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Review

Phosphorylation-dependent regulation of plant chromatin and chromatin-associated proteins

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Abbreviations: BES1, BRI1-EMS suppressor 1; **BIN2**, Brassinosteroid-Insensitive 2; **BR**, brassinosteroid; **BZR1**, brassinazole-resistant 1; **CDK**, cyclin-dependent kinase; **CDKD**, cyclin-dependent kinase activating kinase ; **CDPK**, calcium-dependent protein kinase; **ChIP**, chromatin immunoprecipitation; **CK2**, casein kinase 2; **CTD**, C-terminal regulatory domain ; **GFP**, green fluorescent protein; **HFR1**, Long Hypocotyl in Far-Red Light 1; **Hk**, AT-hook DNA-binding motif; **HMG**, high mobility group proteins; **HR/AM**, high resolution and accurate mass; **MAPK**, mitogen-activated protein kinase; **MYBL2**, myeloblastosis family transcription factor-like 2; **NLS**, nuclear localization signal ; **RBR**, retinoblastoma-related; **RLK**, receptor-like kinase; **SMC**, structural maintenance of chromosomes; **SUMO**, Small Ubiquitin-like Modifier; **TBP**, TATA box-binding protein; **T-DNA**, transfer DNA

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Abstract

In eukaryotes, most of the DNA is located in the nucleus where it is organized with histone proteins in a higher-order structure as chromatin. Chromatin and chromatin-associated proteins contribute to DNA-related processes such as replication and transcription as well as epigenetic regulation. Protein functions are often regulated by post-translational modifications (PTMs) amongst which phosphorylation is one of the most abundant PTM. Phosphorylation of proteins affects important properties such as enzyme activity, protein stability or subcellular localization. We here describe the main specificities of protein phosphorylation in plants and review the current knowledge on phosphorylation-dependent regulation of plant chromatin and chromatin-associated proteins. We also outline some future challenges to further elucidate protein phosphorylation and chromatin regulation.

1 Introduction

In eukaryotes, nuclear DNA is organized in chromatin, a tightly packed higher-order structure which permits genomic DNA to fit within the nucleus. The fundamental structural unit of chromatin is the nucleosome which is composed of 147 base pairs of DNA that is wrapped almost twice around an octamer of histone proteins. The octamer consists of two copies of each of histone H2A, H2B, H3 and H4 [1]. Chromatin higher-order structure switches between condensed and relaxed states and plays a crucial role in the epigenetic regulation of gene expression. Linker histones, such as H1, also contribute to chromatin compaction. In addition to these core chromatin proteins, many chromatin-associated proteins exist such as DNA polymerases, RNA polymerases, transcription factors, chromatin remodeling and modifying enzymes. These chromatin-associated proteins play key roles in processes such as DNA replication, mRNA transcription or epigenetic regulation. The association of these

proteins to nucleosomes is a dynamic process that relies on protein-DNA and protein-protein interactions. Post-translational modifications (PTMs), notably of histones, are also an important way of regulating chromatin structure and accessibility [2]. Histone PTM crosstalk constitutes a complex mechanism of chromatin regulation and has been reviewed elsewhere [3]; it will therefore not be the topic of this review.

Protein phosphorylation is one of the most abundant PTMs in eukaryotes [4, 5]. The covalent modifications are catalyzed by protein kinases and occur predominantly on serine, threonine and tyrosine residues. Protein phosphorylation is a reversible process that is mediated by protein phosphatases. Phosphorylation can have diverse consequences on a protein, such as regulating its enzymatic activity or subcellular localization. Considering its functional importance, the identification of the phosphorylation sites has become an important task in biology. The characterization of phosphorylation sites on histones and chromatin-associated proteins is one of the requirements towards a comprehensive understanding of the mechanisms of replication, transcription and epigenetic regulation. Once phosphorylation sites have been identified, it is possible to determine their biological functions.

In this review, we first describe the protein kinase and phosphatase families in plants and their plant specific features. We also describe the phosphorylation modifications, their molecular effects, the methods to identify and functionally characterize these and the nuclear and chromatin protein phosphorylation resources in plants. We then present some known examples of phosphorylation-dependent regulation of histones and chromatin-associated proteins in plants and finally discuss what will be required to understand the roles of protein phosphorylation in chromatin regulation.

2 Plant kinases and phosphatases

Protein phosphorylation is one of the most abundant PTMs found in eukaryotic organisms and plays important roles in signal transduction, with protein kinases and phosphatases often functioning as switch-like regulators [4, 5]. Protein kinases and phosphatases have been extensively studied, their structure/function relationships have been reported [6-10] and several articles and reviews are available [11-18]. In the context of this review, we will point out some specificities of plant protein kinases.

