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Signal processing by protein tyrosine phosphorylation in plants

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Abbreviations: PTM, post-translational modification; Ser, serine; Thr, threonine; Tyr, tyrosine; PTK, protein tyrosine kinase; Ds, dual-specificity; PTP, protein tyrosine phosphatase; MAPK, mitogen-activated protein kinase; PAO, phenylarsine oxide; TLK, tyrosine-specific protein kinase-like kinase; MS, mass spectrometry; CID, collision-induced dissociation; ECD, electron capture dissociation; ETD, electron transfer dissociation; BR, brassinosteroid; ABA, abscisic acid; SH_2, Src homology 2

Protein phosphorylation is a reversible post-translational modification controlling many biological processes. Most phosphorylation occurs on serine and threonine, and to a less extend on tyrosine (Tyr). In animals, Tyr phosphorylation is crucial for the regulation of many responses such as growth or differentiation. Only recently with the development of mass spectrometry, it has been reported that Tyr phosphorylation is as important in plants as in animals. The genes encoding protein Tyr kinases and protein Tyr phosphatases have been identified in the *Arabidopsis thaliana* genome. Putative substrates of these enzymes, and thus Tyr-phosphorylated proteins have been reported by proteomic studies based on accurate mass spectrometry analysis of the phosphopeptides and phosphoproteins. Biochemical approaches, pharmacology and genetic manipulations have indicated that responses to stress and developmental processes involve changes in protein Tyr phosphorylation. The aim of this review is to present an update on Tyr phosphorylation in plants in order to better assess the role of this post-translational modification in plant physiology.

Introduction

Proteins can undergo various post-translational modifications (PTMs) that affect their conformation, their activity, their stability and their localization. Thus, PTMs are recognized as regulators of many cellular processes. A few hundred types of PTMs have been described so far such as acetylation, thiolation, adenylation, ribosylation and phosphorylation.[1] Protein phosphorylation is one of the most studied PTMs. One-third of all eukaryotic proteins are presumed to be phosphorylated.[2,3] Phosphorylation is the result of the antagonistic action of protein kinases (PKs) and protein phosphatases (PPs) that allow to add or remove respectively a phosphate group in a protein. It has been shown that the human genome encodes at least 518 PKs[4] and approximately 148 PPs[5] indicating the importance of protein phosphorylation. In *Arabidopsis thaliana*, more than 800 PKs[6] and 150 PPs[5] have been identified. Phosphorylation is mostly found on hydroxylated amino acids such as serine (Ser), threonine (Thr) and tyrosine (Tyr). The classification of PKs and PPs has been established following the amino acids they are specifically targeting.[7] The protein Ser/Thr kinases and protein Tyr kinases (PTKs) strictly phosphorylate Ser/Thr and Tyr residues respectively, whereas dual-specificity PTKs (DsPTKs) act on both Ser, Thr and Tyr. Similarly, protein Ser/Thr phosphatases and protein Tyr phosphatases (PTPs) dephosphorylate phospho-Ser/Thr or phospho-Tyr respectively and dual-specificity PTPs (DsPTPs) remove the phosphate group from Ser, Thr and Tyr. First experiments have reported that the majority of phosphorylation occurs on Ser and Thr residues whereas Tyr phosphorylation accounts only for 0.05%.[8] Recently, phosphoproteomics studies based on more accurate detection of phosphopeptides have shown that phosphorylation on Ser, Thr and Tyr residues occurs at a ratio of 88:11:1.[3] It clearly appears that Tyr phosphorylation is minor compared to Ser and Thr. Nevertheless, Tyr phosphorylation plays a crucial role as it regulates many cellular processes in animal cells like division, growth and differentiation.[9] Until recently, very few studies have tried to elucidate the involvement of Tyr phosphorylation in plant cells. This is due to the lack of a typical *PTK* gene in plants and also to the fact that few *PTP* genes have been identified in the genome of Arabidopsis.[10] Sensitive proteomic approaches have however, confirmed the existence of protein Tyr phosphorylation in plants. The combination of peptides enrichment methods with sensitive mass spectrometry (MS) instrumentation has allowed the identification of low abundance phosphopeptides, in particular Tyr-phosphorylated peptides. This review first presents the early studies on Tyr phosphorylation in plants and the different PTKs and PTPs which have been identified. Then, the latest proteomic studies identifying the Tyr-phosphorylated plant proteins and the physiological processes regulated by Tyr phosphorylation in plants are discussed.

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Discovery of Protein Tyrosine Phosphorylation and First Studies in Plants

Detection by radioactive labeling. In 1980, Ser and Thr were the only known phosphorylated amino acids in proteins. Hunter and Eckart[11] were studying polyomavirus middle T and Rous sarcoma virus associated kinase activities and discovered that a third amino acid, identified as Tyr, could also be phosphorylated. The impact of this observation proved to be fundamental for the study of regulation of many physiological processes. Indeed, 30 years later, this PTM has emerged to be one of the most crucial mechanisms of regulation in animal cells.[9] First experiments were based on radioactive labeling by ^{32}P-ortho-phosphate of proteins in the cells, separation of proteins by electrophoresis and partial acid hydrolysis. Following thin-layer chromatography of the hydrolyzates and autoradiography, phospho-Ser, phospho-Thr and phospho-Tyr could be detected. The phospho-Tyr residues had escaped detection previously as they were 3,000-fold less abundant than the phosphorylated-Ser and -Thr, and because phospho-Thr and phospho-Tyr were difficult to separate by traditional electrophoretic procedures.[11] In plants, protein Tyr phosphorylation was initially demonstrated by the ability of kinases to autophosphorylate on Tyr, Ser and Thr in vitro.[12-18] The kinase STY13 autophosphorylates on Tyr, Ser and Thr in vitro.[19] Using this radioactive labeling approach, it was also shown that mitogen-activated protein kinases (MAPKs) and somatic embryogenesis receptor-like kinase can transphosphorylate in vitro artificial substrates on Tyr.[16,18,20,21]

