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Review

Phosphoproteomics perspective on plant signal transduction and tyrosine phosphorylation

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ABSTRACT

Plants and animal cells use intricate signaling pathways to respond to a diverse array of stimuli. These stimuli include signals from environment, such as biotic and abiotic stress signals, as well as cell-to-cell signaling required for pattern formation during development. The transduction of the signal often relies on the post-translational modification (PTM) of proteins. Protein phosphorylation in eukaryotic cells is considered to be a central mechanism for regulation and cellular signaling. The classic view is that phosphorylation of serine (Ser) and threonine (Thr) residues is more abundant, whereas tyrosine (Tyr) phosphorylation is less frequent. This review provides an overview of the progress in the plant phosphoproteomics field and how this progress has lead to a re-evaluation of the relative contribution of tyrosine phosphorylation to the plant phosphoproteome. In relation to this appreciated contribution of tyrosine phosphorylation we also discuss some of the recent progress on the role of tyrosine phosphorylation in plant signal transduction.

Contents

1. Introduction

1.1. Plant signal transduction

Extracellular signals are perceived by cells through plasma membrane (PM) receptors that transduce the signals to an intracellular signal transduction cascade that ends in the activation of transcription of the appropriate set of genes. The transduction of the signal relies in most cases on post-translational modification of the signaling proteins and the generation of so called second messenger molecules. The best-studied and understood post-translational modification is protein phosphorylation, which can lead to changes in conformation, protein–protein interaction and protein activity. In eukaryotic cells protein phosphorylation occurs predominantly on serine, threonine and tyrosine residues, but has also been described to occur on aspartate en histidine residues.

Over the last decade the field of plant signal transduction has seen a tremendous development. The analysis of protein phosphorylation has gone from protein by protein basis to high throughput

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analysis of phosphorylation at a proteome scale. Protein phosphorylation occurs predominantly on Ser, Thr and Tyr residues which contain a hydroxyl group that can accept a phosphoryl group. This reversible reaction is catalyzed by protein kinases. Protein dephosphorylation is catalyzed by protein phosphatases. Plants have an extensive family of protein kinases and protein phosphatases, with nearly 1000 genes encoding kinases in Arabidopsis and 1467 genes in rice and 300 and 132 genes encoding protein phosphatases in Arabidopsis and rice, respectively (Dardick et al., 2007; Initiative, 2000; Jung et al., 2010; Kerk et al., 2006; Singh et al., 2010). The combined activity of kinases and phosphatases, which are in dynamic equilibrium, determines the level of phosphorylation for individual substrate proteins. Moreover, kinases and phosphatases are frequently regulated by negative feedback loops that attenuate the signaling back to basal levels (Brandman and Meyer, 2008). Initial efforts to study protein phosphorylation in plant signal transduction made use of a pharmacological approach to inhibit protein kinases and phosphatases. The mostly general inhibitors, such as staurosporine and K252a, could be used to show a role for protein kinase activity in stimulus-induced responses. Using this approach elicitor induced ion fluxes across the plasma membrane, generation of second messengers, defense gene expression and phytoalexin production were shown to require protein phosphorylation (Menke et al., 1999; Scheel, 1998). This was complemented by identification and cloning of specific kinases that played a role in plant defense responses and disease resistance (Martin et al., 1993; Song et al., 1995; Zhang and Klessig, 1997). At the turn of the century individual research effort were mostly geared towards identification of protein kinases/phosphatase and the stimuli that induced their activation (Asai et al., 2002; Kovtun et al., 2000; Nuhse et al., 2000; Romeis et al., 1999; Zhang et al., 1998). Reverse genetic approaches based on RNAi and virus induced gene silencing (VIGS) allowed the functional analysis of kinases and phosphatases (Jin et al., 2002; Menke et al., 2004; Romeis et al., 2001), but identification of substrates and specific sites of phosphorylation were still scarce. The available methods were limited to biochemical approaches, such as solid state phosphorylation screens to identify the corresponding substrates or Y2H analysis to identify interacting partners of protein kinases and phosphatases (Qiu et al., 2008). The kinase-dependent phosphorylation of the potential substrates was tested in vitro with recombinant or purified kinase substrate pairs (Menke et al., 2005).

2. Plant phosphoproteomics

2.1. Towards plant phosphoproteomics

A first real step towards larger scale phosphoproteomic approaches was the combined use of 2-dimensional gel electrophoresis (2-DE) and spot identification by mass spectrometry. This approach initially made use of radiolabeled protein samples obtained by pulse chase labeling of cells with ^{32}P ortho-phosphate (Peck et al., 2001). Detection and identification of phosphorylated peptides was however hampered by the reduced effectiveness of ionization of phosphorylated peptides in complex peptide mixtures in mass spectrometers. Never the less in vivo phosphorylated proteins were identified allowing the functional analysis of these phosphoproteins and eventually connecting to the upstream protein kinase (Merkouropoulos et al., 2008).

2.2. Phosphopeptide enrichment

The first major step forward towards phosphoproteomics came with the development of enrichment strategies for phosphorylated peptides. Even though about a third of all proteins can be phosphorylated at any time in the cell, phosphorylated proteins are relatively low in abundance. Moreover phosphoproteins with regulatory functions and involved in signal transduction are present at sub-stoichiometric levels and are transient in nature. Phosphorylation events on signaling proteins are highly regulated by the conserted action of protein kinases and protein phosphatases. A small change in stoichiometry between non-phosphorylated and the phosphorylated version of individual signaling proteins can have very significant consequences for the signaling output. These transient and small changes in phosphorylation status thus further hampers the identification of phosphorylated proteins and peptides in complex protein or peptide mixtures. Furthermore, ionization of phosphopeptides (phops) in mixtures with non-phosphopeptides is suppressed and limits their selection and fragmentation in mass spectrometers. Thus enrichment of phosphopeptides from complex mixtures was a prerequisite to the successful large-scale identification by mass spectrometry. Therefore achieving phosphopeptide enrichment was a significant step forward and made it possible to not only identify in vivo phosphorylated proteins on a larger scale, but also to confidently identify the residue that was modified (Blagoev et al., 2003; Ficarro et al., 2002; Nuhse et al., 2003; Pinkse et al., 2004). The most commonly used enrichment methods are based on affinity purification of phosphoryl-group containing peptides and include immobilized metal affinity chromatography (IMAC), strong cation exchange chromatography (SCX) and metal oxide affinity chromatography (MOAC). IMAC uses metal ions such as Fe(III) or Ga(III) immobilized by chelation on a solid substrate. When used at a low pH these positively charged ions can interact selectively with negatively charged phosphoryl groups. After washing under low pH conditions, selectively bound phops are eluted from the IMAC columns using a buffer with a more alkaline pH. Ficarro and collegues were the first to report on the use of IMAC to enrich complex tryptic digestion mixtures for phops and analyzed the enriched fraction by nanoflow HPLC/electrospray ionization mass spectrometry (nano-LC-ESI-MS). They applied this method to characterize the phosphoproteome of whole lysate of yeast cells and identified 383 phosphorylation sites on 216 phops. A large proportion of the identified phops were multi-phosphorylated peptides, and singly phosphorylated peptides were underrepresented. The preferential enrichment of multi-phosphorylated peptides over singly phosphorylated peptides by IMAC was noted in several independent studies. A modified and refined IMAC strategy was used by Nuhse et al. (2003) who analyzed Arabidopsis suspension cultured cells treated with the peptide elicitor flg22. In addition to IMAC they used strong anion exchange (SAX) fractionation to reduce the complexity of the tryptic digest prior to IMAC enrichment. This resulted in a larger proportion of singly phosphorylated peptides in addition to the multi-phosphorylated peptides. With this combined approach the authors reported enrichment up to 75% in phops. In a follow-up study the authors reported a total of 300 phosphorylated sites on 200 phops from plasma membrane associated protein, most of which were newly identified (Nuhse et al., 2004). A drawback of IMAC is the negatively charged carboxyl groups present in peptides that compete with the phosphoryl groups for binding to the metal ions. However, at low pH carboxyl groups are less likely to carry a negative charge and these conditions are more selective for phosphoryl group affinity interactions. A popular and successful alternative to IMAC is metal oxided based affinity purification of phops. Metal oxide (TiO_2 or ZiO_2) columns also have a tendency to bind both phosphoryl and carboxyl groups but by loading the peptides on the column in the presence of acids, such as dihydroxybenzoic acid or lactic acid, the phosphoryl affinity is enhanced. MOAC is also more selective towards mono-phosphorylated peptides (Larsen et al., 2005; Pinkse et al., 2004). Metal oxide purification of phops was successfully used in a number of

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studies involving plant phosphoproteomics approaches (Benschop et al., 2007; Kersten et al., 2009; Stulemeijer et al., 2009) Benschop et al., used the metal oxide TiO_2 in combination with strong cation exchange (SCX) to enrich phops from tryptic digests of plasma membrane associate proteins of Arabidopsis. With this combined approach 1172 phops were successfully identified, of which the vast majority were singly phosphorylated peptides.

