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Using phosphoproteomics to reveal signalling dynamics in plants

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To ensure appropriate responses to stimuli, organisms have evolved signalling networks that rely on post-translational modifications of their components. Among these, protein phosphorylation has a prominent role and much research in plants has focused on protein kinases and phosphatases, which, respectively, catalyse phosphorylation and dephosphorylation of specific substrates. Technical limitations, however, have hampered the identification of these substrates. As reviewed here, novel mass spectrometry-based techniques have enabled the large-scale mapping of *in vivo* phosphorylation sites. Alternatively, methods based on peptide and protein microarrays have revealed protein kinase activities in cell extracts, in addition to kinase substrates. A combined phosphoproteomic approach of mass spectrometry and microarray technology could enhance the construction of dynamic plant signalling networks that underlie plant biology.

Reversible phosphorylation of proteins

In all organisms, proteins can occur in numerous isoforms that each carry different post-translational modifications. Hundreds of post-translational modifications (http://www.expasy.org/cgi-bin/ptmlist.pl), such as phosphorylation, acetylation, glycosylation and ubiquitylation, amplify the complexity of proteomes. Among all post-translational modifications, phosphorylation has been studied most intensively [1].

Studies suggest that one-third of all proteins are modified by phosphorylation [2]. The phosphorylation of specific sites in proteins can have major effects on protein structure that underlie changes in enzyme activity, substrate specificity, choice of binding partners, intracellular localization and protein stability. Each protein can be modified by different protein kinases at multiple sites, which enable dynamic regulation of its function [3]. In many signalling pathways, reversible phosphorylation is essential for the maintenance of signalling amplitude, duration and specificity.

Plants have >1000 protein kinases [4,5], approximately twice the number found in mammals [6]. Although plant genomes encode many of the animal-type kinases and hundreds of receptor-like kinases (RLKs), they lack the typical animal transmembrane receptor-like tyrosine kinases. Plant protein kinases regulate multiple processes, including metabolism [7,8], the cell cycle [9], stress and hormone responses [7,10–14], stomatal closure and development [15,16], and cytokinesis [17].

Because substrates for few plant protein kinases have been identified, the view of signalling pathways and their interconnections is fragmented. The need for large-scale approaches to elucidate complex signalling networks has caused a technological development that has led to improvement in our understanding of signalling pathways in yeast and animals [3]. Although these techniques are rarely used for plant research, striking discoveries have already been made. Here, we focus on mass spectrometry- and peptide and protein chip-based methods, and their potential to unravel plant signal transduction pathways. These techniques have advantages and drawbacks, but we propose that a combination of all these methods could be used as a systematic approach to advance our understanding of the signalling networks that regulate diverse processes in plants.

Large-scale identification of *in vivo* phosphorylation sites by mass spectrometry

Until recently, mapping phosphorylation sites in a protein was difficult and tedious. However, this situation changed as a result of the development of a novel method that opened the way for large-scale phosphorylation site mapping [18]. This method consists in the isolation of phosphopeptides by immobilized metal affinity chromatography (IMAC) followed by mass spectrometric analysis, and enables hundreds of *in vivo* phosphorylation sites to be mapped to specific residues. Mass spectrometry is a powerful way to analyse (phospho)peptides [19,20] or even entire proteins [21], and a new generation of highly sensitive and accurate mass spectrometers have facilitated the sequencing of phosphopeptides. Phosphoproteomic approaches using these machines have recently culminated in the description of >5500 sites [22,23]. Other researchers have used an optimized protocol to identify 1252 phosphorylation sites from as little as 30 μg of yeast protein [24]. The corresponding yeast proteins that were found to be phosphorylated are expressed at a level of <50 to 1 200 000 copies per cell, indicating that the technique enables the detection of low abundant proteins [24].