Immunochemical detection. Developments in the immunochemical field have provided monoclonal antibodies that recognize phosphorylated-residues regardless of the surrounding sequences.[22] Therefore, antibodies raised specifically against phospho-Tyr, enabled to detect the phospho-Tyr residues in proteins by western blot test. Tyr phosphorylation was first detected in *Pisum sativum* using these specific antibodies.[23] Immunoblotting with anti-phospho-Tyr antibodies have permitted detection of plant proteins phosphorylated on Tyr in several other species.[17,19,24-37]

Involvement of PTKs and PTPs by pharmacological approaches. One way to probe specifically elements of signal transduction pathways is by perturbating these pathways through the loss of function of one of these elements. Pharmacological approaches enable a fast, reversible and dose-dependent inactivation of single components in intact cells organisms. Inhibitors that have been designed to specifically block PTKs and PTPs, have been used in plants. PTKs inhibitors such as genistein, lavendustin A,[38,39] erbstatin and tyrphostins,[40] and PTPs inhibitors such as phenylarsine oxide (PAO),[41] dephostatin[42] and ortho-vanadate[43] have permitted to identify plant PTKs and PTPs and to specify the physiological processes in which they are involved. Studies based on genistein have reported PTK activity in *Catharanthus roseus*[44] and in *Pisum sativum*.[45] Genistein has been used to show involvement of PTKs in the ABA signaling pathways leading to stomatal closure and to *RAB18* gene expression in *Arabidopsis thaliana*.[33] Genistein and tyrphostin AG18 altered root hair growth and development suggesting a role for PTKs in the organization of cortical microtubules.[46] In the pathway leading to the production of inositol-1,4,5-trisphosphate in *Citrus limon,* implication of PTKs has been shown using genistein, lavendustin A and erbstatin.[47] Lavendustin A also inhibits a DsPTK activity in *Zea mays*.[48] Tyrphostins inhibit the activity of a DsPTK in *Pisum sativum*.[45] PTP activities sensitive to PAO have been reported in *Lycopersicon esculentum*,[49] in *Mimosa pudica*[36] and in *Vicia faba*.[50] PAO was used to show the implication of PTPs in the ABA-induced expression of *RAB16* in barley aleurone cells[51] and of *RAB18* expression in *A. thaliana* suspension cells.[33] The same inhibitor allowed to implicate PTPs in ABA-activated stomatal closure in Arabidopsis.[33,50,52] A PTP activity inhibited by dephostatin has been detected in soybean.[53] Using sodium ortho-vanadate, it has been shown that a PTP is implicated in the copper-dependent transduction pathway leading to cell death[34] and in the dynamics and organization of microtubules.[46] Once involved by these pharmacological approaches in several plant physiological processes, the genes encoding these enzymes were identified by analysis of the plant genomes using bioinformatics.

Identification of PTK and PTP Genes in Plant Genomes

***PTK* genes.** Although Tyr kinases are separated into two broad classes, PTKs and DsPTKs, they all share a common catalytic domain of approximately 250 amino acids[19] (**Fig. 1**). Eleven major conserved subdomains are evident, out of which they are residues specifically conserved in either Ser/Thr or Tyr kinases and may play a role in correct recognition of hydroxylated amino acid.[19,54] In the N-terminal end of the catalytic region, the subdomain II contains an invariant lysine residue which has been recognized as essential for maximal enzyme activity as it helps to anchor ATP (**Fig. 1**). In the middle of the catalytic region, the subdomain VII has an aspartic acid residue believed to be important in the catalytic activity of the kinase (**Fig. 1**). The subdomain VI confers Ser/Thr specificity but also Tyr specificity. Subdomain VIII appears to play a major role in recognition of peptide substrates and subdomain XI has a specific consensus motif CW$(X)_6$RPXF (**Fig. 1**). These last subdomains both confer PTK specificity. A first genomic survey with CW$(X)_6$RPXF sequence motif of subdomain XI revealed the existence of 57 Arabidopsis Tyr kinases possessing 11 kinase subdomains.[19] The catalytic domains of all the identified Arabidopsis kinases have motifs for Ser/Thr in subdomains VI and PTK motifs in subdomain XI. These datas suggested that all the kinases belong to a DsPTK family. This survey could not detect Tyr kinase specificity alone in the complete genome of Arabidopsis.[19] This is similar to yeast which has DsPTKs but seems to lack PTKs.[55] More recently, another survey has shown that three plant species seemed to encode putative Tyr-specific kinases.[56] Two *A. thaliana* putative Tyr-specific kinases lack the conserved lysine residue of subdomain II and the conserved aspartate of the activation loop in subdomain VII.[56] This suggests that these two enzymes are catalytically inactive. Two rice species were found to encode six and seven putative Tyr-specific kinases. Out of the six predicted PTK-strict

