Opinion

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Protein tyrosine phosphorylation in plants: more abundant than expected?

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Protein phosphorylation in eukaryotes predominantly occurs on serine (Ser) and threonine (Thr) residues, whereas phosphorylation on tyrosine (Tyr) residues is less abundant. Plants lack classic Tyr kinases, such as the epidermal growth factor receptor, that govern Tyr phosphorylation in animals. A long-standing debate questions whether plants have any Tyr-specific kinases and, although several protein kinases with both Ser/Thr and Tyr specificities exist, data supporting the existence of other such kinases are scarce. As we discuss here, mass-spectrometry-based analyses now indicate that Tyr phosphorylation is as extensive in plants as it is in animals. However, careful inspection of available data indicates that these promising mass spectrometry studies have to be interpreted with caution before current ideas on Tyr phosphorylation in plants are revised.

Tyrosine phosphorylation in plants

Many proteins are phosphorylated on serine (Ser), threonine (Thr) and/or tyrosine (Tyr) residues. In contrast to Ser/Thr phosphorylation, Tyr phosphorylation is less common. Animals contain a large family of receptor Tyr kinases, which interact with ligands at the plasma membrane and subsequently mediate Tyr phosphorylation of an extensive array of downstream targets. Plant genomes do not encode such receptor Tyr kinases, and this observation indicates that Tyr phosphorylation in plants occurs less frequently in plants than in animals [1]. By searching for Tyr-kinase-specific sequence motifs, a bioinformatic screen for Tyr kinases in plants identified several potential dual-specificity kinases (DSKs) but no true protein Tyr-specific kinases (PTKs) [2]. The situation in plants might thus be similar to that in yeast, which has DSKs but seems to lack PTKs [3]. However, other bioinformatic screens predicted two PTKs in the *Arabidopsis thaliana* genome [4], or 34 putative PTKs and five DSKs, indicating that ~4% of all *A. thaliana* kinases can phosphorylate Tyr residues [5] (http://www.bio.unipd.it/molbinfo/PTKtable.html). Based on the few bioinformatic screens available, it thus cannot be concluded how many plant PTKs and DSKs exist; however, improved search algorithms and high-throughput experiments could provide a more decisive answer to this question.

Recently, the use of mass spectrometry (MS) has expanded the number of identified Tyr phosphorylation sites in many organisms, including plants. Here, we dis-cuss Tyr phosphorylation in plants in light of these novel discoveries, which show that Tyr phosphorylation is more common in plants than anticipated and enable screening for the responsible protein kinases. We also evaluate the described Tyr phosphorylation sites regarding potential discrepancies associated with the newly developed technological workflows that enable phosphorylation site mapping by MS.

Identification of candidate protein kinases and phosphatases that govern Tyr phosphorylation

After the first description of Tyr phosphorylation of pea proteins in 1986 [6], several studies showed, by using immunoblotting with phosphotyrosine-specific antibodies, that different plant proteins are phosphorylated on Tyr residues [5,7–14]. With only few exceptions, however, the phosphorylated (p)Tyr sites and the kinases involved have remained undiscovered.

Several plant protein kinases autophosphorylate on Tyr, Ser and Thr *in vitro* [2,13,15–20], including two RAF-like kinases identified as STY5 (also known as RAF31) and STY13 (RAF22) [2]. Several mitogen-activated protein kinases (MAPKs; or MPKs) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1 (SERK1) transphosphorylate artificial substrates on Tyr *in vitro* [17,18,21,22]. These studies show that plants have kinases that exhibit dual specificity (towards both Ser and/or Thr in addition to Tyr) *in vitro*. However, the first *in vivo* pTyr sites of these kinases still await identification.

Arabidopsis thaliana has a few protein Tyr-specific phosphatases (PTPs) and 22 dual-specificity phosphatases (DSPs) [23,24]. These numbers are much lower than in humans, who contain >100 members of the PTP super-family (including ~60 DSPs) [23]. The difference between the numbers of PTPs and DSPs are particularly striking considering the fact that *A. thaliana* has twice as many protein kinases than do humans [25]. This implies either that the pTyr component of plant phosphoproteomes is limited or that plant PTPs and DSPs target more sites.