Firstly, plant genomes encode for a huge number of protein kinases compared to other eukaryotes. For example, about 1,000 genes are predicted to code for protein kinases in the model plant *Arabidopsis thaliana* (Arabidopsis) and about 1,400 in *Oryza sativa* (rice), while the genomes of *Saccharomyces cerevisiae* and *Homo sapiens* code for about 120 and 500 protein kinases, respectively [19-23]. In a recent comparison of protein kinases from 25 plant species with other eukaryote models [14], it was clearly shown that the protein kinase superfamily expanded in the different plant lineages and that elevated rates of tandem and whole genome duplication as well as a tendency to retain duplicates contributed to this expansion in plants [14, 24]. It is tempting to speculate that many more phosphorylation events occur in plant species compared to other eukaryotes. Some mechanisms such as subfunctionalization of duplicated genes or conversion of part of the duplicated genes to pseudogenes could nonetheless decrease the expansion effects. However, the appearance of novel phosphorylation sites does not necessarily indicate the creation of new regulatory mechanisms [25-27].

Besides the protein kinase superfamily expansion, a second peculiarity is the functional divergence of some plant protein kinases that is especially evident for the calcium-dependent protein kinase (CDPK) and receptor-like kinase (RLK) families which underwent

both an important expansion and a subsequent functional divergence allowing plants to perceive and transduce extracellular signals. For example, about 600 RLKs and related kinases and about 35 CDPKs are encoded by the Arabidopsis genome while none of them are present in the yeast genome [19, 28].

A third specificity lies in the fact that only a small number of strictly nuclear kinases are currently known in plants in comparison to animals [29]. The nucleus is surrounded by a double membrane that is punctuated by nuclear pores which allow the diffusion or active transport of molecules between the cytosol and the nuclear compartment [30, 31]. Diffusion tends to be inefficient for molecules larger than 20-40 kDa [32]. Proteins generally do not diffuse through nuclear pores and regulation of nucleocytoplasmic shuttling mainly occurs through phosphorylation and dephosphorylation of cargo proteins [32]. Dahan *et al.* reported that most of the currently known nuclear plant kinases are also located and activated in the cytosol suggesting a regulation of nucleocytoplasmic shuttling [29].

Given these briefly described features of plant protein kinases, it is quite probable that the plant nuclear phosphoproteomes will only partially overlap with the ones of other eukaryotes and that plant-specific phosphorylation-dependent regulations of chromatin and chromatin-associated proteins may occur.

3 Protein phosphorylation

3.1 Molecular effects of protein phosphorylation

The direct effects on proteins due to the phosphorylation (HPO_3) are the increment of protein mass by 80 Da, the local input of a negative charge and a local surface change. Phosphorylation may modify the global structure of a protein by conformational changes and may also alter its interactions with other molecules (protein, DNA, RNA, etc.). For example, dual phosphorylation of the activation loop of mitogen-activated protein kinases (MAPKs) modifies their protein conformation rendering the protein kinases active [33]. In terms of interactions with other molecules, many linear motifs include phosphorylated residues [34-36]. Besides linear motifs, the negative charge brought about by phosphorylation or multiple phosphorylations also constitutes a mechanism of interaction regulation [37-40]. From a more functional point of view, the phosphorylation of an amino acid residue can have multiple consequences. For example, phosphorylation may activate/inhibit the activity of an enzyme [41], allow/prevent trans-interactions [39], change subcellular localization [42] or regulate protein stability [43]. In addition, phosphorylation may also regulate proteins as part of PTM crosstalk, as it will be described in part 5.1 [44] (Fig. 1).

3.2 Identification and functional characterization of phosphorylation sites

Although there are differences in the size and function of protein kinase families between plants and animals, the methods to identify protein phosphorylation sites are identical. They range from large-scale techniques, such as phosphoproteomics and protein microarrays, to more targeted techniques, such as in-gel kinase assays. They range from *in vivo* approaches to

in vitro approaches and may or may not allow precise localization of the phosphorylation sites on the proteins. These techniques will not be described here but are presented in numerous articles and reviews [45-58]. Once a precise phosphorylation site has been identified, however, two important questions remain: which particular protein kinase is responsible for the modification and what is the functional role of this modification.

Depending on the initial approach taken for identifying a given phosphorylation site, the protein kinase may be already known, for example by *in vitro* kinase assays. Otherwise, identifying the responsible protein kinase is a difficult task. The analysis of the stretch of amino acids adjacent to the phosphorylated residue may allow the identification of a known kinase motif and some of these motifs were indeed characterized in plants and other eukaryotes [6, 59-66]. However, the identification of the particular kinase remains difficult because protein kinases belonging to the same family and even different protein kinase families share the same signature, while the phosphorylation motifs targeted by numerous protein kinases have not been determined yet. Quantitative phosphoproteomic approaches have allowed to associate some protein phosphorylation events to a biological context [67-73]. More recently, quantitative phosphoproteomic studies comparing different genotypes have even allowed the *in vivo* identification of direct probable substrates of kinases [74-79].