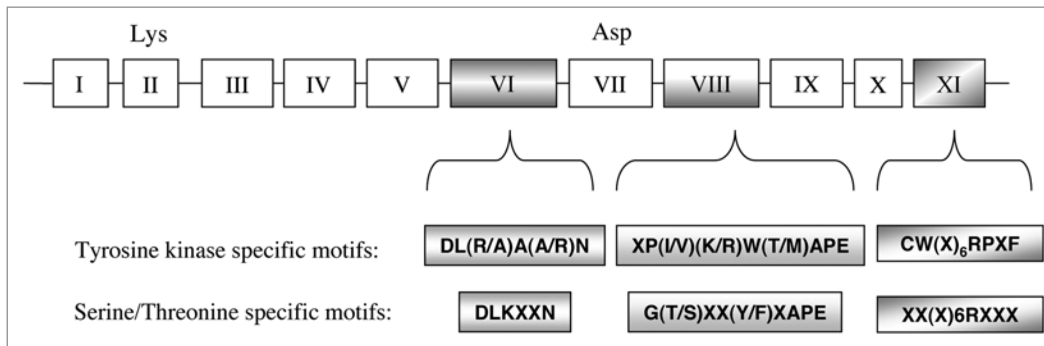

Figure 1. Catalytic domain of the serine/threonine and tyrosine protein kinases (adapted from Rudrabhatla et al.[19]). The catalytic kinase domain of around 250 amino acids contains 11 conserved subdomains (I to XI). At the N-terminal extremity, the subdomain II has a conserved lysine residue believed to be involved in ATP binding. In the central part, an aspartic acid residue in subdomain VII is important for the catalytic activity of the kinase. Subdomains VI and VIII are specifically conserved in either serine/threonine or tyrosine kinases. Consensus DL(R/A)A(A/R)N is specific of tyrosine kinases whereas the consensus DLKXXN is serine/threonine-specific. Subdomain VIII has a tyrosine-specific consensus XP(I/V)(K/R)W(T/M)APE and a poorly conserved serine/threonine-specific consensus G(T/S)XX(Y/F)XAPE. In subdomain XI, the consensus CW(X)$_6$RPXF in subdomain XI is tyrosine-specific.

of *Oryza sativa* ssp. *Indica,* only three of them were found to include all the residues known to be essential for catalytic activity. Out of the seven putative Tyr-specific kinases of *Oryza sativa* ssp. *Japonica,* four were found to have all residues known to be required for catalytic activity.[56] Other possible candidates would be Tyr-specific protein kinase-like kinases (TKLs), which are especially abundant in plants. There are 776 TKLs in Arabidopsis and nearly 1,000 in *Oryza sativa*, compared to 55 in humans.[56] Plant TKLs' function remains unknown, but the large number of TKLs in plants may suggest that they carry out important and diverse plant-specific functions. Plant receptor Tyr kinases have not been reported. In animals, they all share the same basic topology: an extracellular ligand-binding domain, a single transmembrane domain and a cytoplasmic domain which contains the kinase activity.[57]

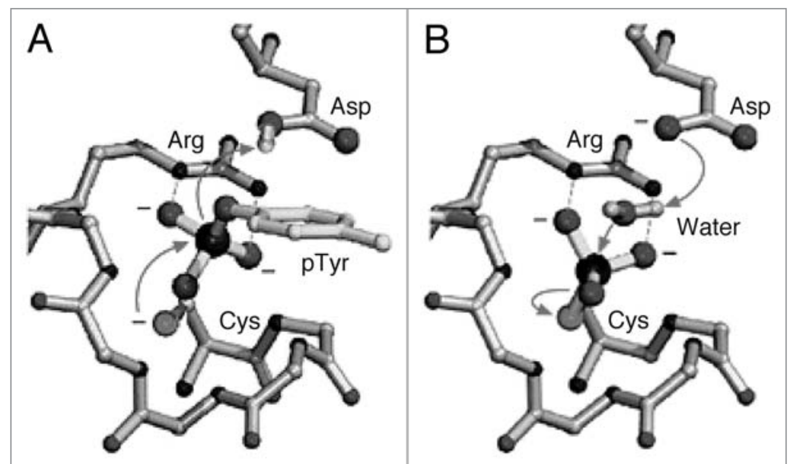

Figure 2. Catalytic mechanisms of protein tyrosine phosphatases (adapted from Denu et al.[59]). The model is derived from Yersinia protein tyrosine phosphatase and the backbone atoms of the active site loop from cysteine to arginine are shown as a ball-and-stick model. (A) Enzyme-Substrate complex: The dianion of the phosphoryl group is coordinated by the nitrogens of the arginine. The catalytic cysteine thiolate does a nucleophilic attack on the phospho-tyrosine substrate and forms the phosphoenzyme intermediate. (B) Hydrolysis of the phosphoenzyme intermediate: The phosphate is covalently bound to the cysteine. The aspartic acid activates a water molecule which hydrolyzes the phosphoenzyme intermediate. The arginine allows to position the substrate for the nucleophilic attack. Reprinted with permission from Denu et al., Cell 1996; 87:361-364 from Elsevier.[59]