Based on the use of inhibitors of Tyr kinases and phosphatases generally used in animal systems, a recent study indicates that Tyr phosphorylation has an important role in plant signalling [26]. Several other reports implicated Tyr phosphorylation in disease-resistance signalling. For instance, the plant pathogen *Pseudomonas syringae* injects the virulence factor HopPtoD2, which is a PTP, into its host cytoplasm [27,28]. Its catalytic activity suppresses programmed cell death [27,28], just like that of another *P.*

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syringae virulence factor, HopAO1, which is also predicted to be a PTP [29]. MAPKs are potential targets of these virulence factors and, although HopAO1 does not target MPK3 and MPK6 *in vivo* [29], several plant PTPs and DSPs are able to dephosphorylate MAPKs [30–32].

The identification of pTyr-containing proteins and the occurrence of a family of PTPs and DSPs indicated that Tyr phosphorylation in plants is more abundant than assumed. This raised several questions, including: how abundant is Tyr phosphorylation in plants? What are the responsible plant protein kinases, and what substrates and sites are their targets? To be able to answer these questions, the individual *in vivo* pTyr sites need to be identified on a global scale. Because of recent advances in the field, MS has become an optimal technique to obtain such information.

MS-based mapping of Tyr phosphorylation sites
Early estimations based on phosphoamino acid analysis of ^{32}P-labelled cells by partial acid hydrolysis indicated a low occurrence of Tyr phosphorylation in animals (0.05%), whereas phosphorylation on Ser (90%) and Thr (10%) were predicted to be more abundant [33]. However, medium- and large-scale (several hundred to >10 000 sites per study) MS-based studies recently indicated that the frequency of Tyr phosphorylation ranges from 2% to 3% in animals [34–36] and <1% in yeast [3,37,38]. A few MS-based phosphoproteomic screens carried out on *A. thaliana* samples indicated that Tyr phosphorylation in plants is similar to those in yeast: 0% (0 on 400 sites) [39], 0.7% (two on 303 sites) [40] and again 0.7% (11 on 1678 sites; the identity of the pTyr sites was not revealed in this work) [41]. These studies collectively seemed to confirm the predicted low occurrence of Tyr phosphorylation in plants.

However, a recent study described 94 pTyr sites on a total of 2172 phosphorylation sites in *A. thaliana* proteins [42], indicating that 4.3% of all phosphorylation events occur on Tyr residues – a figure that is higher than predicted for animals. Another group published 14 pTyr sites among a total of 105 sites in proteins from the moss *Physcomitrella patens* (13%), indicating that massive Tyr phosphorylation occurs [43]. Other experiments can be performed to confirm unambiguously the Tyr phosphorylation sites identified by MS-based phosphoproteomic screens. For instance, pTyr-containing phosphopeptides can be synthesized and analysed by MS to show that these peptides fragment similarly as the original phosphopeptides identified in the screens [44]. Otherwise, sites can be mutagenised and mutated proteins can be compared with wild-type proteins by immunoblotting with anti-pTyr antibodies [40]. Tyr phosphorylation of proteins can be shown by phosphoamino acid analysis of ^{32}P-labelled cells by partial acid hydrolysis. However, this technique only shows the global pSer, pThr and pTyr levels of a purified protein and does not give information about which sites are phosphorylated. Thus, although global MS analysis is the most appropriate method to indicate pTyr sites on a global scale, additional experiments are required to prove that a particular pTyr site assignment is correct.

The large discrepancy in predictions of the abundance of Tyr phosphorylation in plants inspired us to have a closer look at the published datasets. Some of the differences in the plant studies could be explained by the fact that either subcellular fractions or whole-cell extracts were analysed, or by the use of different phosphopeptide affinity purification protocols. However, several peculiarities related to Tyr phosphorylation were noted, which are important when interpreting the datasets.

