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REVIEW

Phosphoproteomics as a tool to unravel plant regulatory mechanisms

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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.

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thaliana genome are predicted to encode protein kinases (The Arabidopsis Genome Initiative 2000). An intriguing difference with animal genomes is the absence of clearly recognizable tyrosine kinases. Moreover, the kinase complement of *A. thaliana* is more complex than that of humans, which consists of about 500 genes (Manning et al. 2002). Many plant protein kinases have been identified that are crucial in resistance to a variety of stresses including cold stress and pathogen invasion. Determination of the molecular events occurring during adaptation to stresses will enhance our understanding of biological processes in plants.

The identification of phosphorylation sites has been technically very difficult in the past. Technological advances on the basis of phosphopeptide isolation by immobilized metal-affinity chromatography (IMAC) followed by tandem mass spectrometry [MS/MS (MS2) or MS/MS/MS (MS3)] have emerged as a novel tool to determine phosphorylation sites in yeast, animals and plants (Chen and White 2004, Laugesen et al. 2004). Contrasting with the rapid expansion of large-scale phosphoproteomic studies performed on yeast and animals, similar work on plants is still limited to a few studies (Heintz et al. 2004, Glinski and Weckwerth 2005, Nühse et al. 2003a). In this minireview, we briefly describe the role of phosphorylation in plant stress illustrated by examples of abiotic and biotic stresses: cold and pathogen stress. The remaining part focuses on the recent developments in the field of phosphoproteomics and its promise to unravel plant phosphoproteomes and their dynamics during stress.

Reversible phosphorylation in cold stress signalling

The transcripts of several protein kinases and phosphatases are upregulated during cold stress (Kreps et al. 2002, Seki et al. 2002), and thus adaptation to this stress may be a concerted action of different members of these families. The involvement of phosphorylation in cold signalling has been clearly established by the role of protein kinases and phosphatases in this stress. Several rice calcium-dependent protein kinases are induced by low temperature (Abbasi et al. 2004, Martin and Busconi 2001, Saijo et al. 2000) and overexpression of their genes enhances cold tolerance (Abbasi et al. 2004, Saijo et al. 2000).

Calcium influx and the hormone abscisic acid (ABA) are essential factors in cold signalling as well as other signalling cascades. The *A. thaliana* protein Ser/Thr kinase AtCIPK3 is transcriptionally activated by different stresses and is involved in the downstream activation of cold-, hyperosmotic- and ABA-related genes (Kim et al. 2003). AtCIPK3 might be activated by cold-induced calcium levels through interaction with a calcium calcineurin B-like calcium sensor (Kim et al. 2003). The ABA-activated protein kinase (AAPK) phosphorylates the AAPK-interacting protein 1 (AKIP1), a heterogeneous nuclear RNA-binding protein. Phosphorylation of AKIP1 increases its affinity for dehydrin mRNA, and the binding stabilizes the mRNA, providing a mechanism of regulating protein level of dehydrin (Li et al. 2002). Dehydrins are implicated in defence against stresses. The *A. thaliana* dehydrin ERD14 itself is also phosphorylated upon cold treatment, which increases its binding affinity for calcium (Alsheikh et al. 2003). The phosphorylation of rice calreticulin and maize ribosomal protein S6 is increased upon cold treatment (Li et al. 2003, Williams et al. 2003), and are thus also substrates of the phosphorylation cascade activated by cold. In contrast to the positive role of kinases in cold signalling, the C-terminal domain phosphatase-like AtCPL1 negatively regulates cold-induced gene expression (Koiwa et al. 2002).

A common feature of plant stress-induced pathways is the signalling through so-called mitogen-activated protein kinase (MAPK) modules (Nakagami et al. 2005). They consist of MAPK kinase kinases (MAPKKKs) that phosphorylate and thereby activate MAPK kinases (MAPKKs) that in their turn phosphorylate and activate MAPKs. Recently, the MAPKK MKK2 has been recognized as an important mediator of cold stress signalling in *A. thaliana* (Teige et al. 2004). MKK2 is activated by cold and several other stresses, and overexpressor and knock-out lines show enhanced and diminished tolerance to cold treatment, respectively. Also MKK1 is activated by cold stress (Matsuoka et al. 2002). The stress-activated MAPK, SAMK is activated within 10 min after cold (and other stress) treatment (Jonak et al. 1996). The induction of cold-activated gene expression is mediated by cytoskeleton destabilization and increased membrane rigidity and depends on calcium (Sangwan et al. 2001). Strikingly, all these factors are required for cold-induced SAMK activity (Sangwan et al. 2002). The SAMK pathway is repressed by the alfalfa MAPK phosphatase 2C (Meskiene et al. 1998). Altogether, these findings show that MAPK signalling plays an important role in cold stress adaptation in plants.