***PTP* genes.** Notwithstanding that the identification of plant PTKs is poorly documented, studies on *PTP* genes are more abundant. The overall protein sequence of Tyr-specific PTPases and DsPTPases share little homology but these phosphatases all contain the CX$_5$R motif in their catalytic domain: (V/I)HCXAGXGR(S/T).[58] This catalytic core has a conserved cysteine residue, which acts as a nucleophile, to displace the phosphate group from the substrate and form the phosphoenzyme intermediate[59] (**Fig. 2**). Several genomic surveys with the CX$_5$R motif have identified one PTP-strict and 24 DsPTPs in Arabidopsis.[5] In humans, more than 100 members of PTP superfamily which includes 60 DsPTPs have been reported in reference 5. Among these 100 human PTPs, 11 are catalytically inactive and 16 dephosphorylate either glycogen,[60] mRNA[61] or phosphoinositides[58] and not proteins.

Some Arabidopsis PTPs have also been shown to be inactive. Inactive PTPs display PTP-like domain structures but possess mutations in the cysteine or arginine residue of the CX$_5$R motif, in an upstream conserved aspartate residue or in combinations of the three. Such alterations are thought to disrupt the enzymatic activity of the phosphatases yet potentially maintain interaction with phosphorylated substrates.[62] For example in plants, PASTICCINO2 (PAS2) is a PTP-like member with an inactive phosphatase catalytic site. It functions as an anti-phosphatase by binding a kinase and preventing its dephosphorylation by other activating phosphatases.[63] It has been shown finally that PAS2 is a dehydratase which functions in the elongase complex required for the production of very-long-chain fatty acids.[64] Another plant

PTP has been reported to act on starch. PTPKIS1/SEX4/DSP4 is a DsPTP interacting with plant SNF1-related kinase AKIN11,[49] that has a carbohydrate-binding domain allowing it to bind starch granules.[65-67] A genetic screen for plants producing excess starch has permitted the identification of a mutant impaired in this *DsPTP* gene. In the chloroplast, SEX4 dephosphorylates starch. SEX4 resembles to animal laforin[62] that regulate glycogen accumulation indicating striking parallels in the regulation of starch metabolism in plants and glycogen metabolism in animals.

The remainder of the PTPs in humans are protein-specific and dephosphorylate phospho-Tyr, and in some cases, phospho-Ser and phospho-Thr. They have been grouped in three classes. Class I includes the classic and dual-specificity enzymes and have a common PTP domain structural fold. They are by far the largest group of PTPs and are further divided into subfamilies. The classic enzymes (receptor and non-receptor) are given the name of PTPs as they all dephosphorylate Tyr residues only. The receptor-like PTPases all contain an extracellular domain of variable length, a single transmembrane region, and one or two cytoplasmic PTPase catalytic domains. No typical PTP receptor has been identified in plants. In *A. thaliana*, there is only one Tyr-strict phosphatase which is non-receptor protein. This only Tyr-strict phosphatase, AtPTP1, was characterized using a systematic PCR approach and sequence alignment.[68] It contains a typical signature motif present in all other PTPases. In Class I, animal DsPTP are divided into the MAPK phosphatases (MKPs), slingshots, phosphatases of regenereting liver (PRL), atypical DsPTPs, cell division cycle 14 (CDC14) phosphatases, tensin homologue (PTEN) and myotubularins (MTMs).[69] In *A. thaliana*, no slingshots, PRLs or CDC14 have been reported. Five MKPs have been identified, three DsPTPs have been classified as atypical and nine have not been classified.[5] Four PTEN containing SH_2 (Src homology 2) domain and PTB (phospho-Tyr-binding) domain have been identified as a tumor suppressor. This subclass contains the catalytic core of PTPs and has a structural domain with high similarity to a cytoskeleton protein called tensin. PTEN proteins have a low DsPTP activity towards proteins whereas they have a high activity against phosphatidyl inositol 3,4,5-triphosphate.[70] In Arabidopsis, two MTMs phosphoinositides phosphatases have been reported in reference 5 and 69. Class II and III are represented by the low-molecular-mass PTP isoforms and CDC25 phosphatases respectively. One Class II PTP has been reported in *A. thaliana* but Class III PTPs are not represented.[69]

Recent development in MS has allowed the efficient identification of the PTKs' and PTPs' protein substrates.

Identification of Phosphorylation Sites by Mass Spectrometry

Estimation of plant Tyr phosphorylation. Phosphoproteome analysis by large-scale MS-based studies have shown that Tyr phosphorylation in animal cells ranges from 2% to 3%.[3,71,72] The proportion of phospho-Tyr residues in human cells is estimated between 1.8 and 6.0%, depending on the analyzed samples.[3,72,73] This PTM concerns less than 1% of proteins in yeast.[55,74] Phosphorylation estimations by MS approaches have to be considered with caution as they are established on the basis of the phosphopeptides which were detected by MS and thus, they only represent a part of all the phosphopeptides existing within the cell.