Although mass spectrometry is a useful technique for large-scale phosphopeptide analysis, it currently does not give full coverage of the phosphoproteome because, for

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Table 1. Phosphopeptide and phosphoprotein isolation methods used in plants

Method	Species	Subcellular compartment	Number of phosphorylation sites identified	Refs
TiO_2	Spinach *(Spinacia oleracea)*	Stroma membrane	7	[61]
SCX-TiO_2	*Arabidopsis*	Plasma membrane	>1000	[27]
IMAC	*Arabidopsis*	Thylakoid membrane	5	[62]
	Arabidopsis	Thylakoid membrane	3	[63]
	Arabidopsis	Nucleus and cytosol	7	[64]
	Arabidopsis	Nucleus and cytosol	79	[65]
	Arabidopsis and *Medicago truncatula*	Cytosol	2 and 3	[66]
	Physcomitrella patens	Total cell lysate	>112	[67]
	Chlamydomonas reinhardtii	Total cell lysate	>328	[68]
	Chlamydomonas reinhardtii	Thylakoid membranes	1	[69]
	Chlamydomonas reinhardtii	Thylakoid membranes	8	[70]
	Chlamydomonas reinhardtii	Thylakoid membranes	19	[71]
	Chlamydomonas reinhardtii	Thylakoid membranes	4	[72]
SAX-IMAC	*Arabidopsis*	Plasma membrane	>300	[35]
MOAC	*Arabidopsis*	Total cell lysate	16	[73]

Abbreviations: IMAC, immobilized metal affinity chromatography; MOAC, metal oxide affinity chromatography (with, in this case, aluminium hydroxide [$Al(OH)_3$]); SAX, strong anionic exchange; SCX, strong cationic exchange; TiO_2, titanium dioxide.

example, phosphopeptide fractions derived from whole cell extracts are too complex to resolve on a mass spectrometer. Organelle purification [25–27] and prefractionation of protein or peptide samples [22,23,26–28] are required to increase coverage. For instance, target class-specific purification identified many novel phosphorylation sites in human protein kinases that were not found in large-scale phosphoproteomic analyses [29]. Several techniques have been used in plants to identify phosphoproteins and phosphorylation sites (Table 1). Other studies have focused on the identification of phosphoproteins rather than that of phosphorylation sites, and revealed components of phosphorylation cascades participating in seed filling and pathogen defense [30–32].

Different techniques for the enrichment of phosphopeptides and phosphoproteins have been described, of which IMAC and titanium dioxide (TiO_2) are two of the most successful ones [3]. Although these methods have been used to unravel phosphoproteomes of yeast, animals, plants and a bacterium [3,33], each method is biased towards a subfraction of the phosphoproteome [34]. Thus, for complete coverage of the phosphoproteome, different methods have to be combined. Several studies have been done on plants but have often described only a few sites (Table 1). The first large-scale plant study demonstrated that many RLKs are phosphorylated inside and outside their kinase domains [35]. Importantly, phosphorylation sites in *Arabidopsis* proteins are often conserved in orthologues of other plant species [35], suggesting that the knowledge gained from this model plant is transferable to crop species [36].

Quantitative mass spectrometry-based phosphoproteomics

A quest for mass spectrometry-based methods that enable the quantification of phosphopeptides has resulted in several useful techniques. The iTRAQ^{TM} Reagent [37,38] and stable isotope labelling by amino acids in cell culture (SILAC) [39] have been used most successfully. These techniques label peptides at the final stage before mass spectrometry *in vitro* or label proteins during cell growth *in vivo*, respectively, and enable the measurement of changes of individual phosphorylation sites during a time-course of a particular treatment or during different treatments. By using a SILAC approach, Olsen *et al.* [22] recently unravelled the phosphorylation cascade downstream of the human epidermal growth factor (EGF) receptor, identifying 6600 phosphorylation sites and following through a time-course of 20 min after receptor activation. The entire (known) kinase cascade from the membrane receptor to the nucleus was visualized, leading to the differential phosphorylation of hundreds of proteins, including components of vesicle trafficking, the ubiquitylation machinery and, ultimately, multiple transcription factors. Although not comprehensive, this study revealed many novel links for the already well-studied EGF signalling pathway, demonstrating the power of quantitative mass spectrometry.