Comparative analysis of modified and improved IMAC and TiO_2 methods revealed a significant bias of each method for a non-overlapping portion of the phosphoproteome (Thingholm et al., 2009). This and other comparative studies (Bodenmiller et al., 2007; Ndassa et al., 2006) made it clear that in order to obtain a more complete phosphoproteomic analysis IMAC and TiO_2 based methods should be combined by using them sequentially or in parallel on the same samples. In addition to the methods described above, additional methods, both commercial and in house developed, and modifications to existing protocols have been described. For further in dept reading on these subjects we recommend reviews by Kersten et al. (2006), Kersten et al. (2009) and Thingholm et al. (2009).

2.3. Separation and detection of phosphoproteins and phosphopeptides

Sample preparation and enrichment of phosphopeptides are two important steps in successful proteomics experiments which are then followed by one or two dimensional separation of the protein/peptide mixture prior to mass spectrometric identification. Broadly speaking these separation techniques can be divided into gel-based separation of proteins, such as one and two-dimensional gel electrophoresis, and chromatography based peptide separation such as strong cation/anion exchange followed by (online) reverse phase liquid chromatography. One and two-dimensional gel electrophoresis (1- or 2-DE) have long been the method of choice to separate protein mixtures into discrete bands of protein or protein spots. Protein bands in 1-DE often still consist of multiple proteins and phosphorylated forms of proteins often cause only minor shifts in the electrophoretic mobility as compared to the non-phosphorylated version. In 2-DE proteins are separated on charge by isoelectric focusing as well as on molecular weight in the second dimension, resulting in highly complex patterns of proteins spots, which mostly consist of individual proteins. Furthermore, phosphorylated proteins give rise to the so called 'string of pearls' patterns in which each spots represents a different number of phosphorylated residues in the protein isoform, as recently shown for the brassinolide-regulated change in phosphorylation of BRZ1 (Tang et al., 2008a,b). During the first dimensional separation different hyperphosphorylated BZR1 forms migrated to a position at the acidic end of the gel whereas different hypophosphorylated forms of BZR1 migrated to positions in the gel with a more basic pH. Since the phosphorylation status has little affect on migration in the second dimension the different phosphorylated forms of BZR1 gave rise to a row of spots (Tang et al., 2008a,b), resembling a 'string of pearls'. Visualization of phosphoproteins in gel based separation techniques is essential for subsequent isolation and identification of the protein by mass spectrometry. Initially this was achieved by pulse chase labeling of cells with radioactive phosphate (32P-ortho-phosphate) and autoradiography of gel, which was followed by excision of the correct spots from analytical gels (Peck et al., 2001). Radioactive labeling has since then become more or less obsolete with the development of the phosphoprotein staining dye ProQ diamond which allows detection of phosphoproteins through fluorescent laser scanning (Jin et al., 2006). This method is compatible with concomitant total protein staining with Sypro Ruby stain which fluoresces with a different spectrum. A third and quantitative method of labeling uses covalently linked Cy dyes that are cross-linked to protein samples prior to separation. Up to three Cy dyes (Cy2, Cy3 and Cy5) each with different excitation and emission spectra can be simultaneously used (Tang et al., 2008a,b; Tannu and Hemby, 2006; Tonge et al., 2001). This allows different conditions to be compared on a single 2-DE gel. This circumvents the running and comparison of individual gels with concomitant technical variation. Following separation and isolation of protein spots, which can be done automated with the use of robotics, the proteins are identified of Maldi-TOF or Q-TOF mass spectrometers. An in depth overview of different types of mass spectrometers and their usage can be found in Boersema et al. (2009a,b).

Chromatography based separation is mostly done on peptide mixtures and uses two qualities, charge state of the peptide and its hydrophobicity. The most commonly used method that is based on charge state affinity purification for phosphoproteomics applications is strong cation exchange (SCX). Fractionation of peptide mixtures by SCX is based on the interaction of positively charged groups on the peptides with the negatively charged groups in the SCX resin. Peptides are loaded onto the column under low salt, low pH conditions and eluted by an increasing salt gradient. The additional negative charge on phosphopeptides causes a weaker interaction and elution under lower salt conditions as compared to the same peptides without the phosphoryl group. The initial fractions from SCX are thus enriched in phops while later fractions are almost completely depleted of phops. Many studies have used SCX to prefractionate phosphopeptide mixtures into deconvoluted fractions that are than further enriched for phopsphopeptides by IMAC or MOAC (Table 1). The enriched phosphopeptide fractions are finally further fractionated by reverse phase LC, which separates peptides based on hydrophobicity. The reverse phase LC fractionation is done online with the mass spectrometer and is usually referred to as ESI-nano-LC-MS, where ESI stands for electro spray ionization and nano denotes the elution in nanoliter per second. Elution from the nano-LC goes straight into the ionization chamber where the eluting droplets are vaporized and ionized by high voltage. The ionized peptides are then accelerated into MS by a powerful electric field. In this first stage MS the peptide ions are separated by mass charge (m/z) ratios and the m/z ratios are recorded in a so called survey scan. The mass spectrometer then selects the most abundant peptide ions for tandem MS using data dependent analysis. The selected peptide ion, or precursor ion, is subjected to fragmentation, usually by collision-activated dissociation (CAD). The resultant fragment ions are recorded in a product-ion scan. Because each survey scan contains multiple precursor ions, several product-ion scans are done for each survey scan. This is possible because the cycle of survey and product-ion scans is completed very fast in comparison to the elution time of the peptides. The resulting product-ion spectra are recorded and matched to the precursor ion m/z value. During separate data analysis this information is used to deduce the peptide amino acid sequence and detect possible post-translational modifications. Most commonly used mass spectrometers in tandem MS phosphoproteomics are, Ion traps, LTQ-Orbi traps and Fournier-Transform ion cyclotron resonance mass spectrometers (FT-ICR-MS). These mass spectrometers have been developed over the last decade and can now operate at sub-femtomolar detection and with low ppm mass accuracy. For an overview of mass spectrometers and fragmentation methods the reader is referred to reviews by Boersema et al. (2009a) and Schulze (2010). The rapid development of enhanced sensitivity and increase mass accuracy has made it possible to obtain larger amounts of phosphoproteomic data from more complex samples but also increasingly smaller amount of proteins. This opens up the measurement of more diverse samples than sub-cellular fractionated suspension cultured cells which up to recently have been the main model system for many plant studies (Table 1). These developments have also contributed to the more comprehensive

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Table 1
Overview of plant phosphoproteome studies using shotgun MS approaches.