First MS studies performed in plants predicted a low occurrence, between 0% and 0.7%, of Tyr phosphorylation as in yeast.[75-77] Improvement of both technology based on MS and phosphopeptide enrichment methods have allowed to explore more efficiently the plant phosphoproteome. Out of 2,172 phosphorylation sites analyzed in *A. thaliana*, 94 have been shown to occur on Tyr residues.[35,78] In Arabidopsis suspension cells, the relative abundances of phospho-Ser, phospho-Thr and phospho-Tyr were estimated to be 85%, 10.7% and 4.3% respectively.[35] This study reported an unexpected high proportion of Tyr phosphorylation in plants compared to the previous studies, close to the proportion found in human phosphoproteome. A phosphoproteome analysis developed by Reiland et al.[79] of Arabidopsis seedlings identified a similar number of phosphorylation sites. The phosphoproteome of *A. thaliana* shoots and rosette leafs was studied using a LTQ-Orbitrap mass spectrometer for phosphopeptide detection combined with immobilized metal affinity chromatography (IMAC) and titanium dioxide (TiO_2) phosphopeptide enrichment. This survey[79] identified 2,349 phosphopeptides and reported that 88% of all phosphorylation events occur on Ser and 11% on Thr similarly to the observations made by Sugiyama et al.[35] Nevertheless, the amount of Tyr phosphorylation was estimated to only 0.3% of the phosphopeptides detected. This discrepancy in the rate of phospho-Tyr peptides could be explained by the differences between the Arabidopsis suspension cells and Arabidopsis seedlings. Also, the recovery rate of phospho-Tyr peptides could be different because of the enrichment techniques and the detection limitations of liquid chromatography coupled to MS. However, even if some of the putative Tyr phosphorylation sites reported by Sugiyama et al.[35] may need to be reconsidered,[78] numerous other studies have confirmed their estimations. Indeed, Nakagami et al.[80] have reported that the contributions of phospho-Ser, phospho-Thr and phospho-Tyr sites were estimated to be respectively 82.7%, 13.1% and 4.2% in Arabidopsis and to be 84.8%, 12.3% and 2.9% in *Oryza sativa*. All the phosphopeptides for *Oryza sativa* and Arabidopsis are available on web-based RIKEN database called Plant Phosphoproteome Database (phosphoproteome.psc.database.riken.jp).[35,80] In *A. thaliana*, a proteomic study mapping the phosphorylation sites of soluble proteins identified 122 phospho-Ser, 44 phospho-Thr and 15 phospho-Tyr which results in a contribution of 67.4% to 24.3% to 8.3% for phospho-Ser:phospho-Thr:phospho-Tyr.[81] There are 14 phospho-Tyr sites that have been identified among 105 phosphosites in proteins from the moss *Physcomitrella patens* which indicates a Tyr phosphorylation rate of 13%.[82] A phosphoproteome study[83] has shown that the proportion of phospho-Tyr in *Medicago truncatulata* is 1.3% similar to the estimations from Nakagami et al.[80] Phosphorylation sites have been characterized from *M. truncatulata* root proteins including both cell lysates and membrane-enriched fractions and using metal affinity chromatography and tandem MS. The Medicago

phosphoproteomic database contains more than 3,457 phosphopeptides (phospho.medicago.wisc.edu).

Finally, a global survey has analyzed all the datas from Arabidopsis. A phosphorylation sites prediction was obtained for 500,000 residues in *A. thaliana* (203,622 Ser, 174,301 Thr, 120,983 Tyr). Experimental evidence from biochemical approaches or MS was obtained for 12,000 residues (9,406 Ser, 2,352 Thr, 699 Tyr) covering about 5,000 proteins in Arabidopsis.[84] A Phosphoproteomic database was created from all these compiled datas to form the largest database called PhosPhAT (phosphat.mpimgolm.mpg.de). This database contains 5,170 Arabidopsis phosphoproteins and 32,601 phosphosites. This global survey conclusion was that the percentage of validated unique phospho-Ser, phospho-Thr and phospho-Tyr is 76%, 17% and 1.3% respectively.

Enrichment of phosphopeptides. Large-scale studies of phosphoproteomes were first based on protein separation by 2D-gel electrophoresis and detection of the phosphoproteins using radioactive labeling with ^{32}P-ortho-phosphate,[85] antibodies raised specifically against the phosphoresidues[22] or a phosphoprotein staining dye called ProQ diamond.[86] These very sensitive techniques allowed to detect phosphoproteins but not to identify them. Protein identification was permitted by MS analysis of the protein spots detected in gel. Comparatively to biochemical approaches, MS allows a more specific detection of phosphoproteins as it maps the phosphorylation sites and thus identifies precisely the residues which are phosphorylated in the protein. On the other hand, MS detection is less sensitive than classical biochemical techniques. Indeed, western blot test appears more sensitive for detection of peptides compared to MS by one or two orders of magnitude. Standard peptides are detected in a range of 10–100 fmoles by MS whereas detection by immunochemical method coupled to chemiluminescence is achieved for less than 0.1 fmoles.[33] Numerous limitations have been reported for the efficient identification of phosphoproteins by this combined approach of 2D-gel electrophoresis separation with MS analysis. A first limitation is due to the biochemical technique used for protein separation itself. Indeed, the number of proteins that could be analyzed at one glance is limited as 2D-gel electrophoresis could not separate on a single gel more than 1,000 proteins. A second limitation is linked to the preparation of the samples for their analysis by MS. Trypsin is the commonly used enzyme for protein digestion in gel and generates tryptic peptides which have to be eluted from the 2D-gel in order to be analyzed in the mass spectrometer. Selective suppression of phosphopeptides seems enhanced when peptides mixture are extracted from gel probably due to the presence of contaminants from the gel. This leads to a low abundance of the phosphopeptides that can not be detected efficiently. A third limitation is inherent to the physico-chemical properties of the phosphopeptides themselves. Protein phosphorylation is a transient state which is low represented within the cell and thus phosphopeptides are naturally low represented. Furthermore, ionization of phosphopeptides in mixtures with non-phosphorylated peptides is suppressed which hampers their analysis by MS.