In a recent paper [27], a quantitative mass spectrometry-based approach revealed the first large-scale analysis of phosphorylation events on plasma membrane proteins downstream of two receptors in *Arabidopsis*. One receptor is FLAGELLIN SENSING 2 (FLS2) that recognizes the bacterial flagellin-derived peptide flg22, the second is the receptor for the fungal elicitor xylanase [27]. Quantification was based on metabolic labelling of *Arabidopsis* cells with either 'light' $^{14}NO_3$ or 'heavy' $^{15}NO_3$, which are taken up and incorporated as ^{14}N or ^{15}N into amino acids, respectively. This technique enables peptides derived from control (^{15}N-labelled) versus stress-treated cells (^{14}N-labelled) to be compared. The mass spectrometric approach identified one or more changing phosphorylation sites (either increased or decreased in abundance) in 76 plasma membrane-associated proteins, revealing novel *in vivo* phosphorylation events that occur after pathogen perception in plants [27]. Together with previous reports [40–42], this research enables the construction of the first map of *in vivo* phosphorylation events in plants (a subset of the sites changing after FLS2 activation is shown in Box 1). Although certainly not comprehensive, these data largely confirm the model proposed by Gómez-Gómez and Boller [43], which demonstrates, for instance, the phosphorylation of NADPH oxidase D (RBOHD) (Box 1). Benschop *et al.* [27] showed that flg22 also induces changes in the phosphorylation of specific ion channels and pumps, protein kinases and vesicular trafficking machineries. FLS2 activation triggers the differential phosphorylation of three auxin transporters, which

suggests a direct link between pathogen signalling and auxin transport. Taken together with an earlier report [44], this mass spectrometry-based study provides insight into what is happening at the plasma membrane. The next step could be the elucidation of intracellular signalling events downstream of FLS2 compared to other plasma membrane receptors, such as the brassinosteroid hormone receptor BRASSINOSTEROID INSENSITIVE 1.

Forward genetics has revealed components of signalling pathways, but is hampered by redundancy and lethality. Quantitative phosphoproteomics could reveal a major part of signalling events within a pathway and includes redundant components, and components that are required for viability. Moreover, snapshots of the *in vivo* phosphoproteome of a plant can be taken at different time points after application of a stimulus. These benefits are revealed by the work by Benschop *et al*. [27], which demonstrates that protein phosphorylation often occurs on family members that might have otherwise been missed by classical genetic screens for components of signalling pathways. In fact, components of the cascade downstream of FLS2 have been suggested mainly by observations of changing ion fluxes and by using kinase and phosphatase inhibitors. Verification of the biological significance of the phosphorylation events has to prove the many hypotheses generated by phosphoproteomic research in plants.

Identification of protein kinase substrates and activities by peptide and protein microarrays

In addition to the mass spectrometry-based approaches, peptide and protein arrays have also been used to study signalling pathways in animal and yeast research. A combination of protein and peptide chips has revealed the substrate specificity of most yeast protein kinases [45]. Using yeast proteome chips, protein kinases show remarkable specificity in *in vitro* kinase reactions [46]. By testing 87 protein kinases individually on ~4400 proteins on the chip, on average 47 proteins were phosphorylated [46]. Even though not all of the 122 potential yeast protein kinases were tested, out of the 4400 proteins tested, a total of 1325 were phosphorylated, close to the estimation that approximately one-third of eukaryotic proteomes are phosphorylated [2]. Importantly, related protein kinases phosphorylated different sets of proteins, suggesting that protein chips are useful tools in the identification of specific protein kinase substrates [46].

Peptide and protein arrays can be used, for example, for kinase profiling and identification of protein interaction partners [47,48]. Two plant protein array-based studies have suggested potential casein kinase 2 (CK2) and mitogen-activated protein kinase (MAPK) substrates [49,50]. By testing 768 proteins as substrates for CK2 and 1690 for the MAPKs, MPK3 and MPK6, a few known and many novel potential substrates were identified [49,50]. Moreover, the comparison of substrate specificities of the closely related MPK3 and MPK6 kinases showed that they have largely overlapping but also distinct sets of substrates [51]. Further research is needed to evaluate the *in vivo* significance of these putative substrates. Although protein arrays have the advantage of enabling high-throughput analysis of protein kinases, a drawback is that phosphorylation site identification is only possible with specific antibodies against the phosphorylated epitope (Table 2).