Reversible phosphorylation in plant defence signalling

Pathogen-derived elicitors induce many phosphorylation events, and among the early targets are two cytosolic proteins and a plasma membrane syntaxin (Nühse

et al. 2003b, Peck et al. 2001). Candidate kinases are for instance calcium-dependent protein kinases, which are rapidly activated upon specific activation of defence responses (Romeis et al. 1999). Pto, which is an intracellular protein Ser/Thr kinase involved in resistance against bacterial speck disease, interacts with and phosphorylates both the protein Ser/Thr kinase Pti1 and the transcription factor Pti4 (Martin et al. 2003). Phosphorylation of Pti4 increases its binding to promoter elements of genes that are activated during disease-resistance responses.

As general stress regulators, MAPK pathways are also involved in defence responses in plants. Complete MAPK cascades have been identified in *A. thaliana*, tomato and tobacco, downstream of pathogen receptors (Nakagami et al. 2005). These MAPK modules act as positive regulators of signalling. In contrast, components of MAPK modules can also be negative regulators of defence responses (Nakagami et al. 2005). The recently discovered protein kinase OXI1 is an upstream activator of MAPKs and is essential for basal resistance against virulent pathogens (Rentel et al. 2004).

Besides protein phosphorylation, also protein dephosphorylation is important during defence signalling. A protein Ser/Thr phosphatase inhibitor mimics part of the response to pathogen-derived elicitors (Felix et al. 1994), suggesting that during the absence of pathogens, phosphatases may dephosphorylate positive regulators of defence responses. A genetic approach showed that protein Ser/Thr phosphatase 2A (PP2A) acts as a negative regulator of defence responses (He et al. 2004). Protein phosphatases are also involved in downstream signalling by the receptor-like kinase (RLK) FLS2 (Asai et al. 2002, Gómez-Gómez and Boller 2000). In conclusion, evidence is accumulating for the involvement of reversible protein phosphorylation in plant disease resistance.

Approaches to study phosphorylation events in plants

Although many plant protein kinases have been shown to be involved in signalling cascades, the number of their substrates that have been identified is still limited. For instance, although MAPKs are activated in response to numerous stress conditions, only two studies have described the identification of their in vivo substrates in plants (Andreasson et al. 2005, Liu and Zhang 2004). In addition, laborious site-directed mutagenesis of potential phosphorylation sites has often been used to determine the targeted sites within each substrate. More global studies are clearly needed to fully understand plant-signalling pathways. Recently, a protein array-based study has suggested a set of potential MAPK substrates (Feilner et al. 2005). Although the potential of this technique is evident, it still requires in vivo studies to verify the findings as well as to identify the phosphorylation sites.

Focused analyses of the roles of specific kinases and substrates have given a fragmented picture of plant-signalling pathways. As a global approach, a novel field has emerged that has the potential to speed up the unravelling of phosphorylation-dependent processes: phosphoproteomics. In spite of its technical drawbacks (for instance, the need of a fully sequenced genome, high equipment costs and high expertise required), phosphoproteomics has already firmly established itself as a method to uncover phosphorylation sites on hundreds of proteins in yeast, animals and *A. thaliana*.

Enrichment strategies for mass spectrometry-based phosphoproteomic analysis

Several problems are encountered during the examination of phosphopeptides by MS (Mann et al. 2002). Most importantly, phosphoproteins are generally of extremely low abundance within the proteome. These features have prompted the development of phosphopeptide (or phosphoprotein)-purification methods from complex mixtures. Several methods are based on the chemical substitution of the phosphate moiety of phosphopeptides by a stable group that can then be used to specifically purify the peptide (Chen and White 2004, Laugesen et al. 2004). Affinity purification of phosphoproteins with phospho-specific antibodies prior to MS has been tested in several studies. Although success with anti-phosphoserine/threonine antibodies is limited (Grønborg et al. 2002), antiphosphotyrosine antibodies are suitable for specific affinity purification as illustrated by many studies. Because of the low occurrence of tyrosine phosphorylation in animal cells (about 0.05% against more than 99% on serine and threonine residues), phosphotyrosine-containing peptides/proteins need to be specifically purified from complex mixtures. Immunoprecipitation with antiphosphotyrosine antibodies coupled to liquid chromatography-mass spectrometry (LC-MS) identified 57 (Ficarro et al. 2005), 64 (Salomon et al. 2003), 66 (Brill et al. 2004), 104 (Zhang et al. 2005) and 628 phosphotyrosine sites (Rush et al. 2005). In plants, such a study has never been performed, but a few studies have suggested the occurrence of tyrosine phosphorylation (for instance of MAPKs). Whether tyrosine phosphorylation is more abundant than assumed in plants remains to be shown.