To circumvent these difficulties, especially in large scale proteomic studies, specific purification strategies have been elaborated. They allow the enrichment of a small proportion of peptide fragments containing the phosphorylated residues once the phosphoproteins have been proteolytically cleaved. A first approach based on immunoaffinity has been developed. The antibodies recognizing phospho-Tyr can be immobilized on a solid support to allow the enrichment of Tyr-phosphorylated proteins from complex cell extracts. This approach was used successfully in a large-scale phosphoproteomic study intending to establish the profiling of phospho-Tyr peptides from tissu extracts.[87] Another approach called IMAC achieves enrichment of phosphopeptides using Fe(III) immobilized on nitrilotriacetate support.[74] This enrichment was used to analyze Arabidopsis suspension cell cultures treated with the bacterial pathogen flagellin.[88] Metal Oxide Affinity Chromatography (MOAC) is based on a separation using TiO_2 that has amphoteric ion-exchange properties which make it suitable for phosphopeptides enrichment.[77,89,90] Different reversible covalent binding techniques have been proposed to convert phosphorylated groups into properties amenable to affinity purification. One method involves the activation of the phosphate group to a reactive phosphoramidate that can be subsequently coupled to glass beads or to a dendrimer.[91,92] Several groups have been taken advantage of the negatively charged phosphate moiety of phosphopeptides to enrich them via interaction chromatography methods such as strong anion exchange or strong cation exchange. These methods can be combined with IMAC or TiO_2 to improve recovery yields of phosphopeptides.[77,88]

Phosphopeptide identification by MS. Downstream of the enrichment step, the improvement of the mass spectrometers has allowed a more efficient identification of the phosphopeptides. Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) permits estimation of peptide mass with very high accuracy. It is now possible to differentiate between nonmodified and phosphorylated peptides directly by their mass as they are determined with an accuracy better than ±0.1 ppm.[93] Soft ion fragmentation methods have been developed in order to optimize the analysis of phosphopeptides.[72] Liquid chromatography tandem MS based approaches including collision-induced dissociation (CID) as fragmentation method were first employed for deciphering large scale phosphoproteome. CID is based upon collision of selected digest peptide as multiprotonated molecules with gas (Nitrogen, Argon, Helium, etc.) at low energy (10–100 eV).[94] Upon collision, peptide ions undergo isomerization reaction involving the mobility of one or more protons to sites that are thermodynamically less stable. As a result, amide bonds are cleaved forming b and y ions series.[95,96] For phosphopeptides, a potential difficulty arises from the labile nature of this modification and the amide bond, undergoing a predominant neutral loss of H_3PO_4 (~98 Da) and to a lesser extend of HPO_3 (~80 Da).[97,98] Today, the sensitivity and the reliability are significantly improved when the information of MS/MS and MS/MS/MS are combined for the phosphopeptide identifications.[99,100] More recently new fragmentation modes have been developed based on the interaction of ion with electron (electron capture dissociation, ECD) or with radical anion (electron transfer dissociation, ETD).[101-103] Both interactions are fast and cleave randomly along the peptide backbone (so called c and z ions) while side chains and modifications such

as phosphorylation are left intact. The combination of these new fragmentation modes with mass analyzers offering high resolution and mass accuracy such as FTICR and Orbitrap offers valuable tools for deciphering phosphoproteome. The ability to fragment peptide ions while simultaneously preserving the labile phosphoester bonds provides ETD with an inherent advantage in large-scale phosphoproteome analyses compared to currently most used CID method. All previous large-scale plant phosphoproteomic studies have relied solely on CID during tandem mass spectrometry (MS/MS).[102,103] Medicago phosphoproteome analysis utilized the ETD technology and confirmed that ETD is more efficient than CID in phosphorylation-site localization.[83]

Physiological Processes Regulated by Tyrosine Phosphorylation in Plants

In animal cells, many physiological roles of Tyr phosphorylation such as regulation of growth, differentiation or oncogenesis, have been reviewed in reference 9. On the other hand, evidence for regulation by Tyr phosphorylation in plant physiological processes is just starting to emerge. This section presents an overview of the signaling pathways and the biological responses shown to be under the control of Tyr phosphorylation in plant cells.