There are many software applications available that predict upstream kinases for substrate phosphorylation sites, however, they rely on data from non-plant systems

Box 1. Early phosphorylation events during flagellin signalling in *Arabidopsis*

Phosphorylation events that are induced or reduced during bacterial flg22-induced signalling [27,40–42] are shown in Figure I (see next page). Changes in phosphorylation states were caused directly or indirectly by signalling induced by the activation of the receptor-like kinase (RLK) FLS2. Several observed phosphorylations could explain previously suggested links between flg22-activated defence responses [43]. For instance, phosphorylation of the NADPH oxidase subunit D (RBOHD/At5g47910) might trigger the production of reactive oxygen species that are associated with pathogen defence. Phosphorylation of the autoinhibited Ca^{2+}-ATPase 10 (ACA10/At4g29900) might block its activation by calmodulin [74], potentially leading to increased cytosolic calcium concentration and the activation of calcium-dependent protein kinases (CDPKs). An increased calcium concentration might activate the two CDPKs CPK7 (At5g12480) and CPK10 (At1g18890) that are differentially phosphorylated after FLS2 activation [27]. In the cytosol, PHOS43 (At2g17390) and MAP kinase 6 (MPK6/At2g43790) become phosphorylated on yet unidentified site(s) within three minutes after flg22 application [41,42]. BOTRYTIS-INDUCED KINASE 1 (BIK1/At2g39660), which is involved in pathogen defence [75], is phosphorylated in its N-terminal myristoylation motif, which might affect its plasma membrane localization. The differential phosphorylation of two cellulose synthase subunits CESA1 (At4g32410) and CESA3 (At5g05170) suggests a possible link between flg22 signalling and cell wall synthesis.

Several potential regulators of vesicular trafficking are differentially phosphorylated during early flg22 signalling [27,40]. The phosphoserine in ScD (At1g61250) replaces a conserved asparagine in the third Asn-Pro-Phe (NPF) motif conserved in non-plant secretory carrier membrane proteins (SCAMPs), whereas the phosphoserine in ScC (At2g20840) replaces the proline in the first NPF motif [76]. ScC and ScD are named according to Fernández-Chacón and Südhof [76], and conserved and non-conserved NPF repeats in ScC and ScD are indicated in black and grey, respectively (Figure I). NPF motifs recruit Eps15 homology (EH) domain proteins that function in endocytosis in animals [77], but whether this occurs in plants in unknown. An EH domain-containing protein (At1g21630) was also found to be phosphorylated [27]. Therefore, it can be speculated that phosphorylation of ScC and ScD regulates their interaction with this EH domain protein and, consequently, endocytosis of signalling components such as FLS2. The plasma membrane syntaxin SYP122 (At3g53400) is a potential regulator of exocytosis that becomes phosphorylated within three minutes after FLS2 activation [40] (although SYP122 probably contains three phosphorylation sites, only one is depicted because one does not know how many are induced). SYP122 is phosphorylated *in vitro* by a calcium-dependent kinase [40], which might be CPK7 or CPK10. For reasons of simplicity, ScC and ScD are not drawn in the intracellular vesicles, where they will probably also localize. The auxin transporters P-GLYCOPROTEIN 1 (PGP1/At2g36910), PINFORMED 1 (PIN1/At1g73590) and PIN6 (At1g77110) become differentially phosphorylated by flg22 treatment. ABC transporters were named according to Sanchez-Fernandez *et al.* [78]. For further details and other responding phosphoproteins in the pathways, see Ref. [27]. Abbreviations: AHA1, *Arabidopsis thaliana* H^+-ATPase 1 (At2g18960); ATH14, ABC2 HOMOLOGUE 14 (At5g61740); MDR13, MULTIDRUG RESISTANCE 13 (At3g28345); PEN3, PENETRATION 3 (PDR8/At1g59870); PM, plasma membrane; PDR7, PLEIOTROPIC DRUG RESISTANCE 7 (At1g15210); WBC3, WHITE-BROWN COMPLEX HOMOLOGUE 3 (At2g28070).