A few successful studies have described the isolation of phosphopeptides from complex mixtures with strong

cationic exchange (SCX) chromatography (Ballif et al. 2004, Beausoleil et al. 2004) or strong anionic exchange (SAX) chromatography followed by IMAC (Nühse et al. 2003a). Coupling these techniques to MS-based peptide identification proved to be very successful. SCX was used to identify spectacular numbers of >500 and 2002 phosphosites from mammalian cells. However, a drawback of SAX or SCX is the failure to efficiently capture multiply phosphorylated peptides (Beausoleil et al. 2004, Nühse et al. 2003a).

As a phosphopeptide-purification method, IMAC has already been used for two decades (Andersson and Porath 1986). IMAC is based on the high affinity of trivalent metal ions (Fe^{3+} is mostly used) for phosphate groups. However, only recently, IMAC has been successfully used in large-scale phosphoproteomic analysis, leading to the identification of numerous phosphorylation sites (Ficarro et al. 2002). An important improvement to the protocol added by Ficarro et al. (2002) was the conversion of carboxylic acid groups to methyl esters prior to IMAC purification of peptides. This step avoids the non-specific binding of peptides containing acidic residues to the IMAC material. Thus, the addition of this step greatly enhanced the specificity for phosphorylated over non-phosphorylated peptides (Brill et al. 2004, Ficarro et al. 2002), although this chemical modification may not be a prerequisite for high specificity (Nühse et al. 2003a). In our hands, the purity increased substantially by introducing the esterification step. In conclusion, IMAC promises to be a valuable tool to specifically isolate phosphopeptides from complex mixtures and recent studies fruitfully described the use of this method for the large-scale analysis of phosphorylation sites.

Global identification of in vivo phosphorylation sites using IMAC coupled to mass spectrometric technology

Ficarro et al. (2002) successfully implemented enrichment of phosphopeptides using IMAC from yeast whole-cell protein extracts that have been digested with trypsin. Coupling of esterification-IMAC to subsequent identification of peptide sequences by tandem MS allowed the identification of 383 phosphorylation sites in yeast peptides (Ficarro et al. 2002) and 238 sites in human peptides (Kim et al. 2005). The power of the combined use of IMAC and MS for plant work has been demonstrated by the large-scale analysis of phosphopeptides from *A. thaliana* plasma membrane extracts (Nühse et al. 2004). This first plant global phosphoproteomic study identified more than 300 phosphorylation sites, of which only a few were known. In a study of the phosphoproteome of the moss *Physcomitrella patens*, 253 phosphopeptides were detected during the esterification-IMAC-MS approach, but the sequence of only a few could be determined (Heintz et al. 2004). In a small-scale study of *A. thaliana* thylakoid membrane-associated proteins, Hansson and Vener (2003) identified several novel phosphorylation sites using the same approach. The work of Nühse et al. (2004) provided the first insights into the nature of protein phosphorylation in plants. For instance, the extensive phosphorylation of RLKs showed complex regulation of these proteins, suggesting that phosphorylation sites may be key determinants of signalling specificity of these receptors. The analysis of plasma membrane phosphoproteins also showed that regions surrounding the phosphorylation sites can often be grouped into conserved phosphorylation motifs that may be targeted by similar kinases. Such analyses will accelerate the identification of the responsible kinase targeting the subset of phosphorylation sites determined in studies aiming at the identification of the total phosphoproteome.

We have adapted the technology as described by Ficarro et al. (2002) to identify in vivo phosphorylation sites of intracellular *A. thaliana* proteins. Our experimental setup consisted of the rough separation of intracellular proteins into a cytosolic and a nuclear fraction (Fig. 1). The aim was to unravel the phosphoproteome within these extracts. In a complex mixture containing up to 300 μg of protein, polypeptides were cleaved with trypsin and esterified. Subsequently, phosphopeptides were isolated by Fe^{3+}-IMAC. In Fig. 2, the efficiency of phosphopeptide isolation from a complex mixture by IMAC is depicted. It is clear that most (abundant) peptides are not captured by IMAC, but only the low abundant phosphopeptide fraction. Sequence information was provided by fragmentation of each peptide by the collision-induced dissociation (CID) on a quadrupole linear ion trap mass spectrometer (Finnigan LTQ), yielding tandem MS (MS/MS) spectra. In addition to the MS/MS spectra, the Finnigan LTQ enables a data-dependent third stage of MS (MS/MS/MS; specifically of peptides that are selected when a loss of phosphate is observed during MS/MS), leading to spectra with more sequence-specific peptide-fragmentation patterns. This is an advantage since phosphopeptides generally give poor-quality fragmentation patterns when fragmented by CID. To circumvent this problem, phosphopeptides can be dephosphorylated before MS analysis. However, this leads to loss of information about the location and amount of phosphorylation sites. Each phosphopeptide fragmentation spectrum has to be manually verified, which is often labour intensive. From MS/MS and MS/MS/MS spectra obtained in a single run, we could make confident assignments for more than 130