Developmental processes and adaptation to environmental signals. Tyr phosphorylation has been involved in the development of the male and female gametophytes in higher plants. The *AtDsPTP1* cDNA has been isolated from an Arabidopsis cDNA library following homology with mammalian PTPs and its expression is restricted to stamens and pollen.[104,105] Another DsPTP, which gene *AtPTEN1* is specifically expressed in pollen grains, is essential for pollen development. Indeed, suppression of *AtPTEN1* gene expression by RNA interference causes pollen cell death after mitosis.[70] Some *DsPTK* genes are expressed specifically in late flowering stage and have been reported to be highly expressed in stamens.[19] Finally, PAO and genistein arrest pollen germination and pollen tube growth confirming involvement of PTKs and PTPs in these processes.[106]

Embryo development and seed germination are also modulated by Tyr phosphorylation. Changes in the protein Tyr phosphorylation occurs during embryogenesis in *Coco nucifera*[32] and in *Daucus carota*.[24] A PTK has been involved in the establishment of zygotic polarity in Fucus as genistein provokes an alteration of the embryo pattern.[107] A group of *DsPTK* genes is expressed specifically during germination and early seedling stages.[19] Reyes et al.[108] have shown that PAO enhances the inhibition of germination of Arabidopsis seeds. PTKs and PTPs have been implicated in the control of germination as ABA modulates the Tyr phosphorylation of seed proteins during this stage.[33] Also, the mutant *phs1-3* impaired in a *DsPTP* gene, is hypersensitive to ABA during germination.[109]

Some developmental aspects regulated by phytohormones involve Tyr phosphorylation. Brassinotseroids (BRs) regulate many mechanisms of plant growth and development. Brassinolide induces phosphorylation of many proteins on Tyr residues in *Pisum sativum*.[31] BRI1 (Brassinosteroid Insensitive 1), the receptor of BRs, is a leucine-rich repeat kinase located in the plasma membrane that interacts with the cytosolic protein BAK1 (BRI1-associated kinase 1). Oh et al.[110] reported that BRI1 and BAK1 autophosphorylate on Tyr residues and thus are DsPTKs. The autophosphorylation of BRI and BAK1 are necessary for root growth inhibition induced by BRs.[110] It was also shown that autophosphorylation/dephosphorylation of the kinase Brassinosteroid Insensitive 2 (BIN2) on Tyr-200 is a critical switch in downstream regulation of BR signaling.[111] Furthermore, upon BR perception, BRI1 phosphorylates the BRI1 kinase inhibitor 1 (BKI1) on Tyr-211 to displace the kinase inhibitor into the cytosol where it is active.[112] Another phytohormone, auxin, also influences many processes such as vascular development, apical dominance, lateral root formation, gravitropism and phototropism. The *ibr5* Arabidopsis mutant which is impaired in a *DsPTP* gene, is less sensitive to auxin and exhibits the characteristics of some auxin mutants such as a long root and a short hypocotyl when grown in light, fewer lateral roots, aberrant vascular patterning and an increased leaf serration.[113] In a proteomic study, Tyr phosphorylation of the auxin receptor, TIR1, has been reported in reference 35. The double mutant *ibr5/tir1* enhances auxin resistance compound compared to that of either parent.[114] All these datas clearly suggest the involvement of Tyr phosphorylation in the developmental aspects controlled by BRs and by auxin.

Response to stresses. Water deficit occurs as a result of drought, salinity, low temperature or heat. Plants respond to these stresses with similar signaling mechanisms, biochemical and metabolic responses. Tyr phosphorylation has been implicated in the abiotic stress responses. In *Arachis hypogaea*, a *PTK* gene expression and its activity are induced by cold and salt.[17] Cold, salt and heat induce high levels of gene expression of 14 *DsPTK* genes and downregulate three *DsPTK* genes.[19] The only member of classical PTP in Arabidopsis, AtPTP1, was upregulated by salt and downregulated by cold treatment.[53,68] MAPK phosphatase 1 (MKP1) is a DsPTP that participates to the response to salt stress. This was shown by expression profiling of wild-type vs. *mkp1* mutant lines and increased resistance to salinity of *mkp1* mutant plants.[115]

During water stress, transpirational loss of water is reduced as stomata close in response to ABA. Using specific inhibitors, PTKs and PTPs have been involved in the signaling pathway leading to stomatal closure.[33,50,52] The *phs1-3* mutant affected in a *DsPTP* gene, presented a deregulation of the ABA-dependent stomatal closure.[109] Perception of water deficit gives rise to increases in internal ABA concentrations. Involvement of Tyr phosphorylation have been observed in ABA signaling as the DsPTP PHS1 is a negative regulator of ABA transduction pathway whereas IBR5, another DsPTP, has been shown to regulate positively this pathway.[109,113]

Moreover, Tyr phosphorylation controls both oxidative stress tolerance and the response to genotoxic stress. Reactive oxygen species are produced during the normal operation of respiratory and photosynthetic electron transport. They are toxic for the plant as they induce the production of highly destructive species. Plants have elaborated mechanisms to counter the action of these compounds. AtDsPTP2 also called MKP2 has been shown to regulate positively the physiological responses to oxidative stress generated during ozone treatment.[116] The *mkp2* mutant plants inhibit hypersensitivity to oxidative stress induced by methyl viologen during germination, confirming the role of this DsPTP as a positive regulator.[117] Besides the stress of increased light intensity, plants

are subjected to stress from the ultraviolet (UV) wavelengths in incident irradiation. UV-C is the most potentially damaging component as it modifies DNA and proteins. Plants use both restorative and repair mechanisms to counter this stress. Screens for UV-sensitive mutants in Arabidopsis led to the identification of the DsPTP MKP1 as essential for UV resistance, that interacts with stress activated MAPK3, MAPK4 and MAPK6.[118,119] The Arabidopsis *mkp1* mutant which was shown to be resistant to elevated salinity, is also hypersensitive to UV radiation.[115,118]