The characterization of the functional role(s) of a phosphorylation event usually requires three steps: 1) the mutation of the phosphorylated amino acid to a non-phosphorylatable amino acid (e.g. substitution of Ser by Ala) and/or to a phospho-mimicking amino acid (e.g. substitution of Ser by Asp), 2) the *in vivo* replacement of the wild type protein by the non-phosphorylatable version and/or by the phospho-mimicking version, 3) the phenotypical comparison of the wild type organism and the mutated organism, especially by looking at the cellular function(s) of the protein. It should be noted that the last step is the most difficult and can be complicated by different aspects: the cellular function(s) of a

protein can be unknown or hard to measure. Alternatively, the function of a phosphorylation site can be difficult to detect because its effect is for example 'diluted' in multisite phosphorylations or phosphorylation 'hotspots' [37, 38, 80, 81] and finally the phosphorylation site might not be functional [25-27].

The picture of phosphorylation events provided by a given discovery-based phosphoproteomics study is always far from complete, even when working on a highly enriched subcellular fraction. In particular, if one phosphosite is identified in a given protein, one cannot rule out the possibility that other phosphosites, not covered by exploratory MS/MS analyses, exist in the same protein. Functional assessment of the role of a given protein phosphorylation may then remain difficult due to the availability of partial information only. To obtain a more exhaustive picture of the phosphosites decorating a protein, one may envision performing targeted MS/MS analyses of putative phosphorylated versions of proteolytic peptides predicted for this protein. With the advent of newer-generation MS instruments, such as the quadrupole-Orbitrap architecture, hundreds of peptides can be targeted within a single LC-MS/MS experiment [82]. In addition, full MS/MS spectra with high resolution and accurate mass (HR/AM) are generated within this instrument and may then contain sufficient fragment ions to precisely localize the phosphorylated residues and to pinpoint the existence of several phospho-isoforms [83]. Such an approach would, however, most probably require purifying the protein of interest to favor high sequence coverage and limit risks of co-fragmenting other nearly isobaric peptides.

3.3 Nuclear and chromatin protein phosphorylation resources in plants

Considerable efforts have been made over the past years to uncover plant phosphoproteomes but the task is huge and many islands were sparsely visited. This is particularly true for the nucleus although this subcellular compartment is the seat of several important biological processes. Even though phosphoproteomics is not the only means to identify phosphorylation sites, this approach is currently the most efficient one to identify large numbers of phosphoproteins with precisely localized phosphorylation sites. Such approaches recently explored plant nuclear phosphoproteomes [84-87] and these results were discussed in different reviews [55, 88-90]. Besides, many proteins have multiple subcellular localizations and may not be detected in the nucleus because the physiological or environmental conditions are not appropriate, suggesting that whole cell studies and reports on other subcellular compartments also contain relevant information for the comprehension of nuclear-localized processes.

Several databases have been created which provide information on plant protein phosphorylation sites, notably PhosPhAt (http://phosphat.mpimp-golm.mpg.de/index.html) [91] and P3DB (http://www.p3db.org/) [92]. They constitute useful resources and some of them also offer different bioinformatics tools to handle the data. None of them, however, specifically gathers information on plant nuclear or chromatin phosphoproteomes. Besides, it should be mentioned that many databases exist which collect more specific data on plant nuclear proteins, such as AtNoPDB (http://bioinf.hutton.ac.uk/cgi-bin/atnopdb/home) that provides a description of experimentally identified Arabidopsis nucleolar proteins [93], ChromDB (http://www.chromdb.org/) which is an inventory of chromatin-associated proteins from different species based on genomic and transcriptomic data [94], At-Dbome which collects *in silico* annotated Arabidopsis DNA-binding proteins [95] and different plant

transcription factor databases [89], but these databases do not contain protein phosphorylation data. Currently, about twelve thousand phosphorylation sites have been precisely determined experimentally in Arabidopsis (PhosPhAt 4.0, http://phosphat.mpimp-golm.mpg.de/statistics.html). A comparison of the PhosPhAt, ChromDB and At-Dbome databases indicated that 35% of the Arabidopsis chromatin-associated annotated proteins and 26% of the *in silico* annotated Arabidopsis DNA-binding proteins have been experimentally found to be phosphorylated (Fig. 2). Moreover, a comparison between Arabidopsis experimental and predicted phosphorylation sites for the functional category 'DNA' of MapMan [96] showed that only one quarter of predicted phosphosites have been experimentally determined [97]. These data clearly suggest that many phosphosites still need to be experimentally confirmed and this is notably the case for the chromatin-associated proteins.

