Using phosphoproteomics to reveal signalling dynamics in plants

Sergio de la Fuente van Bentem¹ and Heribert Hirt¹,²

¹ Department of Plant Molecular Biology, Max F. Perutz Laboratories, University of Vienna, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria
² URGV Plant Genomics, 2 rue Gaston Cremieux, F-91057 Evry, France

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
Table 1. Phosphopeptide and phosphoprotein isolation methods used in plants

<table>
<thead>
<tr>
<th>Method</th>
<th>Species</th>
<th>Subcellular compartment</th>
<th>Number of phosphorylation sites</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TiO₂</td>
<td>Spinach (Spinacia oleracea)</td>
<td>Stroma membrane</td>
<td>7</td>
<td>[61]</td>
</tr>
<tr>
<td>SCX-TiO₂</td>
<td>Arabidopsis</td>
<td>Plasma membrane</td>
<td>&gt;1000</td>
<td>[27]</td>
</tr>
<tr>
<td>IMAC</td>
<td>Arabidopsis</td>
<td>Thylakoid membrane</td>
<td>5</td>
<td>[62]</td>
</tr>
<tr>
<td></td>
<td>Arabidopsis</td>
<td>Thylakoid membrane</td>
<td>3</td>
<td>[63]</td>
</tr>
<tr>
<td></td>
<td>Arabidopsis</td>
<td>Nucleus and cytosol</td>
<td>7</td>
<td>[64]</td>
</tr>
<tr>
<td></td>
<td>Arabidopsis</td>
<td>Nucleus and cytosol</td>
<td>79</td>
<td>[65]</td>
</tr>
<tr>
<td></td>
<td>Arabidopsis and Medicago truncatula</td>
<td>CYtosol</td>
<td>2 and 3</td>
<td>[66]</td>
</tr>
<tr>
<td></td>
<td>Physcomitrella patens</td>
<td>Total cell lysate</td>
<td>&gt;112</td>
<td>[67]</td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii</td>
<td>Total cell lysate</td>
<td>&gt;328</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii</td>
<td>Thylakoid membranes</td>
<td>1</td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii</td>
<td>Thylakoid membranes</td>
<td>8</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii</td>
<td>Thylakoid membranes</td>
<td>19</td>
<td>[71]</td>
</tr>
<tr>
<td></td>
<td>Chlamydomonas reinhardtii</td>
<td>Thylakoid membranes</td>
<td>4</td>
<td>[72]</td>
</tr>
<tr>
<td>SAX-IMAC</td>
<td>Arabidopsis</td>
<td>Plasma membrane</td>
<td>&gt;300</td>
<td>[35]</td>
</tr>
<tr>
<td>MOAC</td>
<td>Arabidopsis</td>
<td>Total cell lysate</td>
<td>16</td>
<td>[73]</td>
</tr>
</tbody>
</table>

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; TiO₂, 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 (TiO₂) 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 subclassification 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™ 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’¹⁴NO₃ or ‘heavy’¹⁵NO₃, which are taken up and incorporated as ¹⁴N or ¹⁵N into amino acids, respectively. This technique enables peptides derived from control (¹⁵N-labelled) versus stress-treated cells (¹⁴N-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 phosphorylation sites of many proteins, including receptors, kinases, and other enzymes involved in signal transduction pathways. These arrays have the advantage of enabling high-throughput analysis of protein kinases, a drawback is that phosphorylation site prediction is only possible with specific antibodies. Further research is needed to evaluate the possibility of using peptide arrays for drug discovery and target identification in plants.

Box 1. Early phosphorylation events during flagellin signalling in Arabidopsis

Phosphorylation events that are induced or reduced during bacterial flagellin-induced signalling [27,40-42] are shown in Figure 1 (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 flagellin-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 Ca2+-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 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 flagellin 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 cellulase synthesis subunits CES1 (At4g32410) and CES3 (At5g58170) suggests a possible link between flagellin signalling and cell wall synthesis.