In contrast to pSer- and pThr-containing peptides, most pTyr-containing phosphopeptides were reported to have one or more additional phosphosites [42]. Moreover, many combinations of phosphosites were identified in multiply phosphorylated peptides. For instance, for the doubly phosphorylated peptide RYSPPYYSPPR, four different combinations of phosphosite assignments were described: pTyr2 with pSer8; pSer3 with pTyr6; pSer3 with pTyr7; and pSer3 with pSer8 [42]. The differential assignment of phosphosites in independently fragmented peptides is not remarkable *per se*, but to find this many different combinations for a single phosphopeptide is rare [39,40,45,46]. Finally, Tyr phosphorylation was also reported to occur in many splicing factors [42]. Although others have also uncovered numerous phosphorylation sites in these proteins [40,45,46], identifying a set of overlapping splicing factor phosphopeptides, a single pTyr site has not yet been identified [46].

Determining protein phosphorylation sites by MS includes the following: mass spectra of phosphopeptides obtained by MS are aligned with a database of *in silico* fragmented (phospho)peptides, resulting in a top hit followed by lower ranking hits. Each hit comprises a peptide sequence, including the assignment of the phosphate(s) to a particular residue. Often, the top hit is a peptide sequence with a particular amino acid assigned as the phosphosite. The second-ranking hit can be the same phosphopeptide sequence but with the phosphosite mapped to a different amino acid. Visual inspection of the fragmentation spectrum of a peptide can help to ensure that the computational assignment of a phosphate group to a specific amino acid in a phosphopeptide sequence is correct. Because this is not feasible for a large-scale analysis, ~40% of phosphosite assignments must be considered to be ambiguous [47] (Box 1). The Ascore [47] and the post-translational modification score [36] methods were designed to obtain confident phosphosite assignments while avoiding the laborious manual verification of phosphopeptide spectra. Without the use of these methods, some authors have claimed to reach 86% unambiguity, stating that 'phosphorylated sites were unambiguously determined when y- or b-ions between which the phosphorylated residue exists were observed in the peak lists of the fragment ions' [42]. However, the use of this criterion alone can easily lead to mis-assignments. To achieve high reliability in phosphosite assignments, thorough visual inspection of all phosphopeptide spectra is necessary [40,45,46]. Without visual inspection, more information of each spectrum is required, such as the total number of fragments generated [47].

Reassessment of described phosphotyrosine sites
The aforementioned discussion prompted us to scrutinise the Tyr phosphosite assignments in the two MS-based

Box 1. Specialist language used in MS-based phosphoproteomic analysis

Collision-induced dissociation, also termed collision-activated dissociation

Collision-induced dissociation (CID) is a process used for fragmentation of ionised (phospho)peptides in mass spectrometers. A 'precursor ion' (the initial peptide) is bombarded with gas molecules, and the collision energy causes the peptide to fragment. In non-phosphopeptides, this usually occurs at the peptide bonds yielding sequence-specifying 'product ions'. When a peptide breaks into two product ions, it gives an N-terminal fragment, called a 'b ion', and a C-terminal fragment, the 'y ion' (Box 2). In the case of phosphopeptides fragmented by CID, however, the main fragmentation event is the neutral loss of phosphoric acid from phosphoserine or phosphothreonine [48]. This gives a high intensity peak corresponding to the mass of the precursor ion lacking phosphoric acid (e.g. the fragmentation spectrum in Box 1). This hampers both the sequencing of the phosphopeptide and the phosphosite assignment because product ions are much less intense. A new generation of mass spectrometers with extremely high mass accuracy has solved the first problem, but not the second.

Peptide fragmentation spectrum or mass spectrum

Collection of peptide fragment ions detected by the mass spectrometer as a result of peptide fragmentation (Box 2).