4 Phosphorylation-dependent regulation of plant chromatin and chromatin-associated proteins

In the previous parts of this review we highlighted some specificities of plant protein kinases and the considerable efforts that are still required to identify the phosphoproteome of plant nuclei and notably of chromatin. We also explained the difficulty to functionally characterize the molecular effects of protein phosphorylation. In this part, we will discuss known examples of phosphorylation-dependent regulation of plant histones and chromatin-associated proteins.

4.1 Histones

Histones are small basic proteins which together with DNA form the minimal unit of chromatin, the nucleosome. Numerous PTMs of histones have been identified [98] and several of these modifications participate in chromatin regulation via two main mechanisms: direct modulation of chromatin compaction and accessibility and/or change in interaction with other molecules such as histone modifying enzymes [99, 100]. Interestingly, the molecular roles of histone PTMs may not be conserved between organisms suggesting a functional evolution [101, 102].

Plant histones have been shown to be phosphorylated at multiple sites [103]. Correlations were determined between some histone phosphorylations and some chromatin phenotypes and the putative upstream kinases were even identified in some cases. For example, serine 10 and serine 28 of Arabidopsis histone H3 are phosphorylated *in vitro*, and quite probably *in vivo*, by the kinase AtAurora3 and these phosphorylations correlate with chromosome segregation and metaphase/anaphase transition [104]. Likewise, threonine 3 of Arabidopsis histone H3 is phosphorylated *in vitro*, and very probably *in vivo*, by the kinase AtHaspin and this phosphorylation correlates with chromatin condensation [105]. In addition, Kurihara *et al.* showed that AtHaspin could phosphorylate H3T3 and also H3T11 residues *in vitro* [106]. However, a more precise phosphorylation-dependent regulation has only been proposed *in vitro* for serine 10 of Arabidopsis histone H3 [107]. Indeed, H3S10 is phosphorylated by members of the Aurora protein kinase family [108, 109] and recently Demidov *et al.* showed that the activity of Arabidopsis H3K9 dimethyltransferase SUVR4 was reduced when H3S10 had been previously phosphorylated, suggesting an interference of SUVR4 by H3S10 phosphorylation [107].

4.2 Linker histones and HMG proteins

Besides core histones, other proteins have direct roles in chromatin structure and compaction, such as H1 linker histones and high mobility group (HMG) proteins [110-114]. In Arabidopsis, three genes encode H1 linker histones [115]. H1 linker histone phosphorylation has been reported both in plants and animals [116-118], but in plants, no clear phosphorylation-dependent regulation has been shown. HMG proteins are more diverse than H1 linker histones and were classified into several families [114, 119, 120]. They were shown to be phosphorylated and the functions of some plant HMG protein phosphorylations have been described.

Maize HMGA protein (formerly named HMG-I/Y) can efficiently and specifically bind the AT-rich sequence of the gamma-zein promoter *in vitro* [118]. Zhao *et al.* showed that the mitotic cyclin-dependent kinase from developing endosperm (CDK, Cdc2/cyclin B kinase) was able to phosphorylate the maize HMGA protein, *in vitro*, preferentially between the AT-hook DNA-binding motifs (Hk) Hk3 and Hk4, and that this CDK-mediated phosphorylation of HMGA decreased its binding efficiency to the gamma-zein promoter *in vitro* [121].

Stemmer *et al.* showed that maize HMGB1 and HMGB2/3 are *in vivo* phosphoproteins and that maize casein kinase 2 (CK2) alpha kinase can phosphorylate maize HMGB1 and HMGB2/3 on multiple sites within the acidic C-terminal domain *in vitro* [122]. Moreover, Stemmer *et al.* demonstrated that the *in vitro* CK2 alpha phosphorylation of HMGB1 and HMGB2 increases their thermal stability and alters their interactions with DNA [122]. Thomsen *et al.* showed that the maize HMGB1 C-terminal domain phosphorylation by CK2 alpha enhances the interaction of this domain with the basic HMGB1 N-terminal domain, suggesting this global phosphorylation-dependent conformational change would

modify the above mentioned thermal stability and interactions with DNA [123]. Krohn *et al.* showed that the protein Dof2, a member of the plant-specific family of Dof transcription factors, interacted with maize HMGB proteins via their HMG-box DNA binding domain and that this interaction enhanced the binding of Dof2 to its DNA target *in vitro* [124]. In addition, Krohn *et al.* showed that the CK2 alpha-mediated phosphorylation of HMGB1 on its acidic C-terminal domain reduced the Dof2-HMGB1 interaction and abolished the stimulation of Dof2 binding to its DNA target [124].