Species	Tissue	Treatment	Enrichment	Quantitation	Main features	References
Arabidopsis	Cell culture	Phyto-hormone	TiO_2	Label free	Quantitative temporal analysis of 5 phytohormone treatments, 152 differential phops on 136 proteins	Chen et al. (2010)
Arabidopsis and rice	Cultured cells	Untreated	SCX and TiO_2	None	6919 phosphopeptides from 3393 proteins from rice showing significant levels of Tyr phops. Conservation of phosphorylation of orthologues in three species	Nakagami et al. (2010)
Medicago	Root	Untreated	SCX and IMAC	None	Use of CAD and ETD fragmentation, 3457 unique phosphopeptides from 829 proteins, significant Tyr phosphorylation	Grimsrud et al. (2010)
Arabidopsis	Leave and shoot	Light treatment	SCX, IMAC and TiO_2	None	3029 unique phosphopeptides from 1429 phosphoproteins detected, focus on chloroplast phosphoproteins and their cognate kinases	Reiland et al. (2009)
Tomato	Seedling	Hypersensitive response	TiO_2	Label free	50 phosphoproteins identified with 12 differential for HR. Photosynthetic activity is specifically suppressed in a phosphorylation-dependent way during the very early stages of HR development	Stulemeijer et al. (2009)
Arabidopsis	Seedlings and protoplasts	Untreated	SCX and TiO_2	None	Identified 416 phosphopeptides from 345 proteins, used sub-cellular fractionation to obtain substancial enrichment of nuclear phosphoproteins	Jones et al. (2009)
Arabidopsis	Cultured cells	Untreated	TiO_2 IMAC ZrO_2	None	Identified 2597 phosphopeptides from 1346 proteins. First report on pS/pT/pY distribution (85.0, 10.7, and 4.3%) similar to animals systems	Sugiyama et al. (2008)
Arabidopsis	Cultured cells	Untreated	IMAC	None	Identification of 303 phosphorylation sites on 205 proteins. Reported pS/pT/pY ratio of 91.8%/7.5%/0.7%	de la Fuente van Bentem et al. (2008)
Arabidopsis	Cultured cells	Sucrose	IMAC	Label free	Temporal study of sucrose resupply. Sixty-seven phosphopeptides identified, quantified over five time points	Niittyla et al. (2007)
Arabidopsis	Cultured cells	Elicitor	SCX and TiO_2	15N metabolic labeling	Quantification based of metabolic labeling. Phops (1172) from 472 protein, 98 differential phops from 76 proteins. Highly complex differential phosphorylation of individual proteins	Benschop et al. (2007)
Arabidopsis	Cultured cells	Elicitor	IMAC and SCX	iTrac	Quantitative temporal analysis of flg22 signalling using iTrac. 279 phops identified, 11 differential phops on 8 PM proteins. Relevance of phosphorylation for RbohD activity shown	Nuhse et al. (2007)
Arabidopsis	Cultured cells	Elicitor	IMAC and SCX	None	First large-scale phosphoproteomics study in plants, identified 300 phosphorylation sites in 283 phops. Includes motif analysis and phospho site conservation in orthologues	Nuhse et al. (2004)

analysis of phospho-proteome samples. However despite the enhanced performance of state-of-the-art mass spectrometers consecutive analysis of the samples still shows only partial overlap between the replicate datasets (Schulze, 2010).

2.4. Quantification of phosphorylation

Identification of phosphorylated residues on proteins is only the first step in understanding signal transduction based on changes in phosphorylation. Quantification of changes in protein phosphorylation in response to specific stimuli is required to characterize the relationship between the phosphorylation of specific residues in a protein and how it affects the proteins behavior. To this end a number of ways to quantify changes in protein phosphorylation have been developed (Fig. 1 and Table 1). In 2DE based phosphoproteomics the aforementioned labeling with different Cy dyes allows for the quantification of the changes in protein phosphorylation using difference gel electrophoresis (DiGE) (Tang et al., 2008a). By labeling the mock treated sample with Cy3 dye and the brassinolide stimulus induced sample with Cy5 the authors were able to mix and run the two protein samples on one gel. Upon scanning the electrophorized gel for each fluorophore independently and then applying false color imaging and overlaying these false colored images changes in protein phosphorylation can be visualized and quantified in a manner very similar to that used in 2-color DNA microarrays (Tang et al., 2008a). In 2D DiGE based proteomics methods quantification and MS analysis are performed separately. Other quantification protocols rely on identification and quantification in coupled MS and MS–MS runs (Fig. 1). Several methods for quantification including dimethyl labeling, iTRAQ, SILAC and $^{14}N/^{15}N$ metabolic labeling as well as label-free quantification have been described (Benschop et al., 2007; Boersema et al., 2009b; Gruhler et al., 2005; Nuhse et al., 2007; Stulemeijer et al., 2009). The first two methods rely on post extraction chemical labeling of the peptide digests with tags with different stable isotopes (Fig. 1b). Isobaric tags for relative quantification (iTRAQ) labels the peptide digest on lysine residues with one of several available isobaric tags that are behaving chromatographically identical. These isobaric tags are composed of different combinations of stable isotopes and yield reporter ions upon collision-induced dissociation (CID) in the mass spectrometer that can be used for identification and quantification. Since 8 different isobaric tags are available up to 8 samples can be multiplexed and analyzed in a single MS run allowing for easy quantification of e.g. time course experiments. This method was used to analyze a time course experiment of flg22 treated suspension cultured cells to identify divergent dynamics of different phosphorylation sites within individual PM H+-ATPases as well as coordinate regulation of conserved phosphorylated residues in homologuous proteins (Nuhse et al., 2007). Another chemical labeling method that is widely used is dimethyl labeling which has been recently been modified to allow multiplexing of three samples (Boersema et al., 2009b). Although this method is fast and inexpensive and does not require measurements in a low m/z range (a disadvantage of iTRAQ) it has not been reported yet for use in plant quantitative proteomics studies. The most widely used method in quantitative phosphoproteomic experiments is stable isotope labeling with amino acids in cell culture (SILAC). However this method has limited applicability in plants and plant cell cultures due to the fact that plant cells can synthesize the amino acid most commonly used for this type of metabolic labeling experiments and thus only a 70% labeling efficiency can be obtained, which makes quantification difficult (Gruhler et al., 2005). However another metabolic labeling method using ^{15}N nitrate and ammonium salts (Fig. 1a) has been successfully implemented for use in plant cells by Benschop et al.,(2007). $^{14}N/^{15}N$ metabolically labeled Arabidopsis suspension cultured cells were used for quantitative phosphoproteomics experiments to identify differentially phosphorylated

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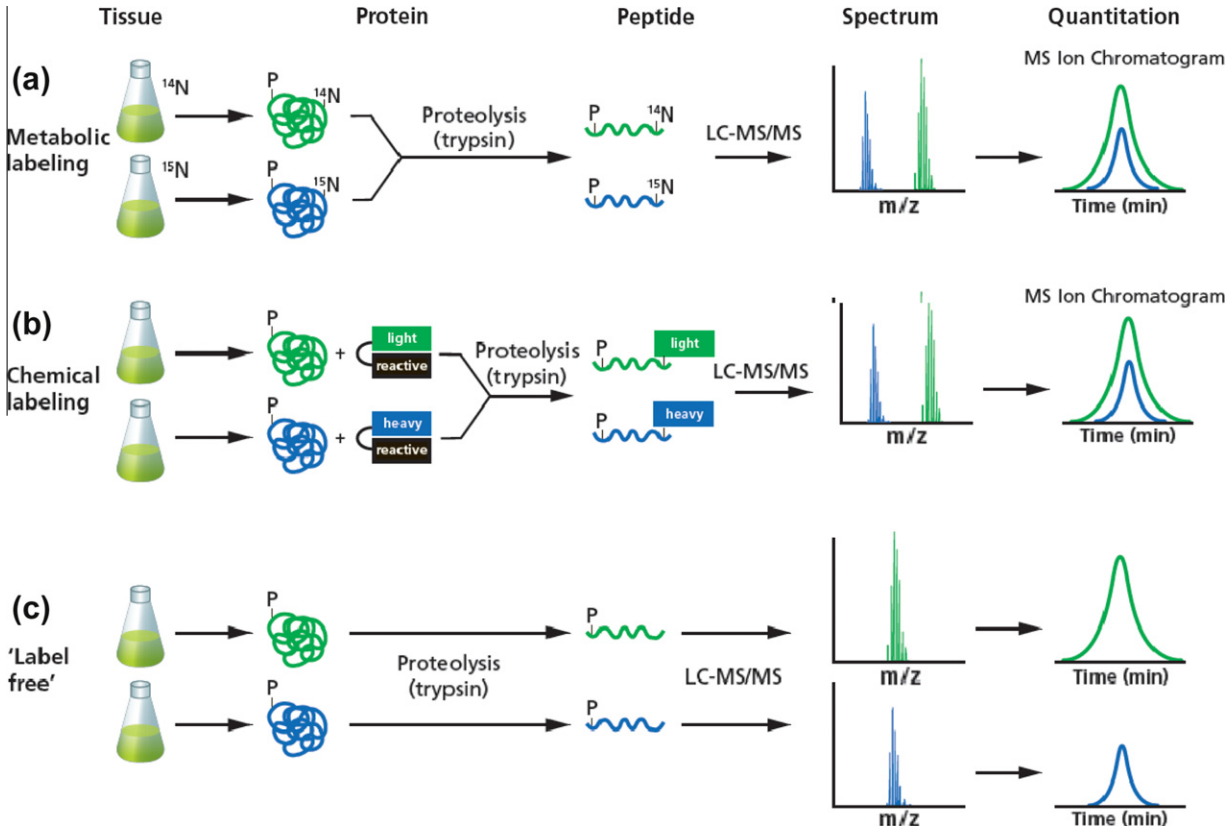