Ambiguous and unambiguous phosphosite assignment

The fragmentation spectrum of a phosphopeptide enables the localization of the phosphorylated residue on the basis of its mass (Box 2). Phosphopeptide fragmentation spectra generated by CID are usually very poor, resulting in ambiguous phosphosite assignments (i.e. the phosphogroup cannot be confidently localized to a particular residue). Moreover, current database search software still cannot account for sequence-dependent ion intensities. This decreases the confidence of automated phosphosite assignment versus visual inspection, which does enable to account for sequence-dependent ion intensities. If the fragmentation spectrum does enable the assignment of the phosphogroup to a specific residue, the site is unambiguous.

Charge state

In the electrospray-ionisation process, which occurs before injection into the mass spectrometer, peptides are protonated [54]. Each proton gives a +1 charge so, depending on the number of protons, the charge state of peptide ions is +1, +2, +3 and so on. Usual charge states are +2 and +3, and individual peptides can occur in different charge states. The higher the charge state the more complex the mass spectrum because usually product ions from all charge states are detected.

Singly, doubly and multiply phosphorylated peptide

A peptide containing one phosphogroup is termed singly phosphorylated; a peptide with two phosphogroups is called doubly phosphorylated, and so on. A peptide with two or more phosphogroups is a multiply phosphorylated peptide.

Kinase consensus motif

A kinase consensus motif is a short conserved sequence around the phosphosite that is preferred by a kinase. Each kinase (family) interacts with one or more residues in the near vicinity of the residue that it phosphorylates. For instance, MAPKs prefer a Pro at the +1 position (the position immediately C terminally of the phosphosite) and target the minimal consensus motifs pSer-Pro and pThr-Pro. This requirement is never 100% strict, and the same kinase consensus motif can be targeted by different kinases. For instance, a pSer-Pro or pThr-Pro motif can also be phosphorylated by cyclin-dependent kinases.

Dual specificity

Protein kinases and protein phosphatases can be divided into enzymes with specificity towards either Ser and Thr (Ser/Thr-specific) or Tyr (Tyr-specific). Some kinases and phosphatases target both Ser/Thr and Tyr residues, and are therefore termed dual-specificity kinases (DSKs) and phosphatases (DSPs).

studies that indicated the pTyr content of plant phosphoproteomes to be higher than 4% [42,43]. To our knowledge, however, the raw data of *P. patens* phosphopeptides [43] is not publicly available. By visual inspection of mass spectra from the study focusing on *A. thaliana* phosphosites [42], 40% of the pTyr assignments were evaluated as correct, 46% as ambiguous and 14% as potentially mis-assigned (Table S1 in the supplementary material online).

The most frequently occurring event during fragmentation of pSer- and pThr-containing phosphopeptides by collision-induced dissociation (CID) in a mass spectrometer is the loss of phosphoric acid from the full-length peptide (Box 1), which complicates the confident assignment of the phosphosite to a particular residue in the peptide. This event occurs because pSer and pThr residues are labile when subjected to CID [48]. In addition, CID of multiply phosphorylated peptides containing more than one pSer or pThr residue complicates phosphosite localization even more because of the strong loss of phosphoric acid from precursor and individual product ions (Box 1). This is not observed with pTyr-containing phosphopeptides, which do not show the strong loss of phosphoric acid because pTyr residues are more stable than pSer and pThr residues [48]. These phosphopeptides, therefore, usually give better fragmentation spectra, making phosphosite assignment relatively easy. In line with this, most of the phosphosite assignments of the singly phosphorylated, pTyr-containing peptides were evaluated to be correct. By contrast, ambiguous or potential mis-assignments were found in many of the multiply phosphorylated peptides that, in addition to a pTyr site, also contained pSer and/or pThr sites (Table S1 in the supplementary material online). An example of a phosphosite mis-assignment in a phosphopeptide is shown in Box 2.