Pedersen *et al.* showed that maize CK2 alpha can phosphorylate Arabidopsis HMGB2 on serine residues within the acidic C-terminal domain *in vitro* [125]. In addition, Pedersen *et al.* mutated these serine residues to alanine or aspartic acid and compared the subcellular localization of the wild-type and mutated proteins fused to green fluorescent protein (GFP) in tobacco protoplasts. While the wild-type and mutated proteins had a similar nucleocytoplasmic distribution, the precise intranuclear localization was different: the wild-type form was present in both the nucleoplasm and the nucleolus while both mutated forms were comparatively less present in the nucleolus and displayed a more pronounced speckled pattern in the nucleoplasm [125]. These results suggested that CK2 alpha-mediated phosphorylation could change the intranuclear distribution of Arabidopsis HMGB2.

4.3 Chromatin remodelers

Chromatin remodeling complexes (remodelers) contribute to the regulation of chromatin-linked processes. They use the energy of ATP hydrolysis to restructure, shift or eject nucleosomes [126]. Four-five different families of chromatin remodelers have been defined and described among eukaryotes [112, 126-128]. Phosphorylation-dependent regulation of chromatin remodelers was reported in animals [126], but not yet in plants.

4.4 Chromatin modifiers

Chromatin modifying enzymes (modifiers) are able to regulate chromatin-linked processes via the addition of covalent modifications to DNA and histones, such as DNA methylation, histone ubiquitination and histone ADP-ribosylation. Many different histone PTMs have been identified but the corresponding catalyzing enzymes are not all known [98]. In plants, DNA methylation, histone methylation and histone acetylation have been the most extensively studied chromatin modifications [2, 129-135].

Brosch *et al.* showed that histone deacetylase HD1-A from germinating maize (now designated ZmHDA1) is phosphorylated *in vivo* and that the dephosphorylated enzyme form has similar substrate specificity for H2B and H3 but a lower specificity for H2A and H4, while the phosphorylated enzyme form has a 2-fold relative increase of specificity for H2A and a decreased specificity for H3 [136].

Kolle *et al.* reported that the enzymatic activity of maize histone deacetylase HD2 was completely abolished *in vitro* after an alkaline phosphatase treatment and showed, inversely, that a phosphatase treatment strongly increased the enzymatic activity of HD1-A, suggesting a phosphorylation-dependent enzyme activation and inhibition, respectively [137].

Servet *et al.* showed that the Arabidopsis histone acetyltransferase GCN5 interacted specifically with the phosphatase 2C AtPP2C-6-6 *in vitro* and *in vivo* and was dephosphorylated by the phosphatase *in vitro* [138]. Moreover, Servet *et al.* compared the global acetylation level of H3K14 and H3K27 residues in wild type, *gcn5* mutant and *pp2c-6-6* mutants showing a reduction of acetylation on both lysine residues in *gcn5* and inversely a slight increase in the *pp2c-6-6* mutants. These results would suggest a phosphorylation-dependent regulation of GCN5 acetyltransferase activity [138].

4.5 Transcription factors

Cells exploit the fast and reversible change in protein phosphorylation as a mechanism to regulate transcription factor activity by modulating their DNA binding activity, cellular localization, stability and interaction with other proteins. Many transcription factors are regulated in a phosphorylation-dependent manner. Only a few of them will be cited as examples.

The expression of the pathogen-inducible transcription factor WRKY33 in Arabidopsis is regulated by two pathogen-responsive MAPKs, MPK3 and MPK6. WRKY33 not only drives the expression of its target genes but also itself by binding to its own promoter forming a positive feedback loop upon phosphorylation by MPK3 and MPK6. Once WRKY33 is activated transcriptionally and post-translationally upon phosphorylation, it in turn switches on the camalexin biosynthetic genes inducing camalexin biosynthesis in response to a pathogen attack [139]. Phosphorylated WRKY33 was also shown to activate ACS2 and ACS6 gene expression, the rate limiting enzymes in ethylene biosynthesis in *Botrytis cinerea*-infected plants. Due to its involvement in multiple stress and defense

responses, Li *et al.* suggest that WRKY33 could function as a master transcriptional regulator downstream of the MPK3/MPK6 cascade [140].