Defense, cytoskeleton organization and response to hormones. The DsPTK BAK1 is the positive regulator of the signaling pathways induced by the BRs but also of the transduction cascade induced by the bacterial pathogen flagellin. This suggested that Tyr phosphorylation might regulate some plant defense aspects. Indeed, phosphorylation on Tyr-610 of BAK1 is critical for BR-signaling and expression of a large number of defense-related genes.[120] Immunoblots and immunoprecipitation analysis with anti-phospho-Tyr antibody showed that a fungal elicitor induces Tyr phosphorylation of a kinase in *Nicotiana tabacum*.[121] MKP1 is implicated in the response to pathogens as an *mkp1* null mutation Columbia (Col) accession, *mkp1* (Col), showed constitutive defense responses including *pathogenesis related* (PR) gene expression, accumulation of salicylic acid and resistance to the bacterial pathogen *Pseudomonas syringae*. A genetic study implicated PTP1 in the negative regulation of plant defense. Indeed, the mutant phenotype *mkp1* (Col) is strongly enhanced by the *ptp1* null mutation indicating redundant function of MKP1 and PTP1.[122] Finally, salicylic acid upregulates eight *DsPTK* genes and suppresses gene expression of three *DsPTKs*.[19]

Genetic studies and pharmacology have reported the involvement of Tyr phosphorylation in the organization of the cytoskeleton. The DsPTP PHS1 controls microtubule organization in *A. thaliana*. A semi-dominant *phs1-1* allele was identified in a screen for mutants with increased sensitivity to a microtubule-destabilizing drug.[123] It has also been shown that both α- and β-subunits of plant tubulin undergo phosphorylation on Tyr residues.[124] Involvement of PTKs and PTPs in the organization of microtubules in primary root cells was shown using specific inhibitors.[46,125] Other components of cytoskeleton are also modulated by Tyr phosphorylation. For example, in *Mimosa pudica*, Tyr phosphorylation of actin is induced by stimulation of the pulvinus and its stimulation is abolished by PAO.[36] Tyr phosphorylation affects pollen germination and polarized pollen tubes via the actin cytoskeleton.[106] *Phaseolus vulgaris* profilin can be phosphorylated on Tyr residues in vivo.[25]

Finally, in a recent large scale proteomic study, very few proteins have been shown to be differentially phosphorylated on Tyr in response to several hormones.[126] Nevertheless, it appears that one protein, the G protein α subunit 1 (GPA1) has a Tyr residue which is phosphorylated in response to all hormones (ABA, auxin, gibberellic acid, jasmonic acid and kinetin). Tyr phosphorylation of Gα subunits is known to activate G protein-coupled receptors.[127] It was suggested that phosphorylation of Gα subunit on Tyr-166 was a common response for multiple hormones and thus constitutes a key switch in the cross-talk between several signaling pathways.[126]

All the studies mentioned in this last chapter have permitted to have a better view of the roles of Tyr phosphorylation in plants. However, they need to be completed in order to establish more precisely the implication of Tyr phosphorylation in these regulatory processes.

Conclusions and Future Prospects

Knowledge about plant PTKs, PTPs and their protein substrates has emerged this last decade. The contribution of protein Tyr phosphorylation to the regulation of a vast array of physiological processes in plant is just starting to be elucidated. However, biochemical studies on PTPs and PTKs will have to confirm their specificity towards Tyr residues. Similarly to PTPs, the functions of the plant PTKs will have to be explored by a reverse genetic approach based on insertional T-DNA mutants. Identification based on enhanced MS technology of more plant Tyr-phosphorylated proteins will allow to complete the existing databases of Tyr-phosphorylated peptides. The next important challenge will be to identify the targets of the Tyr-phosphorylated proteins detected by MS. Lim and Pawson[128] have suggested that Tyr phosphorylation is due to a three part tool kit which includes the PTK, the PTP and the proteins recognizing Tyr-phosphorylated substrates. These last proteins bind to the substrates of the couple PTK/PTP via an SH2 domain that interacts specifically with phospho-Tyr containing motifs.[129] In human cells, there are around 100 of these proteins whereas only four SH2 domain proteins have been reported in Arabidopsis.[78,130] Plants might be similar to yeast that has only one SH2 protein and which is supposed to completely lack Tyr phosphorylation. But, recent proteomic studies have clearly established that Tyr phosphorylation in plants is more abundant than expected. This tends to show that plants probably developed a different strategy from mammalians to read the phospho-Tyr signals. PTB domains are known to bind phospho-Tyr[131] but such domain has not yet been identified in plants. Recently, another type of phospho-Tyr-binding domain has been characterized in animals, the C2 domain of human protein kinase C delta.[132] *A. thaliana* was found to possess 85 proteins containing C2 domain.[56] These questions should be addressed in the near future. The complexity of the signaling networks involving Tyr phosphorylation in plants will also have to be unravelled by positioning each PTK, each PTP and their substrates within each cascade and further precise the functions controlled by these pathways.

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