Fig. 1. Quantitative approaches used for plant phospho-proteomics. (a) Metabolic labeling uses ^{14}N and ^{15}N nitrate and ammonium salt in plant growth media to obtain stable isotope labeled proteins. Light and heavy protein samples are mixed prior to digestion and analysed by LC-MS/MS. (b) In chemical labeling proteins (or peptides) are labeled after extraction with a light or heavy isotope tags after which the isotope tag proteins are mixed and digested prior to LC-MS/MS analysis. In metabolic labeling and chemical labeling light- and heavy-labeled peptides show different *m*/*z* ratios during MS which allows their relative quantification based on the extracted MS ion chromatogram of the elution of corresponding peptides. (c) In label-free quantitation samples are extracted and processed separately including the LC-MS/MS analysis. Alignment of MS Ion chromatograms is essential prior to quantification.

proteins in response to two different elicitors. By using biological replicate samples that were inversely labeled the authors obtained a high confidence set of 96 differentially phosphorylated peptides from 76 proteins. In addition, a large set of differential phops that were observed in only one of the biological replicates was reported. The results demonstrated an important role for vesicle trafficking in response to elicitor induced defense signaling and plant defense response. Interestingly, it also pointed out the highly complex pattern of phosphorylation of the RbohD protein in response to the two different elicitors. RbohD is one of the subunits of NADPH oxidase in Arabidopsis that generates reactive oxygen species in response to stress. Several of the same phosphorylation events were subsequently also reported by Nuhse et al. (2007) and shown to be required for the activation of RbohD. ^{15}N metabolic labeling can also be applied to seedlings and whole plants for the quantitative proteomic analysis of in planta processes, such as proteomic changes in response to oxidative stress in leaf tissue (Bindschedler et al., 2008). MS based quantification of changes protein phosphorylation has also been reported without the use of stable isotopes (Fig. 1c). In this approach, called label-free quantitation, the phosphopeptides are quantified based on MS ion peak area. This approach requires the alignment and calibration of chromatograms of the samples that are compared and requires the accurate determination of mass deviation and retention time variation. Using this approach changes in protein phosphorylation were analysed in tomato plants mounting a hypersensitive response (HR) (Stulemeijer et al., 2009). Using TiO_2 and nano-LC-MS/MS 50 phosphopeptides were identified of which 12 peptides showed changes in abundance upon HR initiation. The results suggest that during initial mounting of an HR photosynthetic activity is suppressed in a phosphorylation-dependent manner. Label-free quantitation has also been successfully used in time course experiments to identify changes in phosphorylation in response to sucrose sensing (Niittyla et al., 2007). In this report differentially phosphorylated residues were identified in PM H+-ATPases that are required for proton pumping activity in response to sucrose feeding. Further in depth reading on quantitative proteomics approaches can be found in Schulze and Usadel (2010).

3. From phosphoproteomic data sets to signal transduction

The technical progress of recent years has been accompanied with an increasing number of large-scale phosphoproteomics data sets, several of which contain quantitative data and some of which also include temporal profiling data (Table 1). Several of the studies reporting these large-scale phosphoproteomic data sets have been discussed in the previous sections and a more complete overview can be found in other reviews on the subject of plant phosphoproteomics (de la Fuente van Bentem and Hirt, 2007; Kersten et al., 2009; Schulze, 2010; Thelen and Peck, 2007). Although large phosphoproteomic data sets are a first step towards understanding plant signal transduction the challenge is to filter those phosphorylation events that are modulated under specific conditions and contribute to the transduction of the perceived signal. There are several ways to approach this challenge, which include generation of quantitative data sets, temporal profiling experiments as well as in depth bioinformatic analysis of the data. A number of large scale quantitative phosphoproteomic data sets have been generated (Benschop et al., 2007; Chen et al., 2010; Niittyla et al., 2007;

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Nuhse et al., 2007; Stulemeijer et al., 2009) and each of these have identified specific phosphoproteins and processes modulated in response to the applied stimulus. A number of these identified phosphoproteins, including RbohD and PM H+ATPases, have been studied in detail in follow up functional analysis and their differential phospho-sites were shown to be required for the modulation of protein activity, as predicted by the initial phosphoproteomic analysis. However, these verifications have to be done on a case by case basis and will take many individual follow up studies to piece together a signal transduction pathway, let alone a complex interconnected signaling network. This is where temporal profiling, preferably in conjunction with relative quantification, will make a difference. Temporal profiling of sufficiently large numbers of phosphoproteins will allow for the inference of signaling pathways and in due time of signaling networks. This requires significantly more data then currently available in the field of plant phosphoproteomics, but in the animal field this type of analysis has already been successfully implemented (Choudhary and Mann, 2010; Lemeer and Heck, 2009). By implementing computational approaches that bring together coexpression data, protein–protein interactions and sub-cellular localization with protein phosphorylation data, a predictive kinase substrate signaling network was build that identified kinase substrate pair in DNA damage signaling in human cells (Linding et al., 2007). In time, with more temporal quantitative phosphoproteomic data sets this type of computational analysis will also become within reach of the plant phospho-proteomic field.

4. Plant phosphoproteomic databases

The availability of these large-scale data sets has let to the creation of a number of phosphoproteomic databases where the data set can be queried and compared. The largest of these is PhosPhAt (http://phosphat.mpimpgolm.mpg.de/) (Heazlewood et al., 2008) which can be queried using Arabidopsis locus identifiers and reports measured and predicted phosphorylation sites for each protein. The computational predictions are trained on the experimentally verified sites contained in the PhosPhAT database. The PhosPhAt database currently contains data on 5170 Arabidopsis phosphoproteins and 32601 phosphosites. The majority of the phosphosites are validated (21.353) whereas the total number of ambiguous phosphosites is 11.248. Out of the total number of phosphosites 12.457 are reported to be unique, indicating that a substantial number of phosphosites have been identified multiple times. The percentage of validated unique pSer in the database is about 76%, pThr represents 17% and pTyr represents 6% of the total validated unique phosphosites. The Plant Phosphoproteome Database (http://phosphoproteome.psc.database.riken.jp) is based on two data sets from the Shirasu lab (Nakagami et al., 2010; Sugiyama et al., 2008) and currently contains data on 5143 rice phops and 6919Arabidopsis phops. The reported ratios of pSer/pThr/pTyr in these two datasets are 84.8%/12.3%/2.9% for rice and 82.7%/13.1%/4.2% for Arabidopsis. A recent addition is the Medicago phosphoproteomics database (http://www.phospho.medicago.wisc.edu) (Grimsrud et al., 2010), which currently contains data of one phosphoproteomics data set from Medicago. This database can be queried based on protein sequence, phosphorylation motif and description and contains 3457 unique phops and shows a pSer/pThr/pTyr distribution of 86%/12.7%/1.3%. An additional resource that is not a phosphoproteomics database persé, but that brings together a wealth of data on proteins kinases and protein phosphatases from many plant species is PlantP (http://plantsp.genomics.purdue.edu/html/).

When considering the data available from plant phosphoproteomic studies and databases the amount of data on phosphorylation on Ser or Thr residues is much more extensive and much fewer phosphorylations on Tyr residues are reported. The reported numbers are very similar to those observed in yeast and animal studies, indicating that overall the phosphorylation machinery is conserved since an early point in eukaryotic evolution. Despite the relatively limited number of pTyr residues reported this specific modification has gained attention in both the animal and plant signal transduction fields. In the next section we will discuss the available data on Tyr phosphorylation in plant systems and put it in perspective in relation to Tyr phosphorylation in animal systems.