The brassinosteroid (BR) signaling pathway plays a key role in regulating several aspects of plant growth and development. The GSK3-like kinase, Brassinosteroid-Insensitive 2 (BIN2) negatively regulates BR signaling by phosphorylating and destabilizing two closely related transcription factors BES1 (BRI1-EMS suppressor 1) and BZR1 (brassinazole-resistant 1) crucial for BR responses [141, 142]. The phosphorylation of the transcription factors BES1/BZR1 targets them for degradation to prevent their nuclear localization and inhibit their DNA-binding activities analogous to the Wnt signaling pathway of metazoans [143-146]. Additionally BES1 acts on a transcription repressor myeloblastosis family transcription factor-like 2 (MYBL2) to down-regulate BR-repressed gene expression. MYBL2 itself is stabilized upon phosphorylation by BIN2. This phenomenon is in sharp contrast to the destabilization of BES1 upon phosphorylation by BIN2 [43].

The Arabidopsis Long Hypocotyl in Far-Red Light 1 (HFR1) encodes a basic helix loop helix type transcription factor that plays a key role in light signaling. CK2 phosphorylates HFR1 on exposure to light and this enhanced phosphorylation stabilizes the protein promoting light signaling. In darkness HFR1 is dephosphorylated by an as yet unknown phosphatase, and is targeted for degradation by the COP1-SPA1 E3 ubiquitin ligase complex [147, 148].

Another intriguing example of a plant transcription factor regulated by phosphorylation is VIP1. The Agrobacterium virulence protein VirE2 was shown to associate with VIP1 for nuclear import of the transfer DNA (T-DNA) during the transformation of plant cells. Agrobacterium infection induces the activation of MPK3 which phosphorylates VIP1 resulting in its translocation from the cytosol to the nucleus. The phosphorylated nuclear localized VIP1 regulates the expression of PR1, a pathogenesis related gene [42]. Not

only VirE2, but VirD2 also plays an important role in the nuclear import of T-DNA. VirD2 harbors a C-terminal bipartite nuclear localization signal (NLS), which interacts with an α-importin nuclear import receptor named AtKAPα. Once inside the nucleus, it interacts with host factors to target the T-strand to specific chromosomal integration sites. Bako *et al.* showed that in Arabidopsis VirD2 is tightly associated with the TATA box-binding protein (TBP). Additionally they also found that in alfalfa cells, VirD2 is phosphorylated by CAK2Ms, a cyclin-dependent kinase-activating kinase. CAK2Ms also phosphorylates the C-terminal regulatory domain (CTD) of the largest subunit of RNA polymerase II which recruits the TATA box-binding protein [149]. The phosphorylation of the CTD of RNA polymerase II is involved at several stages during transcription starting from initiation all the way to termination. In yeast and mammals the combined site-specific PTMs and their various regulatory roles have been well characterized. While plants share a number of fundamental similarities with yeasts and mammals, there exist some plant specific characteristics. One such example is CDKF;1, a plant specific CTD Ser7-kinase involved in phosphorylation of cyclin-dependent kinase activating kinases (CDKDs). It has no counterpart in yeast and mammals. Mutant analysis of cdkf;1 in Arabidopsis showed that the phosphorylation of serine residues of RNA polymerase II CTD during transcription cycle is mainly regulated by the plant specific CDKF;1. Disruption of its activity influences the 3' end processing of a subset of precursor miRNAs and transcripts of essential players in the small RNA biogenesis pathways, resulting in the downregulation of unprocessed and mature small RNAs. In parallel, Arabidopsis CTD phosphatases have also been shown to be involved in the regulation of signaling pathways including processing of miRNAs [150].

4.6 Other chromatin-associated proteins

Apart from regulating gene expression via transcription factors, phosphorylation/dephosphorylation of certain proteins can also influence DNA metabolism, genome integrity, chromosome segregation during mitosis and meiosis, etc. For instance, in pea (*Pisum sativum*) the enzyme DNA topoisomerase I catalyzes the interconversion of topological states of DNA to maintain the superhelical density of DNA in turn contributing to genome integrity. This catalytic activity of topoisomerase I is regulated by phosphorylation of serine residue(s) by CK2 and protein kinase C [151]. Similarly, the plant retinoblastoma-related (RBR) protein is a critical regulator of G1/S phase transition during cell cycle. As in mammals, the function of RBR proteins in plants is regulated by protein-protein interactions and phosphorylation. The plant RBR proteins possess multiple potential CDK phosphorylation sites and an interaction of the CDKs with D-type cyclins is essential to form an active kinase complex that can then phosphorylate the RBR proteins [152].

The cohesin complex is one of the three structural maintenance of chromosomes (SMC) complexes. SMC proteins play an important role in maintaining chromosome structure and dynamics, gene regulation and DNA repair. The cohesin complex is formed by the heterodimerization of SMC1 and SMC3. It is involved in sister chromatid cohesion and chromosome segregation at anaphase during cell division [153, 154]. In yeast it was shown that the loss of cohesin complex proteins is essential for the segregation of homologous chromosomes during meiosis I and sister chromatids in meiosis II. The switch for this loss of cohesin complex proteins is the phosphorylation of the cohesin subunit Rec8 [155]. This step in the process is not completely elucidated in plants yet.