An interesting finding of Ref. [42] is that Tyr phosphorylation is usually found on multiply phosphorylated peptides (Box 1). The authors suggest that Tyr phosphorylation is performed by kinases that require pre-phosphorylation of closely located residues. The Tyr residue is then part of a conserved sequence termed 'kinase motif' that includes the pre-phosphorylated residue at a fixed position. These kinase motifs have been described in plants [40], and casein kinase 2 and glycogen synthase kinase (GSK)3 (also known as shaggy-like kinases) target such motifs [49]. However, although 20 motifs were described as centring the pTyr site, no motifs with pre-phosphorylated sites were found [42], indicating that Tyr phosphorylation does not require pre-phosphorylation of closely located residues.

Usually, the assignment of pTyr sites corresponding to various protein kinases seems to be correct. These sites mainly occur in members of the class 4, group 4.5 MAPK, cell-division-cycle-2-like kinases, casein kinase 2 and GSK kinases (PlantsP database, http://plantsp.genomics.purdue.edu), which share a conserved Tyr residue in their activation loops (Table 1). This residue is the well-known Tyr in the MAPK TxY motif, which is phosphorylated upon stress [12] and has now also been shown to be phosphorylated in other members of this kinase family [40,42]. Autophosphorylation of the equivalent Tyr residue in the GSK3/shaggy-like kinase ASKα was shown to be essential for full protein kinase activity [40].

Box 2. Phosphosite assignment in phosphopeptides

When phosphopeptides are fragmented and analysed by MS, there are usually several possible residues to which the phosphate group can be assigned. In the example shown in Figure Ia, the highest-ranking hit from a database search with Sequest[TM] is depicted as RRSPDpYGAR, where the phosphorylation site was assigned to the Tyr residue. The most intense peak is annotated as the product ion (Box 1) b_5. However, despite a potential strong fragmentation event in between the Asp (D) and the Tyr (Y), the corresponding y_5 fragment is not detected.

In case of the second-ranking peptide, the phosphate was assigned to the Ser residue (Figure Ib). The most intense ion is the parent ion (Box 1) that has lost phosphoric acid (H_3PO_4), which is indicated by $[M+2H-H_3PO_4]^{2+}$, because the peptide is doubly charged (Box 1). This assignment is more convincing because the most prominent peak corresponds to a loss of H_3PO_4 from the precursor ion (Box 1), which is usually observed for pSer- and pThr-containing peptides (Box 1).

Strong fragmentation N terminally of a Pro residue in peptides is usually observed, and this occurred in this example phosphopeptide between the pSer and Pro resulting in a strong y_7 peak (this fragment is therefore more likely to be y_7 than b_6) and the corresponding b_3 ion (Figure Ib). Moreover, strong loss of H_3PO_4 is observed from y_8 and b_3

(Figure Ib), as is usually seen when the phosphoresidue is terminally located on a product ion (Box 1). All these aspects indicate that the correct phosphosite assignment is RRpSPDYGAR and that the highest-ranking hit was incorrect.