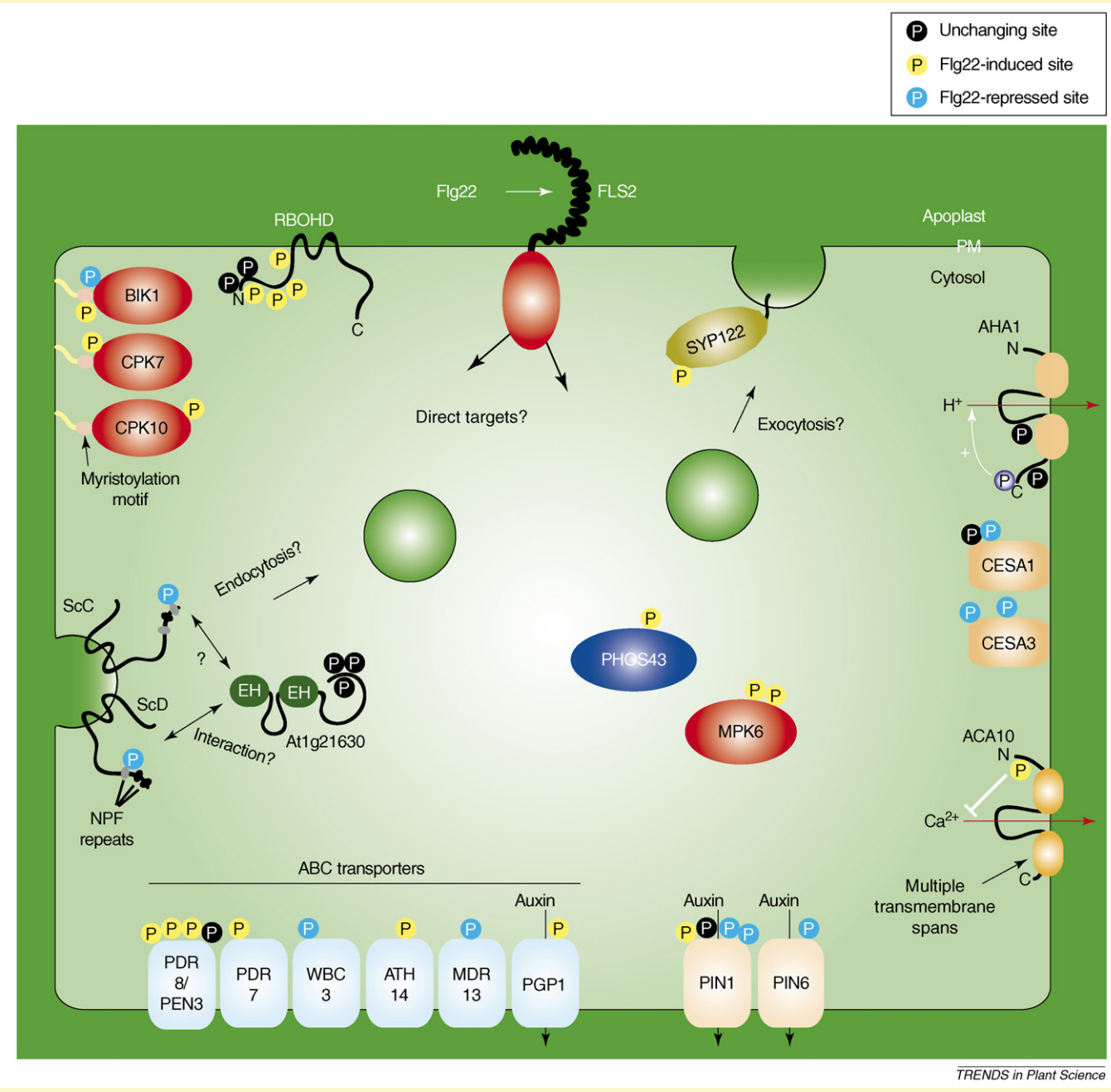

Figure I (Box 1). Early phosphorylation events during flagellin signalling in *Arabidopsis*.

and, thus, give only suboptimal predictions with unacceptable confidence levels. This limitation leaves plant researchers with the difficult task of validating kinase–substrate pairs experimentally. However, as revealed by animal and yeast work, many kinases phosphorylate conserved motifs. Therefore, mass spectrometric and protein chip analyses can be informative about the kinases that are upstream of the identified phosphorylation sites. Moreover, a novel approach has been developed that enables the prediction of the *in vivo* substrate specificity of protein

Table 2. Benefits and drawbacks of different large-scale techniques for phosphoproteomics