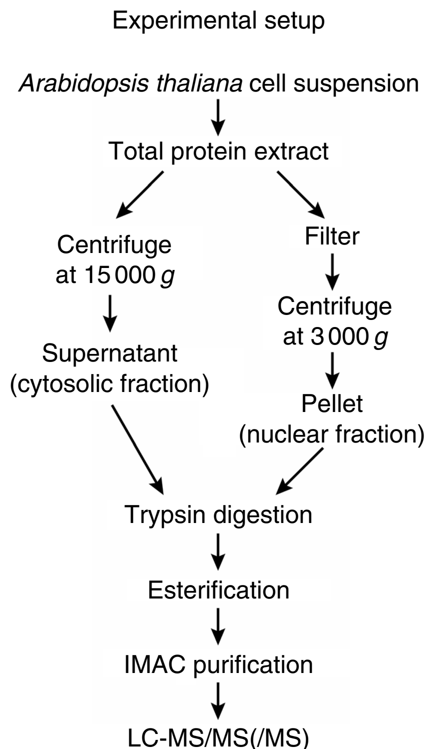

Fig. 1. Schematic representation of an experimental setup of phosphoproteomic studies on *Arabidopsis thaliana*. *A. thaliana* root cell suspensions were isolated by centrifugation and ground in liquid nitrogen to yield total extracts. Several intermediate steps from the protocol are omitted for simplicity. IMAC, immobilized metal-affinity chromatography; LC-MS, liquid chromatography-mass spectrometry.

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 *A. 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

Recent developments in MS-based phosphoproteomics promised to be an excellent instrument to study dynamic phosphoprotein profiling of signalling networks. In animal and yeast systems, several such studies have already been performed and identified many novel stress-induced phosphorylation events (Ballif et al. 2005, Blagoev et al. 2004, Cutillas et al. 2005, Gruhler et al. 2005a, Ibarrola et al. 2004, Kratchmarova et al. 2005, Zhang et al. 2005).

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 *A. 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, *A. thaliana*, labelling was most efficient with $^{13}C_6$-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

