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REVIEW PAPER

Sumoylation and phosphorylation: hidden and overt links

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Abstract

Post-translational modifications are essential mediators between stimuli from development or the environment and adaptive transcriptional patterns. Recent data allow a first glimpse at how two modifications, phosphorylation and sumoylation, act interdependently to modulate stress responses. In particular, many components of the SUMO conjugation system are phosphoproteins, and some regulators and enzymes of protein phosphorylation can be sumoylated. Equally important, however, a number of proteins can be subject to both modifications. These substrates also have the capacity to connect stimuli transmitted via sumoylation with those transmitted via phosphorylation. As a prime example, we review data suggesting that nitrate reductase is a hub that integrates cues from these two modifications. Powerful proteomics approaches allowed the identification of additional common substrates, paving the way for studies to understand, on a broader basis, the cross-talk of phosphorylation with sumoylation and how it contributes to plant growth.

Keywords: Nitrate reductase, phytochrome B, protein modification, protein phosphorylation, SUMO.

Introduction

The role of post-translational modifications (PTMs) of proteins in regulating activity and function in various cellular processes has been well established over the past decades. While many PTMs have been studied in great detail, their interactions and interdependencies have so far attracted much less attention, although mechanistic connections are expected for many of them. Recently, a number of publications have analyzed interconnections between protein phosphorylation and sumoylation.

Protein phosphorylation, the linkage of phosphate to Ser, Thr, or Tyr OH-groups, is one of the most prominent modifications, both in animals and in plants. About one-third of all cellular proteins are estimated to be phosphorylated at a given time (Cohen, 2000). Protein phosphorylation occurs in one step, requiring a single active site. However, the multitude of protein kinases working in plants implies sophisticated regulatory processes and substrate preferences, even though individual kinases usually recognize short (hence relatively widespread) amino acid motifs, and may therefore have a large number of substrates (Kanshin *et al.*, 2017).

In contrast to phosphorylation, modification of proteins by the small ubiquitin-related modifier (SUMO), while being also essential, is less prominent. Analysis in humans indicates that more than 20% of all protein-coding genes produce

sumoylatable isoforms, indicating that this modification also has a wide range of substrates (Hendriks *et al.*, 2017). Covalent attachment of SUMO to its substrates is instrumental in a broad range of plant responses to biological and environmental stimuli (for reviews, see Castro *et al.*, 2012; Novatchkova *et al.*, 2012; Xu and Yang, 2013; Elrouby, 2015; Yates *et al.*, 2016; He *et al.*, 2017; Verma *et al.*, 2018). In particular, SUMO conjugation influences the response to drought, cold, and salt stress (Catala *et al.*, 2007; Miura *et al.*, 2007; Conti *et al.*, 2008; Miura *et al.*, 2013), and impacts on flowering time, pathogen response, and phosphate accumulation (Murtas *et al.*, 2003; Miura *et al.*, 2005; Lee *et al.*, 2007; Jin *et al.*, 2008). Sumoylation relies on an enzyme cascade similar to the one for ubiquitin conjugation, but consisting of a much smaller number of enzyme isoforms. A heterodimeric SUMO activating enzyme (SAE) binds SUMO in a thioester bond, between the carboxyl-terminal Gly of SUMO and its active site Cys, and transfers this small protein to the SUMO conjugating enzyme (SCE) active site. Most plants have one or two SAEs (Novatchkova *et al.*, 2012; Castaño-Miquel *et al.*, 2013), and one SCE (monocotyledonous plants have a second, somewhat distinct isoform; Novatchkova *et al.*, 2012; Augustine *et al.*, 2016). SUMO ligases promote the SUMO transfer from SCE to substrate Lys ε-amino groups, but SCE alone can also modify substrates. Only two SUMO ligases have been identified so far in Arabidopsis (SIZ1 and HPY2/MMS21; Miura *et al.*, 2005; Huang *et al.*, 2009; Ishida *et al.*, 2009). SUMO E4 type ligases with ability to promote SUMO chain formation have also been identified (PIAL1 and PIAL2; Tomanov *et al.*, 2014). Interestingly, a growing number of SUMO-specific proteases have been shown to hydrolyse the SUMO–substrate bond (Novatchkova *et al.*, 2012; Yates *et al.*, 2016). SUMO proteases were suggested to significantly contribute to substrate selectivity of SUMO conjugation, by reversal of sumoylation in a substrate-specific manner (Verma *et al.*, 2018). Figure 1 summarizes enzymes of SUMO conjugate generation and removal known from Arabidopsis.