Method	*In vitro or in vivo*	Site specificity	Direct link kinase-substrate	High throughput quantitative analysis
Peptide chip	*In vitro*	Yes	Yes[a]	Yes
Protein chip	*In vitro*	Yes[b]	Yes[c]	Yes
Mass spectrometry-based	*In vivo*	Yes	No[d]	No

[a]If peptide is sufficient for recognition of the phosphorylation site, and if no scaffold is required.
[b]But only with specific antibodies against the phosphorylated epitope.
[c]If no scaffold is required.
[d]Although phosphorylation motifs might suggest the upstream kinase.

kinases [52]. One advantage of peptide and protein chips is that these kinases can subsequently be tested on the array, thereby enabling the direct discovery of kinase–substrate connections (Table 2). In animals, peptide chips have been used successfully to determine the substrate specificity of single protein kinases, for instance protein kinase A [53]. A large-scale inventory of the site specificity of multiple protein kinases using peptide chips might reveal specific phosphomotifs for different kinases. This will facilitate the process of coupling kinases to substrates identified by mass spectrometry, and enhance the development of bioinformatic programs that either predict substrates for a particular protein kinase or that predict a candidate kinase for an observed phosphorylation site.

Combining mass spectrometry-based methods with peptide microarrays to construct plant signalling pathways

A quantitative mass spectrometry-based approach such as that carried out recently for epidermal growth factor receptor (EGFR) signalling in animal cells [22] requires a large effort for any one condition under examination. An alternative approach to determine the activity of many protein kinases in a cell extract is the use of peptide arrays. One exciting application of this technique relies on, first, the extraction of proteins from cells subjected to a range of stresses and, second, the profiling of global kinase activities against a large number of known *in vivo* phosphorylation sites [53]. This method enables relatively rapid high-throughput quantitative analyses of different conditions (Table 2). This is in contrast to mass spectrometry approaches, which, although possible, still require substantial efforts to compare different samples quantitatively. That being said, mass spectrometry-based protocols using SILAC or iTRAQ^{TM} are being developed that facilitate the quantification of multiple samples in the same experiment.

A drawback of using peptide arrays is that the substrate specificity of some protein kinases (such as that of MAPKs) depends on a kinase-interaction motif that is spatially separated from the phosphorylation site. Thus, the use of peptides might not be easily compatible with substrate binding and substrate specificity of particular classes of protein kinases. However, the specificity of many kinases is