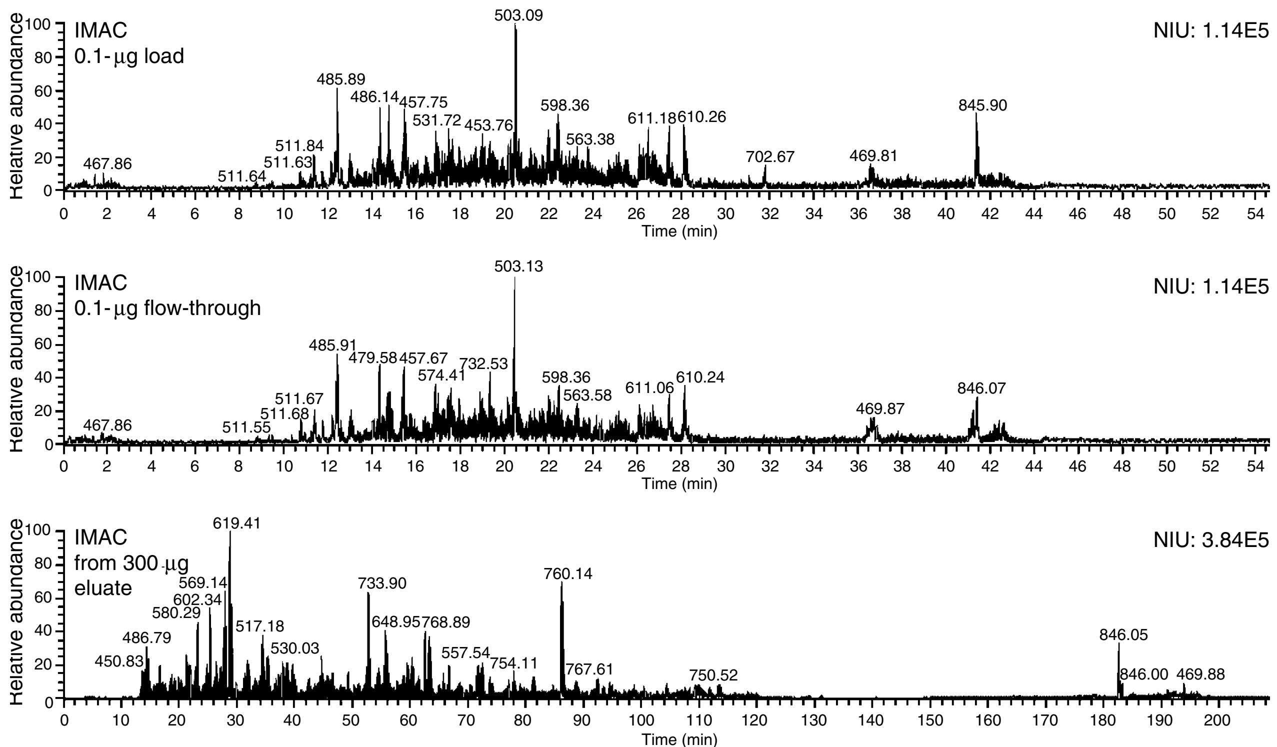

Fig. 2. Specificity of phosphopeptide isolation by Fe^{3+}-immobilized metal-affinity chromatography (IMAC). Base peak chromatograms of full mass spectrometry scans of different IMAC fractions. IMAC load, flow-through and eluate fractions were separated by nano-reversed phase (C18) high-performance liquid 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.

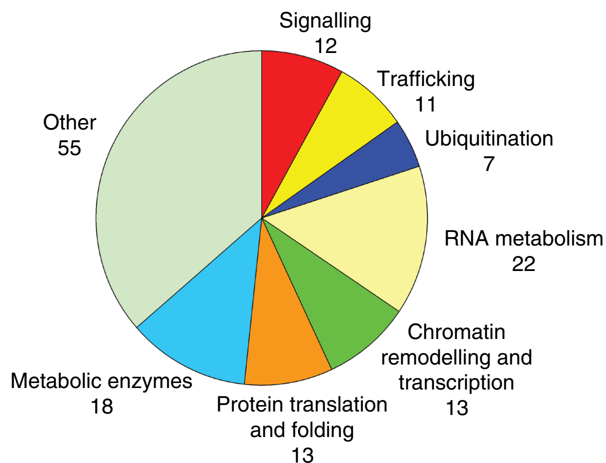

Fig. 3. Functional classification of intracellular phosphoproteins identified in *Arabidopsis thaliana*. Phosphopeptides belonging to 151 phosphoproteins were determined in our analyses of both nuclear and cytosolic extracts of *A. thaliana* cells (Fig. 1). Assignment of phosphoproteins to different classes is based on both their functional domains as predicted by the SMART database and database hits.

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

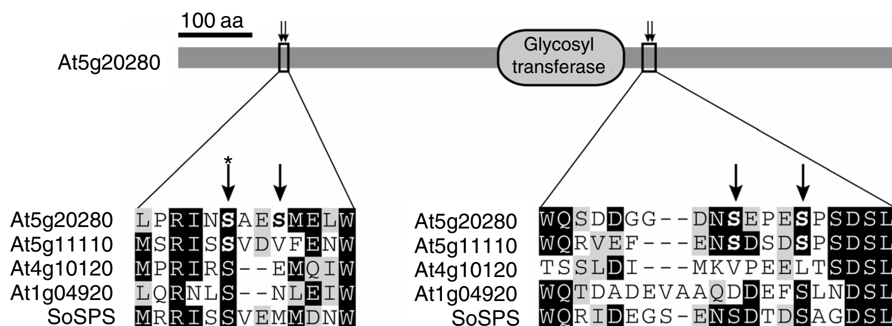

Fig. 4. In vivo phosphorylation sites of sucrose–phosphate synthase isoforms. Structural representation of At5g20280 with positioned phosphorylation sites. The SPS isoform contains a group 1 glycosyl transferase domain. The two regions surrounding the phosphorylation sites of all four *Arabidopsis thaliana* 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).

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.