5. Phospho-tyrosine signaling in plants

There are a number of examples of plant proteins that show phosphorylation on Tyr residues that have been shown to be required for some of the proteins function in vivo. The BRI receptor is one of these proteins, although it was initially characterized as an autophosphorylating serine/threonine kinase. Using anti-phosphotyrosine antibodies it was recently shown that BRI is also phosphorylated on tyrosine residues (Oh et al., 2009b). BRI1 is a PM localized receptor kinase that binds the phytohormone brassinolide (BL) in a complex with its coreceptor BAK1. BL perception requires active BRI1 and activates a phosphorylation-dependent signaling cascade, which was the first cascade to be fully characterized in plants (Kim and Wang, 2010; Tang et al., 2008a,b). Tyrosine phosphorylation on 4 C-terminal residues of BRI1 is important for its kinase activity, whereas phosphorylation of Tyr831 in the juxtamembrane domain causes inhibition of growth and delay in flowering (Oh et al., 2009a). In addition to BRI1, BAK1 and it paralogue BKK1 were also shown to be phosphorylated on tyrosine residues, although the functionality of these events was not shown. However other RLKs, including FLS2, which also forms a complex with BAK1 in flg22 signaling cascade, were also tested in the same experimental set up and did not show Tyr phosphorylation. This demonstrates that Tyr phosphorylation is not a general aspect of RLK signaling in plants. Tyrosine phosphorylation also plays in important role downstream of BRI as it controls the proteasomal degradation of the negatively regulating kinase BIN2, mediated through protein phosphatase BSU1. BIN2 is a GSK3 kinase and autophosphorylates on Tyr200 that is required for kinase activity. This pTyr-dependent activation mechanism is also conserved in mammalian GSK3s. The role of BIN2 in BL signaling is similar to the role played by GSK3ß in Wnt signaling in animal cells. Both kinases phosphorylate their respective targets, BZR1/2 and ß-catenin, respectively, to promote their degradation in the cytosol (Kim and Wang, 2010). Another evolutionary conserved family of protein kinases that require tyrosine phosphorylation for their activation is mitogen activated protein (MAP) kinases. MAP kinases act as part of a three tiered cascade containing MAPK kinases and MAPKK kinases phosphorylating each other. MAP kinases are involved in many signaling cascades in plants leading to diverse responses, including biotic and abiotic stress response, phytohormonal response, embryonic and stomatal patterning, cytokinesis and cytoskeletal organization (Andreasson and Ellis, 2010; Pitzschke et al., 2009). MAP kinases become phosphorylated on the TxY motive of their activation loop by their upstream MAPKK, which can be functionally classified as dual specificity protein kinases (DSK). Reversal of this dual phosphorylation is required to inactivate the MAP kinases and this achieved by dual specificity MAPK phosphatases (Bartels et al., 2010; Humberto et al., 2005).

All of the above mentioned kinases can be functionally classified as DSKs as they can phosphorylate Tyr residues as well as Thr or Ser residues (Oh et al., 2009a,b). However tyrosine-specific kinases (TSKs) are conspicuously low abundant in the annotated genomes of fully sequenced plant species, with 2 putative

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TSKs predicted in Arabidopsis and 6 or 7 putative TSKs in Rice (Miranda-Saavedra and Barton, 2007). Furthermore, tyrosine receptor kinases (TRKs), that play a prominent role in receptor mediated signaling in metazoan systems, are completely absent in plant genomes (Initiative, 2000). Interestingly, plants do have an extended family of receptor like kinases (RLK) but these are distinct from TRKs as defined in metazoans, and only a handful of plant RLKs are phosphorylated on tyrosine residue so far. Plant do have some Tyr-specific phosphatases (PTPs) and several dual specificity protein phosphatases (DSPs) (Luan, 2002; Moorhead et al., 2009), but the PTP family in metazoans is much more extensive and seems to have expanded specifically in this evolutionary branch.

6. pTyr in phosphoproteomic datasets

In non-plant model systems several large-scale phosphoproteomic studies have reported levels of serine, threonine and tyrosine phosphorylation to be around 80–85%, 10–15% and 2%, respectively (Olsen et al., 2006; Swaney et al., 2009). Initial phosphoprotemics approaches in plants however only showed minor numbers of phosphopeptides containing phosphorylated tyrosine residues (Benschop et al., 2007; Nuhse et al., 2007, 2004). This suggested that tyrosine phosphorylation was not as prevalent in plant systems as it was in animal systems. This suggestion was reinforced by the notion that model plant species Arabidopsis and rice, have very few TSKs, no TRKs and only limited numbers of DSKs. The first indication that tyrosine phosphorylation may be more abundant then hitherto acknowledged came with the large-scale phosphoproteomic study by Sugiyama et al. (2008) that reported 4% Tyr phosphorylation in Arabidopsis cells. In their study, and follow-up study by the same group, much more pTyr containing peptides were identified from both Arabidopsis suspension cultured cells and rice suspension cultured cells (Nakagami et al., 2010). The total number of pTyr containing peptides as well as their relative contribution to the plant phosphoproteome were much more significant then reported before in phosphoproteomic studies by three independent labs. In the later three cases only very few pTyr containing peptides were identified in relatively larger scale studies that ranged from a few hundred (de la Fuente van Bentem et al., 2008; Nuhse et al., 2004) to well over a thousand phosphopeptide (Benschop et al., 2007). Re-evaluation of the data contained in the Sugiyama study suggested that ambiguous phospho-site assignment may have contributed to an overestimation of the number of true Tyr phosphorylation sites, but it also indicated that most phosphotyrosine sites in mono-phosphorylated peptides were correct (de la Fuente van Bentem and Hirt, 2009). Nakagami et al. (2010) also reanalyzed their data using PTM score-derived localization probability method (Olsen et al., 2006) resulting in adjusted levels of tyrosine phosphorylation of 1.6% for rice and 2.4% for Arabidopsis. Similar levels of tyrosine phosphorylation were recently also reported in a Medicago phosphoproteomics study on root tissue (Grimsrud et al., 2010). Whereas all previous plant phosphoproteomics studies have used collision-activated dissociation (CAD) during MS/MS to obtain ion fragments from which to deduce peptide sequence information, Grimsrud and colleagues have also used electron-transfer dissociation (ETD) during MS/MS. With CAD the energy to induce dissociation can often result in the preferential lost of phosphoric acid from the phospho-peptide, so called neutral loss, and as a result poor sequence information, especially directly around the phosphorylated site. ETD does not induce dissociation of labile PTMs and the electron transfer results in fragmentation of the peptide backbone, giving superior phospho-site assignment (Swaney et al., 2009). However, only a subset of the phops data could be identified by both fragmentation methods, whereas the majority of the phops were identified by either ETD or CAD based fragmentation (Grimsrud et al., 2010; Swaney et al., 2009). To circumvent this problem, a decision tree-driven tandem MS algorithm was used to select the optimal fragmentation method for each precursor ion (Grimsrud et al., 2010; Swaney et al., 2009).

With the use of improved EDT methodology in plants and the identification of similar levels of pTyr in three plant model species Sugiyama's suggestion that tyrosine phosphorylation is as significant in plants as it is in animal systems seems to be confirmed. The significance of tyrosine phosphorylation for plant signaling has previously also been investigated by immunodetection using pTyr specific antibodies. Ghelis et al. (2008) used such an approach to study the regulation of Tyr phosphorylation in ABA transduction pathways. Proteins extracted from ABA treated Arabidopsis seeds were separated by two-dimensional gel electrophoresis and the changes in tyrosine phosphorylation levels were detected by using an anti-phosphotyrosine antibody on Western blot. A total number of 19 pTyr containing proteins were detected. The changes in pTyr levels of proteins obtained by immunoblot were confirmed by MALDI-TOF-TOF MS (Ghelis et al., 2008). In another study tyrosine phosphorylation of the BRI1 receptor kinase was characterized by using anti-phosphotyrosine antibodies. BRI1 was initially characterized as an autophosphorylating serine/threonine kinase, but recent work by Oh et al. (2009a,b) has shown that BRI1 is also autophosphorylated on tyrosine residues. In our group we have conducted a targeted phosphoproteomics approach to specifically analyze pTyr signaling events. We used pTyr specific antibodies to immunoprecipitate Tyr phosphorylated peptides, implementing a methodology that has been previously described for animal systems (Blagoev et al., 2004; Lemeer et al., 2007). With our approach we were able to identify, with high confidence, 124 pTyr peptides (Mithoe et al., unpublished). Our results are thus also in support of more significant levels of tyrosine phosphorylation in Arabidopsis cells, in contrast to the number of pTyr site identified in our initial study (Benschop et al., 2007).