Cryptochromes are ubiquitous photosensory receptors that mediate photomorphogenic responses viz. inhibition of stem elongation, stimulation of leaf expansion, control of

photoperiodic flowering, coordination of the circardian rhythm and regulation of gene expression [156]. In Arabidopsis, CRY2 is nuclear localized, undergoes a conformational change upon absorption of blue light photons rendering itself amenable for phosphorylation by an unknown kinase. While phosphorylation activates CRY2 to perform its physiological functions it also marks it for degradation by the 26S proteasome complex. It is important to note that blue light dependent phosphorylation and degradation of CRY2 occurs in the nucleus. This strengthens the idea of cryptochromes being the major blue light receptors regulating nuclear gene expression [157].

5 Perspectives on protein phosphorylation and chromatin regulation

5.1 Crosstalk with other PTMs

The nucleus is a complex subcellular compartment made of different membrane-free subnuclear bodies [158, 159] and differences have been observed between animal and plant nuclei [89, 160]. In such an environment, all nuclear processes, chromatin regulation among them, are only dictated by chemical properties of the involved molecules. PTMs of proteins and modifications of DNA, notably, constitute theoretically an almost unlimited source of regulation of nuclear events. The study of crosstalk between phosphorylation and other PTMs should reveal new mechanisms of regulation [161]. Such examples of crosstalk have been reported on both histones and non-histone proteins [5, 44].

The interplay between phosphorylation and various modifications has been described for plant histones. For example, *in vitro* phosphorylation of Arabidopsis H3S10 by AtAurora1 and H3T3 by AtHaspin is modulated by methylation, acetylation and phosphorylation of neighbouring residues [107, 162]. Interestingly, these two histone H3

residues are conserved in mammals and yeast, but some modifications of histone H3 have been specifically identified in plants [163]. It will be interesting to decipher the roles of these plant-specific histone marks and the possible crosstalk between conserved and plant-specific PTMs.

Co-occurrence of Small Ubiquitin-like Modifier (SUMO) and phosphorylation has been uncovered in Arabidopsis: proteomics studies indicated a considerable overlap between the proteins identified to be targets of SUMO and the known substrates of MAPK3, 4 and 6 [164, 165]. MAPK signalling and SUMOylation converge onto common transcriptional regulators, among which are transcription factors such as WRKYs, EIN3, EIL1 and ERF104 that are involved in plant defense [165]. More globally, in Arabidopsis, the SUMOylation level of proteins involved in a large range of nuclear processes was shown to increase during various stresses, such as heat shock, ethanol and oxidative stress. These include proteins implicated in RNA processing, DNA methylation, histone covalent modification, chromatin remodeling and transcription regulation [166] but the mechanisms of the presumed crosstalk remain to be elucidated.

Interplay between phosphorylation and yet other modifications, such as ubiquitination or O-linked N-acetylglucosaminylation, can be expected in plants [167]. To understand in more depth the relationships between several PTMs and PTM sites within a protein, one would have to characterize the full set of modifications present on individual protein molecules, and determine whether variable combinations of PTMs decorate the same protein sequence. Ultimately, the order of addition and removal of the diverse modifications of a protein and the possible causal links between them would have to be deciphered within the considered biological context. To reach this level of description, the more classically employed bottom-up analysis of proteolyzed protein samples can be efficiently complemented by top-down or middle-down approaches [168]. Given the low molecular

weight of histones, such analyses of intact proteins have been largely put in practice [169], they require, however, the purification of reasonably large amounts of the proteins of interest.

5.2 Localization of (phospho)proteins at gene resolution level

In the case of chromatin-associated proteins, one of the future challenges will be to precisely localize the binding of the different versions of a protein (e.g. non-phosphorylated and phosphorylated) to the chromatin and to determine whether these binding sites correlate with particular histone modifications and gene expression.

The current most efficient techniques to localize a protein at the gene resolution level are based on chromatin immunoprecipitation (ChIP) [170-173]. However, ChIP approaches require the use of sensitive and specific antibodies. To reveal the *in vivo* putative differential localization of the different phosphorylated states of a protein would need the use of several antibodies, each recognizing a particular phosphorylation state of the protein of interest. Recent advances made in the field of phosphorylation site–specific antibody production are compatible with the realization of such approaches. Besides, the use of mutated versions of the protein, as described in part 3.2 (e.g. substitution of Ser by Ala, or Ser by Asp), should represent an interesting alternative.