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References

Abbasi F, Onodera H, Toki S, Tanaka H, Komatsu S (2004) OsCDPK13, a calcium-dependent protein kinase gene from rice, is induced by cold and gibberellin in rice leaf sheath. Plant Mol Biol 55: 541–552

Alsheikh MK, Heyen BJ, Randall SK (2003) Ion binding properties of the dehydrin ERD14 are dependent upon phosphorylation. J Biol Chem 278: 40882–40889

Andersson L, Porath J (1986) Isolation of phosphoproteins by immobilized metal (Fe^{3+}) affinity chromatography. Anal Biochem 154: 250–254

Andreasson E, Jenkins T, Brodersen P, Thorgrimsen S, Petersen NH, Zhu S, Qiu JL, Micheelsen P, Rocher A, Petersen M, Newman MA, Bjorn Nielsen H, Hirt H, Somssich I, Mattsson O, Mundy J (2005) The MAP kinase substrate MKS1 is a regulator of plant defense responses. EMBO J 24: 2579–2589

Asai T, Tena G, Plotnikova J, Willmann MR, Chiu WL, Gomez-Gomez L, Boller T, Ausubel FM, Sheen J (2002) MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature 415: 977–983

Ballif BA, Villen J, Beausoleil SA, Schwartz D, Gygi SP (2004) Phosphoproteomic analysis of the developing mouse brain. Mol Cell Proteomics 3: 1093–1101

Ballif BA, Roux PP, Gerber SA, MacKeigan JP, Blenis J, Gygi SP (2005) Quantitative phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling cassette and its targets, the tuberous sclerosis tumor suppressors. Proc Natl Acad Sci USA 102: 667–672

Beausoleil SA, Jedrychowski M, Schwartz D, Elias JE, Villen J, Li J, Cohn MA, Cantley LC, Gygi SP (2004) Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc Natl Acad Sci USA 101: 12130–12135

Blagoev B, Ong SE, Kratchmarova I, Mann M (2004) Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. Nat Biotechnol 22: 1139–1145

Brill LM, Salomon AR, Ficarro SB, Mukherji M, Stettler-Gill M, Peters EC (2004) Robust phosphoproteomic profiling of tyrosine phosphorylation sites from human T cells using immobilized metal affinity chromatography and tandem mass spectrometry. Anal Chem 76: 2763–2772

Chen WG, White FM (2004) Proteomic analysis of cellular signaling. Expert Rev Proteomics 1: 343–354

Cutillas PR, Geering B, Waterfield MD, Vanhaesebroeck B (2005) Quantification of gel-separated proteins and their phosphorylation sites by LC-MS using unlabeled internal standards: analysis of phosphoprotein dynamics in a B cell lymphoma cell line. Mol Cell Proteomics 4: 1038–1051

Feilner T, Hultschig C, Lee J, Meyer S, Immink RG, Koenig A, Possling A, Seitz H, Beveridge A, Scheel D, Cahill DJ, Lehrach H, Kreutzberger J, Kersten B (2005) High-throughput identification of potential *Arabidopsis* MAP kinases substrates. Mol Cell Proteomics 4: 1558–1568

Felix G, Regenass M, Spanu P, Boller T (1994) The protein phosphatase inhibitor calyculin A mimics elicitor action in plant cells and induces rapid hyperphosphorylation of specific proteins as revealed by pulse labeling with [^{33}P]phosphate. Proc Natl Acad Sci USA 91: 952–956

Ficarro SB, McCleland ML, Stukenberg PT, Burke DJ, Ross MM, Shabanowitz J, Hunt DF, White FM (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. Nat Biotechnol 20: 301–305

Ficarro SB, Salomon AR, Brill LM, Mason DE, Stettler-Gill M, Brock A, Peters EC (2005) Automated immobilized metal affinity chromatography/nano-liquid chromatography/electrospray ionization mass spectrometry platform for profiling protein phosphorylation sites. Rapid Commun Mass Spectrom 19: 57–71

Glinski M, Weckwerth W (2005) Differential multisite phosphorylation of the trehalose-6-phosphate synthase gene family in *Arabidopsis thaliana* – a mass spectrometry-based process for multiparallel peptide library phosphorylation analysis. Mol Cell Proteomics 4: 1614–1625

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Mol Cell 5: 1003–1011

Grønborg M, Kristiansen TZ, Stensballe A, Andersen JS, Ohara O, Mann M, Jensen ON, Pandey A (2002) A mass spectrometry-based proteomic approach for identification of serine/threonine-phosphorylated proteins by enrichment with phospho-specific antibodies: identification of a novel protein, Frigg, as a protein kinase A substrate. Mol Cell Proteomics 1: 517–527

Gruhler A, Olsen JV, Mohammed S, Mortensen P, Faergeman NJ, Mann M, Jensen ON (2005a) Quantitative phosphoproteomics applied to the yeast pheromone signaling pathway. Mol Cell Proteomics 4: 310–327