7. Tyrosine phosphorylation in plants, is it more than a numbers game?

The question of how much pTyr may not be as relevant as the question what makes pTyr signaling so special in metazoans and could this also apply to plants. An interesting perspective on this subject was recently provided by looking at the evolution of pTyr signaling (Lim and Pawson, 2010). In metazoans pTYR signaling is regulated by a three-part tool kit; TSK, PTP and Scr Homology 2 (SH2) domains. Lim and Pawson (2010) describe this tool kit as the writer/reader/eraser module that can be used to build complex signaling circuits based on positive and negative feedback loops. This tool kit is only fully developed in metazoans and the unicellular Choanoflagellates that have about 40 PTP, about 100 SH2 and 50–100 TSK. Evolution of this toolkit is described to have occurred in three stages. In the first stage the early eukaryotic ancestor has only a few PTP and a prototypical SH2 domain that is not selective for pTyr. Stage 1 is currently exemplified by the situation in yeast. In stage 2 of the evolution the SH2 domain becomes selective for pTyr and is incorporated into simple multi-domain architecture proteins. This stage in the evolution is also accompanied by more DSK activity. This stage 2 version of the writer/reader/eraser system represents a very primitive form. The PTP family has not diversified yet and TSKs have not evolved, limiting the uses of this tool kit. This very primitive version of the writer/reader/eraser system is currently exemplified by the situation in Slime mold *Dictostelium discoideum*. In stage 3 of the evolution, TSK domains evolve and expand and with concomitant expansion of both the PTP and the SH2

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leads to a writer/reader/eraser system that allows a novel way of signaling based on tyrosine phosphorylation. It is only when TSK domains evolve that the full functionality of the PTP and SH2 domains can be exploited. The benefit of a pTyr specific signaling system that is based on three newly evolved domains is that it could have been implemented on top of existing signaling cascades without interfering with existing signaling networks and would have allowed new and complex signaling input to be transduced. Stage three is exemplified by the current state in metazoans and Choanoflagellates (Lim and Pawson, 2010).

When we survey the available plant genomics data it is immediately apparent that plants have not evolved along the three stage of the writer/reader/eraser system. Plants have only a few PTPs and four proteins with predicted SH2 domains (de la Fuente van Bentem and Hirt, 2009; Williams and Zvelebil, 2004) and few TSKs (Miranda-Saavedra and Barton, 2007). In this respect plants resemble yeast cells, although the latter do not have TSKs, but these may have been lost in the fungal lineage since diverging from the last common eukaryotic ancestor. However since the relative amount of tyrosine phosphorylation is not significantly different from animal systems, plants may have evolved a different strategy to read and write pTyr modifications. In this respect it is interesting that an additional pTyr binding domain has recently been identified in protein kinase C delta from mammalian cells (Miranda-Saavedra and Barton, 2007). Domains similar to C2 domains have also been identified in a substantial number of plant proteins, but the functionality as pTyr binding has yet to be tested (Miranda-Saavedra and Barton, 2007). The observation that C2 domains may be present in plants does leave room for an altogether different system to read and write tyrosine phosphorylation in plants. In such as system the reading could be mediated by C2 domains, the writing by DSK domains and the erasing could be done by PTP domains. To constitute a functional alternative reader/writer/eraser system for pTyr signaling in plants, C2 domains would be expected to be in proteins with either phosphatase domains or kinase domains. However, C2 domains have not been identified in association with either domain in the same protein. Yet another interpretation could be that instead of developing a specific tyrosine phophorylation tool kit to add additional complexity to signaling networks, plant may have chosen to diversify their protein kinase tool kit, resulting also in increase numbers of DSKs and thus more tyrosine phosphorylation. When we take Arabidopsis for example the repertoire of RLK is about 630 and total number of annotated kinases well over a thousand, consistent with diversification of the kinase family as compared to metazoans. This hypothesis implies that the use of tyrosine phosphorylation in plants has not evolved to fulfill a special regulatory role. The future challenge will be to obtain sufficiently large phosphoproteomic data sets, couple them to protein–protein interaction network analyses as well as computational motif analysis. This should point out in the near future whether a plant specific pTyr reader domain exist or whether plants made due with more kinase substrate connections to deal with complex signaling input.

8. Conclusion

The rapid development of the plant phospho-proteomic field is evident from the growing number of publications describing increasingly large-scale phops data sets. Almost all of these publications concern so called shotgun proteomic approaches aimed to discover as many new phosphorylation sites from plant cells from a specific tissue or under a specific condition. In addition to phosphorylation events that may be specific for the tissue or change under the selected condition, the fast majority of the identified phops will not necessarily be related to the question under investigation. Currently, the major challenge for the plant phospho-proteomic field is how to identify the relevant phosphorylation sites from the vast majority of phops. Implementation of quantitative approaches is an important development that will help to identify these relevant phosphorylation sites in proteins of interest to the investigator. A number of limitations of quantitative approaches are known to affect their success rate. First and foremost the ability to consistently observe and identify individual phops during replicate analysis. Since shot gun proteomic approaches use data dependent analysis to select precursor ions for tandem MS analysis the complexity of the mixture of peptide ions in the survey scan and as well as their relative abundance will determine whether a particular peptide ion is selected and identified. Variations in enrichment and fractionation between samples as well as between replicate analysis of the same sample affect this process. The inability to observe and identify a phop during tandem MS analysis is therefore not conclusive evidence of the absence of a phop from a particular sample. This has a significant impact on most commonly used relative quantification approaches as each phop has to be observed and quantified in both samples that are compared (Fig. 1). Similar limitations also apply to temporal analysis and are compounded when quantitative temporal analyses are undertaken. Improved enrichment and fractionation protocols have helped reduced complexity of the samples and enhanced the comprehensiveness of the phosphoproteome analysis. However no single enrichment strategy is capable of completely capturing the full complement of phops in any particular sample. Therefore, further development of improved enrichment strategies and development of novel enrichment strategies is still needed. Development of strategies using phospho-amino acid specific antibodies to specifically isolate phops is one potential novel approach that has been implemented for pTyr containing peptides. Currently this is not yet possible pSer or pThr due to the lack of suitable antibodies. Further development and implementation of fragmentation methods is another area that can help increase the comprehensiveness of phosphoproteome analysis, as recently shown (Grimsrud et al., 2010; Swaney et al., 2009) Fragmentation based on CAD is now complemented with newly developed ETD methodology and each method seems suitable for a specific subset of phops, but only some phops can be identified by both CAD and ETD. Parallel tandem MS analysis using both CAD and ETD based fragmentation will provide the most comprehensive phops identification and in cases where MS instruments can do both a decision tree-driven tandem MS algorithm as implemented by Swaney and colleagues (2009) seems very promising.

On the biological side the methodology to test the relevance of changes in phosphorylation is limiting. Functional analysis based on mutagenesis of phosphorylated residues in proteins is time consuming and is dependent on the availability of knock-out mutant lines to complement with loss- and gain-of-function substitution versions of phosphorylated proteins. These analyses can only be done on a case by case basis. However, it is possible to piece together a complete pathway as was shown for the brassinosteriod signal transduction pathway.

This effort will be aided by novel proteomics efforts, including selective reaction monitoring (SRM) which is a targeted MS approach (Domon and Aebersold, 2010). SRM is a relatively new targeted MS approach that selectively monitors and analysis predefined analytes (peptide ions) in complex mixtures. This makes it possible to focus only on changes in specific (phospho)proteins and ignore all the irrelevant peptide ions. SRM requires detailed pre-existing knowledge on all the reactions that are monitored, including chromatographic retention time of the peptide, m/z ratio of precursor ion and fragmentation induced product-ions for the specific instrumentation used (Domon and Aebersold, 2010). When al this data is acquired it is possible to use SRM to monitor al

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(phospho)proteins that may together constitute a pathway, as was recently done for all yeast kinases and phosphatases (Picotti et al., 2010). This targeted proteomics approach promises to be the next big thing for the (plant) phospho-proteomic field and in time may help to significantly advance plant signal transduction research beyond its current limitations.