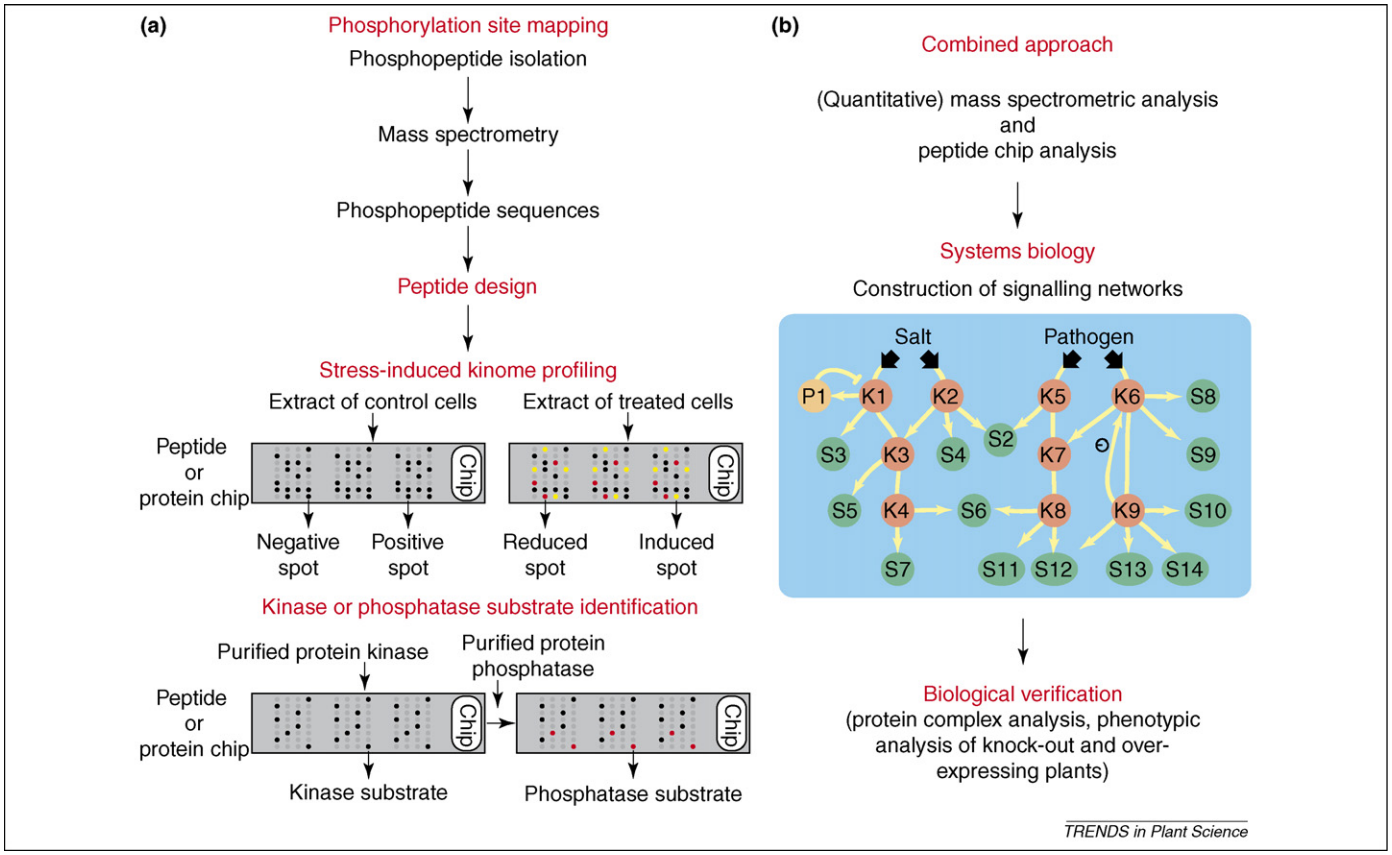

Figure 1. Combined large-scale approaches to unravel phosphorylation-driven signalling networks. **(a)** Combination of mass spectrometry-based and chip analyses to discover protein kinase and phosphatase substrates and activities. On chips, protein kinase activities are measured by the incorporation of radioactive phosphate into the substrate peptide or protein that are spotted in small amounts, in duplicate or triplicate (as shown here). Yellow and red spots indicate peptides or proteins that are more intensely and less intensely phosphorylated, respectively. Using cell extracts, a more intensely phosphorylated ('induced') spot means that a kinase activity in the treated extract towards the peptide or protein is activated. On the contrary, a less phosphorylated ('reduced') spot means that the responsible kinase is inactivated. In case of protein phosphatases, a chip prephosphorylated by a purified kinase (or, alternatively, a cell extract) could be used for target discovery. This is possible by analyzing which phosphorylated peptides or proteins are dephosphorylated on the chip, as indicated here by red spots. **(b)** Scheme for a possible experimental setup combining (quantitative) mass spectrometry-based phosphorylation site mapping and peptide chip analysis to construct signalling pathway structures and crosstalk between pathways. The experimental data, ultimately, need to be combined by systems biology analysis, which translates the separate, large-scale datasets into signalling networks [58]. The predicted connections within and between signalling cascades need to be experimentally verified by, for instance, analysis of protein complexes and analysis of kinase or substrate knockout and overexpressing plants. In the phosphorylation cascade, arrows indicate phosphorylation reactions and the circled minus sign indicates negative feedback phosphorylation. Only phosphoproteins in the signalling network are indicated. Abbreviations: K, protein kinase; P, protein phosphatase; S, substrate. Numbers behind each kinase, phosphatase and substrate are given arbitrarily.