Gruhler A, Schulze WX, Matthiesen R, Mann M, Jensen ON (2005b) Stable isotope labeling of *Arabidopsis thaliana* cells and quantitative proteomics by mass spectrometry. Mol Cell Proteomics 4: 1697–1709

Hansson M, Vener AV (2003) Identification of three previously unknown *in vivo* protein phosphorylation sites in thylakoid membranes of *Arabidopsis thaliana*. Mol Cell Proteomics 2: 550–559

He X, Anderson JC, Pozo Od O, Gu YQ, Tang X, Martin GB (2004) Silencing of subfamily I of protein phosphatase 2A catalytic subunits results in activation of plant defense responses and localized cell death. Plant J 38: 563–577

Heintz D, Wurtz V, High AA, Van Dorsselaer A, Reski R, Sarnighausen E (2004) An efficient protocol for the identification of protein phosphorylation in a seedless plant, sensitive enough to detect members of signalling cascades. Electrophoresis 25: 1149–1159

Hubbard MJ, Cohen P (1993) On target with a new mechanism for the regulation of protein phosphorylation. Trends Biochem Sci 18: 172–177

Ibarrola N, Molina H, Iwahori A, Pandey A (2004) A novel proteomic approach for specific identification of tyrosine kinase substrates using [^{13}C]tyrosine. J Biol Chem 279: 15805–15813

Jonak C, Kiegerl S, Ligterink W, Barker PJ, Huskisson NS, Hirt H (1996) Stress signaling in plants: a mitogen-activated protein kinase pathway is activated by cold and drought. Proc Natl Acad Sci USA 93: 11274–11279

Kim KN, Cheong YH, Grant JJ, Pandey GK, Luan S (2003) CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. Plant Cell 15: 411–423

Kim JE, Tannenbaum SR, White FM (2005) Global phosphoproteome of HT-29 human colon adenocarcinoma cells. J Proteome Res 4: 1339–1346

Koiwa H, Barb AW, Xiong L, Li F, McCully MG, Lee BH, Sokolchik I, Zhu J, Gong Z, Reddy M, Sharkhuu A, Manabe Y, Yokoi S, Zhu JK, Bressan RA, Hasegawa PM (2002) C-terminal domain phosphatase-like family members (AtCPLs) differentially regulate *Arabidopsis thaliana*

abiotic stress signaling, growth, and development. Proc Natl Acad Sci USA 99: 10893–10898

Kratchmarova I, Blagoev B, Haack-Sorensen M, Kassem M, Mann M (2005) Mechanism of divergent growth factor effects in mesenchymal stem cell differentiation. Science 308: 1472–1477

Kreps JA, Wu Y, Chang HS, Zhu T, Wang X, Harper JF (2002) Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. Plant Physiol 130: 2129–2141

Laugesen S, Bergoin A, Rossignol M (2004) Deciphering the plant phosphoproteome: tools and strategies for a challenging task. Plant Physiol Biochem 42: 929–936

Li J, Kinoshita T, Pandey S, Ng CK, Gygi SP, Shimazaki K, Assmann SM (2002) Modulation of an RNA-binding protein by abscisic-acid-activated protein kinase. Nature 418: 793–797

Li Z, Onodera H, Ugaki M, Tanaka H, Komatsu S (2003) Characterization of calreticulin as a phosphoprotein interacting with cold-induced protein kinase in rice. Biol Pharm Bull 26: 256–261

Liu Y, Zhang S (2004) Phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthase by MPK6, a stress-responsive mitogen-activated protein kinase, induces ethylene biosynthesis in *Arabidopsis*. Plant Cell 16: 3386–3399

Mann M, Ong SE, Grønborg M, Steen H, Jensen ON, Pandey A (2002) Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. Trends Biotechnol 20: 261–268

Manning G, Whyte DB, Martinez R, Hunter T, Sudarsanam S (2002) The protein kinase complement of the human genome. Science 298: 1912–1934

Martin ML, Busconi L (2001) A rice membrane-bound calcium-dependent protein kinase is activated in response to low temperature. Plant Physiol 125: 1442–1449

Martin GB, Bogdanove AJ, Sessa G (2003) Understanding the functions of plant disease resistance proteins. Annu Rev Plant Biol 54: 23–61

Matsuoka D, Nanmori T, Sato K, Fukami Y, Kikkawa U, Yasuda T (2002) Activation of AtMEK1, an *Arabidopsis* mitogen-activated protein kinase kinase *in vitro* and *in vivo*: analysis of active mutants expressed in *E. coli* and generation of the active form in stress response in seedlings. Plant J 29: 637–647