Several potential regulators of vesicular trafficking are differentially phosphorylated during early flagellin 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 1). NPF motifs recruit Eps15 homology (EH) domain proteins that function in endocytosis in animals [77], but whether this occurs in plants is 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 SYPI22 (At3g34000) is a potential regulator of exocytosis that becomes phosphorylated within three minutes after FLS2 activation [40] (although SYPI22 protein contains two phosphorylation sites, only one is depicted because one does not know how many are induced). SYPI22 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/At1g35980) and PIN3 (At1g71710) become differentially phosphorylated by flagellin 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); MDR1, MULTIDRUG RESISTANCE 13 (At3g28345); PEN3, PENETRATION 3 (PDR8/At1g58970); PM, plasma membrane; PDR7, PLEIOTROPIC DRUG RESISTANCE 7 (At1g15210); WBC3, WHITE-BROWN COMPLEX HOMOLOGUE 3 (At2g28070).
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

<table>
<thead>
<tr>
<th>Method</th>
<th>In vitro or in vivo</th>
<th>Site specificity</th>
<th>Direct link kinase-substrate</th>
<th>High throughput quantitative analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide chip</td>
<td>In vitro</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Protein chip</td>
<td>In vitro</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Mass spectrometry-based</td>
<td>In vivo</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

*aIf peptide is sufficient for recognition of the phosphorylation site, and if no scaffold is required.

*But only with specific antibodies against the phosphorylated epitope.

*If no scaffold is required.

*Although phosphorylation motifs might suggest the upstream kinase.
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™ 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...
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 kinase 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 vitro 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.

Acknowledgements

We thank Adam Schikora, Concetta Giuliani and Jean Colcombet for their comments on the article. Work in our laboratory is supported by the Austrian Science Foundation, the Vienna Science and Technology Fund and the European Union.

References

14 Takahashi, F. et al. (2007) The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. Plant Cell 19, 805–818
55 Stulemeijer, I.J. et al. (2007) The tomato MAP Kinases LeMPK1, LeMPK2 and LeMPK3 are activated during the Cf-4/Avr4-induced HR and have distinct phosphorylation specificities. Plant Physiol. 144, 1481–1494
Five things you might not know about Elsevier

1. Elsevier is a founder member of the WHO’s HINARI and AGORA initiatives, which enable the world’s poorest countries to gain free access to scientific literature. More than 1000 journals, including the Trends and Current Opinion collections and Drug Discovery Today, are now available free of charge or at significantly reduced prices.

The online archive of Elsevier’s premier Cell Press journal collection became freely available in January 2005. Free access to the recent archive, including Cell, Neuron, Immunity and Current Biology, is available on ScienceDirect and the Cell Press journal sites 12 months after articles are first published.

2. Have you contributed to an Elsevier journal, book or series? Did you know that all our authors are entitled to a 30% discount on books and stand-alone CDs when ordered directly from us? For more information, call our sales offices:

+1 800 782 4927 (USA) or +1 800 460 3110 (Canada, South and Central America)
or +44 (0)1865 474 010 (all other countries)

3. Elsevier has a long tradition of liberal copyright policies and for many years has permitted both the posting of preprints on public servers and the posting of final articles on internal servers. Now, Elsevier has extended its author posting policy to allow authors to post the final text version of their articles free of charge on their personal websites and institutional repositories or websites.

4. The Elsevier Foundation is a knowledge-centered foundation that makes grants and contributions throughout the world. A reflection of our culturally rich global organization, the Foundation has, for example, funded the setting up of a video library to educate for children in Philadelphia, provided storybooks to children in Cape Town, sponsored the creation of the Stanley L. Robbins Visiting Professorship at Brigham and Women’s Hospital, and given funding to the 3rd International Conference on Children’s Health and the Environment.

www.sciencedirect.com