It should be mentioned that some techniques have been recently developed which tackle this question in the opposite way by identifying the proteins which are associated *in vivo* to a specific DNA region [174, 175]. The complementarity of these approaches should allow shedding new light on the understanding of chromatin regulation.

6 Concluding remarks

Protein phosphorylation is among the most abundant and important PTMs in plants, regulating many protein properties. Huge efforts have been made to uncover plant phosphoproteomes, but some subcellular compartments such as the nucleus and chromatin have been poorly explored. Although the identification of the functional consequences of protein phosphorylation is crucial for the understanding of chromatin regulation, only a few cases of phosphorylation-dependent regulation of plant chromatin and chromatin-associated proteins have been described to date. Moreover, PTM crosstalk constitutes an additional level of complexity in the regulation of protein properties and substantial efforts will be necessary to reach a sophisticated understanding of plant chromatin regulation.

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Figure 1. Scheme showing the direct molecular effects of protein phosphorylation and the functional protein consequences. Once a protein has been phosphorylated, its conformation and its interactions with other molecules may be modified. These changes may have diverse functional consequences which may further be modulated by PTM crosstalk. Finally, these events may lead to cellular responses such as signal transduction or transcription regulation.

Figure 1

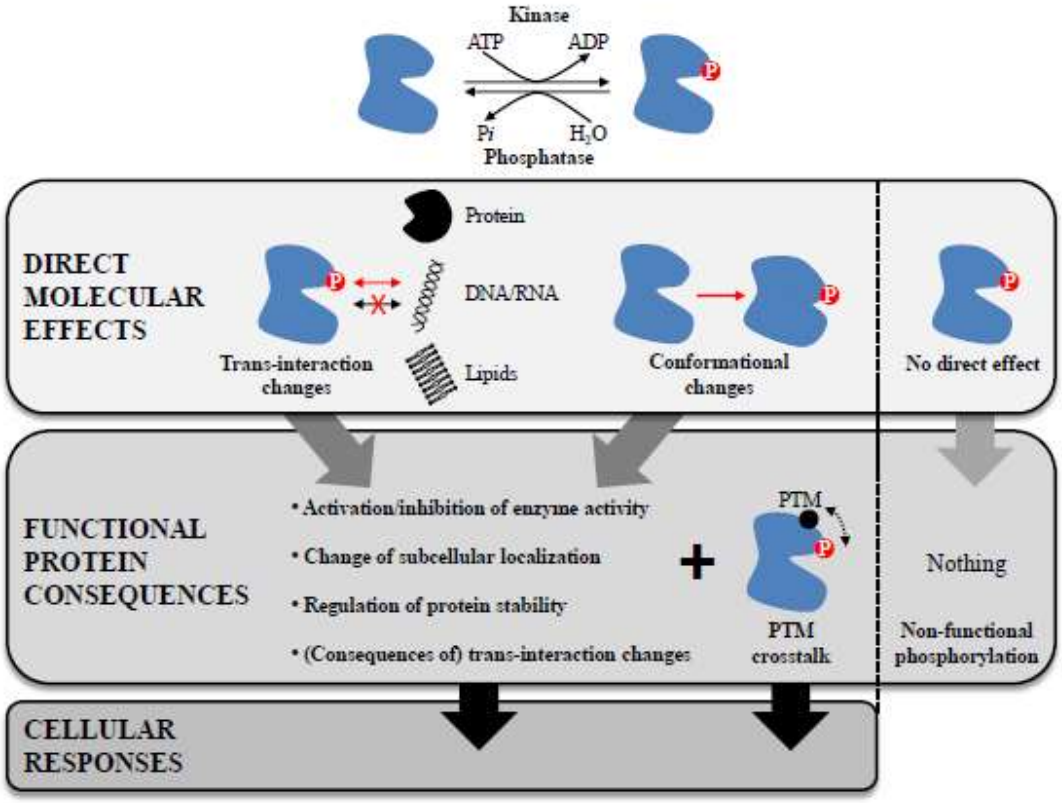

Figure 2. Comparison of the experimentally identified phosphoproteins from Arabidopsis reported in the PhosPhAt 4.0 database (http://phosphat.mpimp-golm.mpg.de/, release phosphat_20130429.csv), the Arabidopsis chromatin-associated annotated proteins from ChromDB (http://www.chromdb.org/, database updated on February 1st 2014) and the in silico annotated Arabidopsis DNA-binding proteins from At-Dbome (Malhotra and Sowdhamini, 2013). Only the generic Arabidopsis Genome Initiative identifiers were considered for the comparison, such as ATXGXXXXX; the isoforms such as ATXGXXXXX.1 and ATXGXXXXX.2, for example, were not considered.

Figure 2