The potential mis-annotation depicted in Figure Ia is observed in case of the phosphopeptide DFNGYRSPPR, in which Tyr5 was assigned as the phosphosite [42] (Table S1 in the supplementary material online). The ion that was more likely to correspond to the parent ion that lost phosphoric acid was assigned to a product ion. In case of the phosphopeptides SYGDMTEMGGGGGGGGRDEK and KPVYNLDDSDDDDFVPKK, the strong loss of phosphoric acid from the peptides is also potentially mis-assigned to a product ion (Table S1 in the supplementary material online). In the case of other peptides, the phosphogroups were independently assigned to different positions, although their fragmentation spectra look similar. For instance, in two independent fragmentation spectra of the singly phosphorylated (Box 1) peptide NATEVPSPDYSQGK, showing a less prominent loss of phosphoric acid, the assigned phosphosites were Ser7 or Tyr10. However, the spectra look similar, which is impossible for peptides that either contain pSer or pTyr residues owing to the difference in loss of phosphoric acid from these peptides.
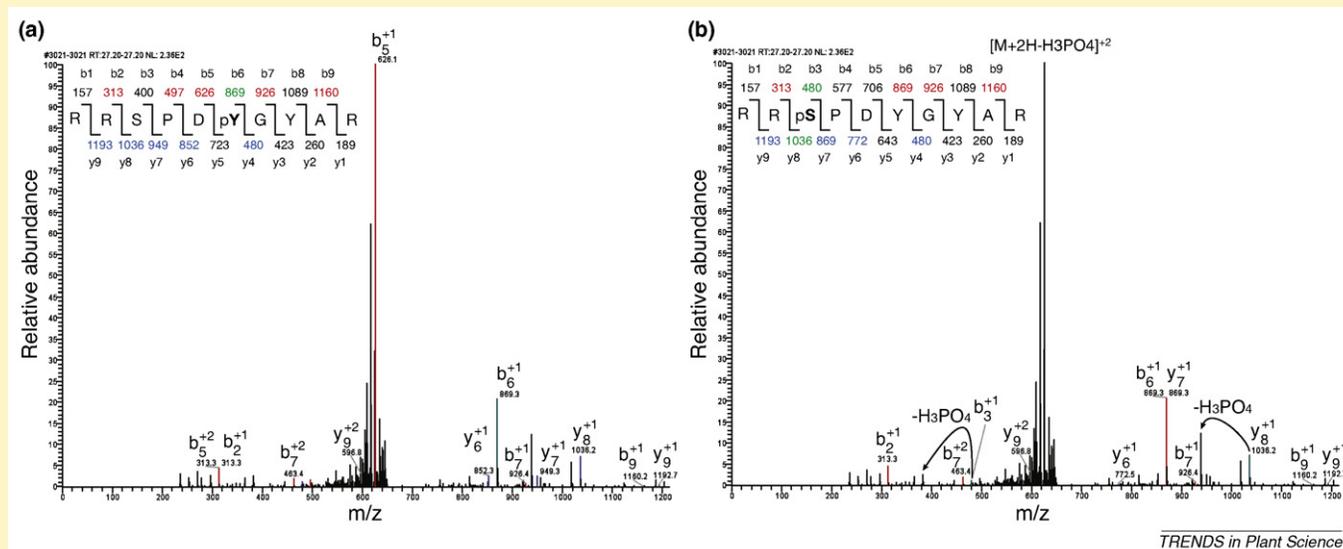

Figure I. Phosphosite assignment within a phosphopeptide by analysis of its fragmentation spectrum.

In contrast to the many convincing Tyr sites in protein kinases [42], Tyr phosphorylation of splicing factors is more questionable. Many of the phosphopeptides of these proteins were previously identified and suggested to be non-Tyr phosphorylated [46]. Numerous pSer and pThr sites have been mapped in animal splicing factors, but no single pTyr site has been found [45]. Strikingly, all splicing-factor phosphopeptides were found several times with the same number of phosphosites [42]. However, independent fragmentations of each phosphopeptide yielded different assignments, giving many combinations of the actual sites within these peptides. For each peptide, there exists one assignment of all phosphogroups to non-Tyr residues (Table S1 in the supplementary material online).

Table 1. Tyrosine phosphorylation sites identified by mass spectrometry in *Arabidopsis thaliana* protein kinases

Protein kinase	Site(s)	Responsible kinase(s)	Activation loop	Refs
MPK4 or MPK11	Tyr203 or Tyr200	MAPK kinase(s) or autophosphorylation	Yes	[17,42]
MPK6	Tyr223	MAPK kinase(s)	Yes	[42]
AtSK11 (also known as ASKα) or AtSK12 (ASKγ) or ASKδ or ASKε or AtGSK1 (ASKι)	Tyr229, Tyr233, Tyr242, Tyr234 or Tyr230	Autophosphorylation	Yes	[40,42]
BIN2 (also known as USU1 and ASKη) or ASKζ	Tyr200 or Tyr232	Autophosphorylation	Yes	[42]
AtK-1 (also known as ASKκ)	Tyr243	Autophosphorylation	Yes	[42]
At3 g25840	Tyr782	Unknown	Yes	[42]
At2 g40120	Tyr425	Unknown	Yes	[42]
At5 g35980	Tyr284	Unknown	Yes	[40,42]
At1 g70520	Tyr178	Unknown	No	[42]
At2 g30940	Tyr181	Unknown	No	[42]
At3 g05140	Tyr154 and Tyr158	Unknown	No	[42]