Meskiene I, Bogre L, Glaser W, Balog J, Brandstotter M, Zwerger K, Ammerer G, Hirt H (1998) MP2C, a plant protein phosphatase 2C, functions as a negative regulator of mitogen-activated protein kinase pathways in yeast and plants. Proc Natl Acad Sci USA 95: 1938–1943

Nakagami H, Pitzschke A, Hirt H (2005) Emerging MAP kinase pathways in plant stress signalling. Trends Plant Sci 10: 339–346

Nühse TS, Stensballe A, Jensen ON, Peck SC (2003a) Large-scale analysis of *in vivo* phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. Mol Cell Proteomics 2: 1234–1243

Nühse TS, Boller T, Peck SC (2003b) A plasma membrane syntaxin is phosphorylated in response to the bacterial elicitor flagellin. J Biol Chem 278: 45248–45254

Nühse TS, Stensballe A, Jensen ON, Peck SC (2004) Phosphoproteomics of the *Arabidopsis* plasma membrane and a new phosphorylation site database. Plant Cell 16: 2394–2405

Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M (2002) Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 1: 376–386

Peck SC, Nühse TS, Hess D, Iglesias A, Meins F, Boller T (2001) Directed proteomics identifies a plant-specific protein rapidly phosphorylated in response to bacterial and fungal elicitors. Plant Cell 13: 1467–1475

Rentel MC, Lecourieux D, Ouaked F, Usher SL, Petersen L, Okamoto H, Knight H, Peck SC, Grierson CS, Hirt H, Knight MR (2004) OXI1 kinase is necessary for oxidative burst-mediated signalling in *Arabidopsis*. Nature 427: 858–861

Romeis T, Piedras P, Zhang S, Klessig DF, Hirt H, Jones JD (1999) Rapid Avr9- and Cf-9-dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound, and salicylate responses. Plant Cell 11: 273–287

Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, Khainovski N, Pillai S, Dey S, Daniels S, Purkayastha S, Juhasz P, Martin S, Bartlet-Jones M, He F, Jacobson A, Pappin DJ (2004) Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. Mol Cell Proteomics 3: 1154–1169

Rush J, Moritz A, Lee KA, Guo A, Goss VL, Spek EJ, Zhang H, Zha XM, Polakiewicz RD, Comb MJ (2005) Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol 23: 94–101

Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J 23: 319–327

Salomon AR, Ficarro SB, Brill LM, Brinker A, Phung QT, Ericson C, Sauer K, Brock A, Horn DM, Schultz PG, Peters EC (2003) Profiling of tyrosine phosphorylation pathways in human cells using mass spectrometry. Proc Natl Acad Sci USA 100: 443–448

Sangwan V, Foulds I, Singh J, Dhindsa RS (2001) Cold-activation of *Brassica napus* BN115 promoter is mediated by structural changes in membranes and cytoskeleton, and requires Ca^{2+} influx. Plant J 27: 1–12

Sangwan V, Orvar BL, Beyerly J, Hirt H, Dhindsa RS (2002) Opposite changes in membrane fluidity mimic cold and heat stress activation of distinct plant MAP kinase pathways. Plant J 31: 629–638

Seki M, Narusaka M, Ishida J, Nanjo T, Fujita M, Oono Y, Kamiya A, Nakajima M, Enju A, Sakurai T, Satou M, Akiyama K, Taji T, Yamaguchi-Shinozaki K, Carninci P, Kawai J, Hayashizaki Y, Shinozaki K (2002) Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray. Plant J 31: 279–292

Syka JE, Coon JJ, Schroeder MJ, Shabanowitz J, Hunt DF (2004) Peptide and protein sequence analysis by electron transfer dissociation mass spectrometry. Proc Natl Acad Sci USA 101: 9528–9533

Teige M, Scheikl E, Eulgem T, Doczi R, Ichimura K, Shinozaki K, Dangl JL, Hirt H (2004) The MKK2 pathway mediates cold and salt stress signaling in *Arabidopsis*. Mol Cell 15: 141–152

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408: 796–815

Williams AJ, Werner-Fraczek J, Chang IF, Bailey-Serres J (2003) Regulated phosphorylation of 40S ribosomal protein S6 in root tips of maize. Plant Physiol 132: 2086–2097

Zhang Y, Wolf-Yadlin A, Ross PL, Pappin DJ, Rush J, Lauffenburger DA, White FM (2005) Time-resolved mass spectrometry of tyrosine phosphorylation sites in the EGF receptor signaling network reveals dynamic modules. Mol Cell Proteomics (Epub ahead of print)

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