studies will reveal hints at novel signalling pathways and regulatory processes that are dependent on phosphorylation.

Although the scale of studies on signalling cascades is increasing rapidly, improvements are required to generate entire signalling webs. This will require better software for both the automated identification of phosphopeptide sequences by MS and efficient structuring of the wealth of data becoming available. The development of a mass spectrometer with a high mass accuracy and a suitable method for phosphopeptide fragmentation will advance global analysis of signalling pathways. Promising fragmentation techniques such as electron-transfer dissociation, which produces a high degree of sequence information of phosphopeptides (Syka et al. 2004), will have to prove their applicability for large-scale phosphopeptide analysis. These phosphoproteomic studies will induce future experiments to determine the function of the phosphorylation sites.

In summary, future studies aiming at global phosphoproteomics will greatly benefit from the recent developments in the field of MS-based technology. As discussed here, these methods are becoming equally applicable for plant studies. The rapidly increasing methodology for quantitative phosphoproteomics is about to revolutionize our conceptual understanding of plant biology and should uncover many unexpected links within the signalling network in plants.

Acknowledgements – This work was supported by grants from the Austrian Science Foundation, the Vienna Science and Technology Fund and the European Union.

References


Fig. 4. In vivo phosphorylation sites of sucrose–phosphate synthase isoforms. Structural representation of At5g20280 with positioned phosphorylation sites. The SPS isoform contains a group 1 glycosyl transferase domain. The two regions surrounding the phosphorylation sites of all four *Arabidopsis thaliana* SPS isoforms and spinach (*Spinacia oleracea*) SPS are aligned. Arrows indicate phosphorylation sites, and pSer residues are in bold lettering. The asterisk indicates the pSer residue that is analogous to the known pSer-158 of spinach SPS (SoSPS).
MAP protein phosphorylation sites in – a mass spectrometry approach.


Arabidopsis thaliana in vivo


Arabidopsis thaliana


Arabidopsis thaliana


Arabidopsis thaliana


Arabidopsis thaliana


Arabidopsis thaliana


Arabidopsis thaliana


plasma membrane


Edited by C. Guy

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