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References

Andreasson, E., Ellis, B., 2010. Convergence and specificity in the Arabidopsis MAPK nexus. Trends Plant Sci. 15, 106–113.
Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., Sheen, J., 2002. MAP kinase signalling cascade in Arabidopsis innate immunity. Nature 415, 977–983.
Bartels, S., Besteiro, M.A., Lang, D., Ulm, R., 2010. Emerging functions for plant MAP kinase phosphatases. Trends Plant Sci. 15, 322–329.
Benschop, J.J., Mohammed, S., O'Flaherty, M., Heck, A.J.R., Slijper, M., Menke, F.L.H., 2007. Quantitative phosphoproteomics of early elicitor signaling in Arabidopsis. Mol. Cell. Proteomics 6, 1198–1214.
Bindschedler, L.V., Palmblad, M., Cramer, R., 2008. Hydroponic isotope labelling of entire plants (HILEP) for quantitative plant proteomics; an oxidative stress case study. Phytochemistry 69, 1962–1972.
Blagoev, B., Kratchmarova, I., Ong, S.E., Nielsen, M., Foster, L.J., Mann, M., 2003. A proteomics strategy to elucidate functional protein–protein interactions applied to EGF signaling. Nat. Biotechnol. 21, 315–318.
Blagoev, B., Ong, S.E., Kratchmarova, I., Mann, M., 2004. Temporal analysis of phosphotyrosine-dependent signaling networks by quantitative proteomics. Nat. Biotechnol. 22, 1139–1145.
Bodenmiller, B., Mueller, L.N., Mueller, M., Domon, B., Aebersold, R., 2007. Reproducible isolation of distinct, overlapping segments of the phosphoproteome. Nat. Methods 4, 231–237.
Boersema, P.J., Mohammed, S., Heck, A.J., 2009a. Phosphopeptide fragmentation and analysis by mass spectrometry. J. Mass Spectrom. 44, 861–878.
Boersema, P.J., Raijmakers, R., Lemeer, S., Mohammed, S., Heck, A.J., 2009b. Multiplex peptide stable isotope dimethyl labeling for quantitative proteomics. Nat. Protoc. 4, 484–494.
Brandman, O., Meyer, T., 2008. Feedback loops shape cellular signals in space and time. Science 322, 390–395.
Chen, Y., Hoehenwarter, W., Weckwerth, W., 2010. Comparative analysis of phytohormone-responsive phosphoproteins in Arabidopsis thaliana using TiO_2-phosphopeptide enrichment and mass accuracy precursor alignment. Plant J. 63, 1–17.
Choudhary, C., Mann, M., 2010. Decoding signalling networks by mass spectrometry-based proteomics. Nat. Rev. Mol. Cell Biol. 11, 427–439.
Dardick, C., Chen, J., Richter, T., Ouyang, S., Ronald, P., 2007. The rice kinase database. A phylogenomic database for the rice kinome. Plant Physiol. 143, 579–586.
de la Fuente van Bentem, S., Anrather, D., Dohnal, I., Roitinger, E., Csaszar, E., Joore, J., Buijnink, J., Carreri, A., Forzani, C., Lorkovic, Z.J., Barta, A., Lecourieux, D., Verhounig, A., Jonak, C., Hirt, H., 2008. Site-specific phosphorylation profiling of Arabidopsis proteins by mass spectrometry and peptide chip analysis. J. Proteome Res. 7, 2458–2470.
de la Fuente van Bentem, S., Hirt, H., 2007. Using phosphoproteomics to reveal signalling dynamics in plants. Trends Plant Sci. 12, 404–411.
de la Fuente van Bentem, S., Hirt, H., 2009. Protein tyrosine phosphorylation in plants: more abundant than expected? Trends Plant Sci. 14, 71–76.
Domon, B., Aebersold, R., 2010. Options and considerations when selecting a quantitative proteomics strategy. Nat. Biotechnol. 28, 710–721.
Ficarro, S.B., McCleland, M.L., Stukenberg, P.T., Burke, D.J., Ross, M.M., Shabanowitz, J., Hunt, D.F., White, F.M., 2002. Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. Nat. Biotechnol. 20, 301–305.
Ghelis, T., Bolbach, G., Clodic, G., Habricot, Y., Miginiac, E., Sotta, B., Jeannette, E., 2008. Protein tyrosine kinases and protein tyrosine phosphatases are involved in Abscisic acid-dependent processes in Arabidopsis seeds and suspension cells. Plant Physiol. 148, 1668–1680.
Grimsrud, P.A., den Os, D., Wenger, C.D., Swaney, D.L., Schwartz, D., Sussman, M.R., Ane, J.M., Coon, J.J., 2010. Large-scale phosphoprotein analysis in Medicago truncatula roots provides insight into in vivo kinase activity in legumes. Plant Physiol. 152, 19–28.
Gruhler, A., Schulze, W.X., Matthiesen, R., Mann, M., Jensen, O.N., 2005. Stable isotope labeling of Arabidopsis thaliana cells and quantitative proteomics by mass spectrometry. Mol. Cell. Proteomics 4, 1697–1709.
Heazlewood, J.L., Durek, P., Hummel, J., Selbig, J., Weckwerth, W., Walther, D., Schulze, W.X., 2008. PhosPhAt: a database of phosphorylation sites in Arabidopsis thaliana and a plant-specific phosphorylation site predictor. Nucleic Acids Res. 36, D1015–1021.
Humberto, M., Marta, F., César, N., María, M., 2005. Protein phosphatases in MAPK signalling: we keep learning from yeast. Mol. Microbiol. 58, 6–16.
Initiative, A.G., 2000. Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature 408, 796–815.
Jin, H., Axtell, M.J., Dahlbeck, D., Ekwenna, O., Zhang, S., Staskawicz, B., Baker, B., 2002. NPK1, an MEKK1-like mitogen-activated protein kinase kinase kinase, regulates innate immunity and development in plants. Dev. Cell 3, 291–297.
Jin, M., Diaz, P.T., Bourgeois, T., Eng, C., Marsh, C.B., Wu, H.M., 2006. Two-dimensional gel proteome reference map of blood monocytes. Proteome Sci. 4, 16.
Jones, A.M., MacLean, D., Studholme, D.J., Serna-Sanz, A., Andreasson, E., Rathjen, J.P., Peck, S.C., 2009. Phosphoproteomic analysis of nuclei-enriched fractions from Arabidopsis thaliana. J. Proteom. 72, 439–451.
Jung, K.H., Cao, P., Seo, Y.S., Dardick, C., Ronald, P.C., 2010. The rice kinase phylogenomics database: a guide for systematic analysis of the rice kinase super-family. Trends Plant Sci. 15, 595–599.
Kerk, D., Conley, T.R., Rodriguez, F.A., Tran, H.T., Nimick, M., Muench, D.G., Moorhead, G.B., 2006. A chloroplast-localized dual-specificity protein phosphatase in Arabidopsis contains a phylogenetically dispersed and ancient carbohydrate-binding domain, which binds the polysaccharide starch. Plant J. 46, 400–413.
Kersten, B., Agrawal, G.K., Iwahashi, H., Rakwal, R., 2006. Plant phosphoproteomics: a long road ahead. Proteomics 6, 5517–5528.
Kersten, B., Agrawal, G.K., Durek, P., Neigenfind, J., Schulze, W., Walther, D., Rakwal, R., 2009. Plant phosphoproteomics: an update. Proteomics 9, 964–988.
Kim, T.-W., Wang, Z.-Y., 2010. Brassinosteroid signal transduction from receptor kinases to transcription factors. Annu. Rev. Plant Biol. 61, 681–704.
Kovtun, Y., Chiu, W.L., Tena, G., Sheen, J., 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proc. Natl. Acad. Sci. USA 97, 2940–2945.
Larsen, M.R., Thingholm, T.E., Jensen, O.N., Roepstorff, P., Jorgensen, T.J., 2005. Highly selective enrichment of phosphorylated peptides from peptide mixtures using titanium dioxide microcolumns. Mol. Cell. Proteomics 4, 873–886.
Lemeer, S., Heck, A.J., 2009. The phosphoproteomics data explosion. Curr. Opin. Chem. Biol. 13, 414–420.
Lemeer, S., Ruijtenbeek, R., Pinkse, M.W.H., Jopling, C., Heck, A.J.R., den Hertog, J., Slijper, M., 2007. Endogenous phosphotyrosine signaling in zebrafish embryos. Mol. Cell. Proteomics 6, 2088–2099.
Lim, W.A., Pawson, T., 2010. Phosphotyrosine Signaling: Evolving a New Cellular Communication System 142, 661–667
Linding, R., Jensen, L.J., Ostheimer, G.J., van Vugt, M.A., Jorgensen, C., Miron, I.M., Diella, F., Colwill, K., Taylor, L., Elder, K., Metalnikov, P., Nguyen, V., Pasculescu, A., Jin, J., Park, J.G., Samson, L.D., Woodgett, J.R., Russell, R.B., Bork, P., Yaffe, M.B., Pawson, T., 2007. Systematic discovery of in vivo phosphorylation networks. Cell 129, 1415–1426.
Luan, S., 2002. Tyrosine phosphorylation in plant cell signaling. Proc. Natl. Acad. Sci. USA 99, 11567–11569.
Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., Tanksley, S.D., 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262, 1432–1436.
Menke, F.L., Kang, H.G., Chen, Z., Park, J.M., Kumar, D., Klessig, D.F., 2005. Tobacco transcription factor WRKY1 is phosphorylated by the MAP kinase SIPK and mediates HR-like cell death in tobacco. Mol. Plant Microbe. Interact. 18, 1027–1034.
Menke, F.L., Parchmann, S., Mueller, M.J., Kijne, J.W., Memelink, J., 1999. Involvement of the octadecanoid pathway and protein phosphorylation in fungal elicitor-induced expression of terpenoid indole alkaloid biosynthetic genes in *Catharanthus roseus*. Plant Physiol. 119, 1289–1296.
Menke, F.L., van Pelt, J.A., Pieterse, C.M., Klessig, D.F., 2004. Silencing of the mitogen-activated protein kinase MPK6 compromises disease resistance in Arabidopsis. Plant Cell 16, 897–907.
Merkouropoulos, G., Andreasson, E., Hess, D., Boller, T., Peck, S.C., 2008. An Arabidopsis protein phosphorylated in response to microbial elicitation, AtPHOS32, is a substrate of MAP kinases 3 and 6. J. Biol. Chem. 283, 10493–10499.
Miranda-Saavedra, D., Barton, G.J., 2007. Classification and functional annotation of eukaryotic protein kinases. Proteins 68, 893–914.
Moorhead, G.B., De Wever, V., Templeton, G., Kerk, D., 2009. Evolution of protein phosphatases in plants and animals. Biochem. J. 417, 401–409.
Nakagami, H., Sugiyama, N., Mochida, K., Daudi, A., Yoshida, Y., Toyoda, T., Tomita, M., Ishihama, Y., Shirasu, K., 2010. Large-scale comparative phosphoproteomics identifies conserved phosphorylation sites in plants. Plant Physiol. 153, 1161–1174.
Ndassa, Y.M., Orsi, C., Marto, J.A., Chen, S., Ross, M.M., 2006. Improved immobilized metal affinity chromatography for large-scale phosphoproteomics applications. J. Proteome Res. 5, 2789–2799.
Niittyla, T., Fuglsang, A.T., Palmgren, M.G., Frommer, W.B., Schulze, W.X., 2007. Temporal analysis of sucrose-induced phosphorylation changes in plasma membrane proteins of Arabidopsis. Mol. Cell. Proteomics 6, 1711–1726.
Nuhse, T.S., Bottrill, A.R., Jones, A.M.E., Peck, S.C., 2007. Quantitative phosphoproteomic analysis of plasma membrane proteins reveals regulatory mechanisms of plant innate immune responses. Plant J. 51, 931–940.
Nuhse, T.S., Peck, S.C., Hirt, H., Boller, T., 2000. Microbial elicitors induce activation and dual phosphorylation of the Arabidopsis thaliana MAPK 6. J. Biol. Chem. 275, 7521–7526.