Table 2. *Arabidopsis thaliana* proteins with predicted[a] SH2 domains

AGI	Name	Predicted function	Region	Refs
At1 g17040[b]	Not determined	Unknown	545–631	[51]
At1 g78540	Not determined	Unknown	572–661	[51]
At1 g63210[c]	Not determined	Related to yeast Spt6 protein. Chromatin remodelling, transcription initiation	1009–1102	
At1 g65440	GTB1 (GLOBAL TRANSCRIPTION FACTOR GROUP B1)	Related to yeast Spt6 protein. Chromatin remodelling, transcription initiation	1225–1318	

[a]By the SMART database [52].
[b]Homologous to At1 g78540.
[c]Homologous to At1 g65440.

Box 3. Outstanding questions

- Is Tyr phosphorylation in plants less abundant than in animals, and what are the pTyr sites in plants?
- Are there Tyr-specific kinases in plants; if yes, how many?
- How many DSKs are there in plants?
- How many Tyr-specific phosphatases and DSPs are there, and what are their target sites? And what are the pTyr sites sensitive to *P. syringae* PTPs that are injected into plant cells?
- Does phosphotyrosine signalling through proteins with SH2 domains also occur in plants?

In agreement with previous analyses showing that plant splicing factors are highly phosphorylated on Arg-Pro, Ser-Pro and Thr-Pro kinase motifs [46] (Box 1), most of these non-pTyr sites are part of such motifs [42]. On the basis of these analyses, further experimental proof is required for the unambiguous assignment of these Tyr phosphorylation sites.

Conclusions and future directions
Although the observation that Tyr phosphorylation in plants is as extensive as it is in animals still needs confirmation, it is becoming clear that it occurs in plants more often than was previously assumed and that it is probably more abundant than in yeast [5]. MS-based determination of phosphorylation sites is a useful way in which to identify Tyr phosphorylation on a large scale. Because of the difficulties to assign the phosphosite by MS, as described earlier, it is important to confirm the suggested pTyr sites by independent techniques such as site-directed mutagenesis and immunoblotting with anti-pTyr antibodies. The next important challenge will be to identify the protein kinases and phosphatases that are responsible for targeting these Tyr sites (Box 3). Once we know the specificities of these kinases, prediction programs can be used to study the comprehensive set of Tyr-directed protein kinases and connect them to their substrates. Some of these substrates might bind to signalling proteins via an Src homology 2 (SH2) domain, which interacts specifically with pTyr-containing motifs [50]. In animals, an extensive network of SH2-based interactions exists. In *A. thaliana*, only two related proteins with a predicted SH2 domain, which is homologous to SH2 domains of animal STAT1 transcription factors, have been reported [51]. The current Simple Modular Architecture Research Tool (SMART) database (http://smart.embl-heidelberg.de) [52] predicts two additional proteins to have SH2 domains (Table 2). Although not predicted by SMART, regions of DELLA transcription factors also share homology with SH2

domains [53]. Based on these predictions, phosphotyrosine signalling in plants might be directly connected to transcriptional regulators (Box 3). Future studies should verify whether the predicted plant SH2 domains are *bona fide* pTyr-binding modules and what their interacting partners are. Collectively, further experiments should unravel Tyr kinase and phosphatase networks, including substrates, the position of each phosphorylation event in signalling cascades and their roles in the biology of plants.

Supplementary data
Supplementary data associated with this article can be found at doi:10.1016/j.tplants.2008.11.003.

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