determined by the amino acids immediately surrounding the phosphorylation site, enabling the use of peptides for specific substrate identification. The use of peptide and protein chip methods for quantitative phosphoproteomics could be complementary to mass spectrometry-based approaches. This is possible by constructing peptide arrays that represent large amounts of *in vivo* phosphorylation sites identified by mass spectrometry. Each of these sites could then function as individual markers to monitor specific signalling pathways in a cell, or even entire organisms, by subjecting them to a particular treatment. On the one hand, this could be achieved by using peptide and protein chips for phosphorylation assays using extracts of untreated and treated cells (or plants) (Figure 1a). The kinome activities of complex plant extracts have recently been monitored successfully using non-plant peptide chips [54]. On the other hand, peptide and protein chips could be used for discovery of targets and target motifs of individual protein kinases and phosphatases (Figure 1a). Substrates identified in these experiments might give clues to the function of the responsible kinases and phosphatases that can be tested by analysing kinase or substrate knockout or overexpressing plants. The use of chips for finding kinase and phosphatase targets might be complicated, because scaffold proteins can be required. Recently, however, optimal kinase phosphorylation motifs of plant MAPKs have been determined by using non-plant peptide chips [55]. This knowledge can be used to find and verify *in vivo* targets of kinases. Currently, no plant peptide chips are commercially available and will have to be developed to enhance plant research in this area.

A combined approach of (quantitative) mass spectrometry and peptide arrays that cover *in vivo* sites (Figure 1b) can be used to test which sites are phosphorylated by protein kinases individually applied on the array [56] or by cell extracts [53]. If an *in vitro* peptide chip approach suggests a signalling pathway, mass spectrometry data could be used to verify whether it occurs *in vivo*. Phosphoproteome examination by mass spectrometry can show which protein kinases are active, because the active state of a kinase is marked by phosphorylation in its activation loop. By following the activity profile of each kinase and the substrates that were suggested by peptide chip analysis, one can verify whether the phosphorylation profiles of the substrates coincide with the activation profiles of their predicted upstream kinases [22]. Therefore, phosphoproteomic analysis combining mass spectrometry-based and peptide array analyses might enable the construction of signalling pathways (Figure 1b).

Knowing kinase substrate specificities and phosphorylation sites (and their dynamics) is not enough to position all players within a signalling pathway. For instance, two related kinases might have the same targets on peptide chips but different or overlapping sets of substrates *in vivo*. Therefore, the knowledge of gene expression and subcellular localization is needed to couple kinase–substrate pairs *in vivo*. However, straightforward *in vivo* methods enable the verification of at least a portion of *in vitro*-identified connections between kinases and their substrates [44,46]. Evidently, signalling networks constructed by a systems biology approach that integrates datasets from large-scale experiments should be verified by testing individual components (Figure 1b). For instance, potential links between kinases and substrates can be examined by analyzing whether they are in the same protein complex *in vivo*. Such analysis has been performed successfully in yeast and confirmed many links suggested by a protein chip approach [57]. In conclusion, peptide chips could be used for high-throughput, large-scale profiling of phosphorylation sites as an independent but complementary technique to quantitative mass spectrometry to build plant signalling pathways.

Conclusions and prospects

Phosphoproteomics in plants has now moved from being descriptive [30] to becoming quantitative, by the use of mass spectrometry and peptide chips. Large-scale approaches combining mass spectrometry, peptide and protein chips for phosphorylation site-specific profiling and identification of protein kinase substrates are likely to reveal novel connections within signal transduction pathways. For reasons of complexity, systems biology approaches are required to construct complex signalling pathways and inter-pathway crosstalk [58]. We envisage that an integrative approach combining mass spectrometry-based mapping of phosphorylation sites with protein and peptide chip analysis will reveal protein kinases and phosphatases, their substrates and their positions within signalling webs (Figure 1).

As other post-translational modifications and mechanisms are also important regulators of signalling cascades, phosphoproteomic information should be combined with data from other studies to reach a comprehensive view of signalling networks. The integration of datasets from large-scale studies on protein–protein interactions and transcriptional regulation with phosphoproteomic data has revealed many novel signalling modules in yeast [57]. Also, in plants, future phosphoproteomic research, together with protein–protein interaction screens, gene expression profiling, chemical genetics and mutant screens, could yield insights into the complexity of entire signalling networks. Importantly, systems biologists are continuously developing programs to translate the massive datasets from large-scale approaches into an understandable picture [59]. In conclusion, the young field of phosphoproteomics has already uncovered many striking findings and promises to reveal many more unexpected signalling capabilities that transform plants from sessile into dynamic organisms.

Note added in proof

Nühse *et al*. [60] recently published flg22-induced changes in the *Arabidopsis* phosphoproteome.

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