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Nuhse, T.S., Stensballe, A., Jensen, O.N., Peck, S.C., 2003. Large-scale analysis of in vivo phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. Mol. Cell. Proteomics 2, 1234–1243.
Nuhse, T.S., Stensballe, A., Jensen, O.N., Peck, S.C., 2004. Phosphoproteomics of the Arabidopsis plasma membrane and a new phosphorylation site database. Plant Cell 16, 2394–2405.
Oh, M.H., Clouse, S.D., Huber, S.C., 2009a. Tyrosine phosphorylation in brassinosteroid signaling. Plant Signal Behav. 4.
Oh, M.H., Wang, X., Kota, U., Goshe, M.B., Clouse, S.D., Huber, S.C., 2009b. Tyrosine phosphorylation of the BRI1 receptor kinase emerges as a component of brassinosteroid signaling in Arabidopsis. Proc. Natl. Acad. Sci. USA 106, 658–663.
Olsen, J.V., Blagoev, B., Gnad, F., Macek, B., Kumar, C., Mortensen, P., Mann, M., 2006. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127, 635–648.
Peck, S.C., Nuhse, T.S., 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.
Picotti, P., Rinner, O., Stallmach, R., Dautel, F., Farrah, T., Domon, B., Wenschuh, H., Aebersold, R., 2010. High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. Nat. Methods 7, 43–46.
Pinkse, M.W., Uitto, P.M., Hilhorst, M.J., Ooms, B., Heck, A.J., 2004. Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns. Anal. Chem. 76, 3935–3943.
Pitzschke, A., Schikora, A., Hirt, H., 2009. MAPK cascade signalling networks in plant defence. Curr. Opin. Plant Biol. 12, 421–426.
Qiu, J.L., Fiil, B.K., Petersen, K., Nielsen, H.B., Botanga, C.J., Thorgrimsen, S., Palma, K., Suarez-Rodriguez, M.C., Sandbech-Clausen, S., Lichota, J., Brodersen, P., Grasser, K.D., Mattsson, O., Glazebrook, J., Mundy, J., Petersen, M., 2008. Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO J. 27, 2214–2221.
Reiland, S., Messerli, G., Baerenfaller, K., Gerrits, B., Endler, A., Grossmann, J., Gruissem, W., Baginsky, S., 2009. Large-scale Arabidopsis phosphoproteome profiling reveals novel chloroplast kinase substrates and phosphorylation networks. Plant Physiol. 150, 889–903.
Romeis, T., Ludwig, A.A., Martin, R., Jones, J.D., 2001. Calcium-dependent protein kinases play an essential role in a plant defence response. EMBO J. 20, 5556–5567.
Romeis, T., Piedras, P., Zhang, S., Klessig, D.F., Hirt, H., Jones, J.D., 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.
Scheel, D., 1998. Resistance response physiology and signal transduction. Curr. Opin. Plant Biol. 1, 305–310.
Schulze, W.X., 2010. Proteomics approaches to understand protein phosphorylation in pathway modulation. Curr. Opin. Plant Biol. 13, 280–287.
Schulze, W.X., Usadel, B., 2010. Quantitation in mass-spectrometry-based proteomics. Annu. Rev. Plant Biol. 61, 491–516.
Singh, A., Giri, J., Kapoor, S., Tyagi, A., Pandey, G., 2010. Protein phosphatase complement in rice: genome-wide identification and transcriptional analysis under abiotic stress conditions and reproductive development. BMC Genomics 11, 435.
Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C., Ronald, P., 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. Science 270, 1804–1806.
Stulemeijer, I.J., Joosten, M.H., Jensen, O.N., 2009. Quantitative phosphoproteomics of tomato mounting a hypersensitive response reveals a swift suppression of photosynthetic activity and a differential role for hsp90 isoforms. J. Proteome Res. 8, 1168–1182.
Sugiyama, N., Nakagami, H., Mochida, K., Daudi, A., Tomita, M., Shirasu, K., Ishihama, Y., 2008. Large-scale phosphorylation mapping reveals the extent of tyrosine phosphorylation in Arabidopsis. Mol. Syst. Biol. 4.
Swaney, D.L., Wenger, C.D., Thomson, J.A., Coon, J.J., 2009. Human embryonic stem cell phosphoproteome revealed by electron transfer dissociation tandem mass spectrometry. Proc. Natl. Acad. Sci. USA 106, 995–1000.
Tang, W., Deng, Z., Oses-Prieto, J.A., Suzuki, N., Zhu, S., Zhang, X., Burlingame, A.L., Wang, Z.Y., 2008a. Proteomics studies of brassinosteroid signal transduction using prefractionation and two-dimensional DIGE. Mol. Cell. Proteomics 7, 728–738.
Tang, W., Kim, T.-W., Oses-Prieto, J.A., Sun, Y., Deng, Z., Zhu, S., Wang, R., Burlingame, A.L., Wang, Z.-Y., 2008b. BSKs mediate signal transduction from the receptor kinase BRI1 in Arabidopsis. Science 321, 557–560.
Tannu, N.S., Hemby, S.E., 2006. Two-dimensional fluorescence difference gel electrophoresis for comparative proteomics profiling. Nat. Protoc. 1, 1732–1742.
Thelen, J.J., Peck, S.C., 2007. Quantitative proteomics in plants: choices in abundance. Plant Cell 19, 3339–3346.
Thingholm, T.E., Jensen, O.N., Larsen, M.R., 2009. Analytical strategies for phosphoproteomics. Proteomics.
Tonge, R., Shaw, J., Middleton, B., Rowlinson, R., Rayner, S., Young, J., Pognan, F., Hawkins, E., Currie, I., Davison, M., 2001. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. Proteomics 1, 377–396.
Williams, J.G., Zvelebil, M., 2004. SH2 domains in plants imply new signalling scenarios. Trends Plant Sci. 9, 161–163.
Zhang, S., Du, H., Klessig, D.F., 1998. Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora* spp.. Plant Cell 10, 435–450.
Zhang, S., Klessig, D.F., 1997. Salicylic acid activates a 48-kD MAP kinase in tobacco. Plant Cell 9, 809–824.

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