Protein phosphorylation and sumoylation have in common that they help to convert stimuli from development or from the environment into cellular responses. In stress responses, these modifications help to buffer dangerous insults resulting from the environment. They are necessary for adaptation to changed environmental conditions, by adjusting transcriptional patterns and metabolism. An interconnection or crosstalk between these modifications apparently occurs by modification of enzymes from the modification pathways themselves. Moreover, a number of recent studies suggest that sumoylation and phosphorylation may frequently impinge on the same proteins, implying the existence of key substrates (hubs) that co-ordinate input from two distinct signalling cascades. Table 1 summarizes those protein substrates for both modifications that are discussed below.

Modification of modifying proteins

An accumulating body of evidence shows that components of the SUMO conjugation machinery are modified by sumoylation, and by other post-translational modifications. SUMO ligase SIZ1 has been identified as an *in vivo* sumoylation substrate. For example, the extent of sumoylation increases upon heat stress (Miller *et al.*, 2013). SIZ1 sumoylation was also reported to increase during dehydration stress (Kim *et al.*, 2017). Sumoylation of SIZ1 may compete with COP1-dependent ubiquitylation and degradation of SIZ1, which was detected upon heat stress, but not dehydration stress in one study (Kim *et al.*, 2017). Increased degradation of SIZ1 upon heat stress seems difficult to reconcile with the known role of SIZ1 in the heat-induced increase in global sumoylation, and with the quantitative data of Miller *et al.* (2013), which show that sumoylation of SIZ1 itself increases during heat stress. One interpretation is that timing is important, so that degradation of SIZ1 plays a role after an initial heat-induced sumoylation and activation of SIZ1, to moderate SUMO conjugation during later stages of heat exposure. In contrast, the extent of *in vivo* sumoylation of SUMO activating enzyme subunit 2 (SAE2) does not vary much during heat stress (Miller *et al.*, 2013). Functional studies are necessary to understand the consequences of these modifications, but it is tempting to speculate that changes in sumoylation are actively involved in managing the transient increase in SUMO conjugation that accompanies most sudden stress applications. Furthermore, SIZ1 and SAE2 are also phosphoproteins, according to the PhosPhAt database (phosphat.uni-hohenheim.de; Heazlewood *et al.*, 2008), and the dynamics and significance of these modifications remain to be elucidated. SUMO chain forming ligase PIAL2 is sumoylated *in vitro* (Tomanov *et al.*, 2014) and listed in PhosPhAt as a phosphoprotein, but neither modification has been functionally investigated. Finally, fractions of SUMO1 and of SUMO2 (Nukarinen *et al.*, 2017; PhosPhAt) have also been identified as phosphoproteins. Again, functional implications of this modification are currently unknown.

Sumoylation of SUMO1 or -2, linking several SUMO moieties into a chain, is also found *in vivo*. SUMO chains can act as a protein degradation signal. This conclusion comes from the existence (in all eukaryotes) of so-called SUMO-targeted ubiquitin ligases (STUbLs; Sriramachandran and Dohmen, 2014). These ubiquitin ligases apparently bind to multiple SUMO moieties when present in close proximity on the same protein (complex), and in particular to SUMO chains (see Fig. 1). Consistent with a specific role for SUMO chains, proteins that enhance SUMO chain formation (SUMO chain forming ligases, also called SUMO E4 ligases) were identified in plants (Tomanov *et al.*, 2014). A SUMO chain-dependent ubiquitylation/degradation pathway must compete with the activity of desumoylating enzymes, which reverse the sumoylation of SUMO to remove SUMO chains from substrates (Fig. 1). Certainly, only a small fraction of proteins are modified by SUMO chains, with subsequent degradation of the modified substrate via SUMO-targeted ubiquitin ligases. However, it is currently unclear whether this can occur to any sumoylated protein with low probability, or whether it is restricted to preferred substrates (Tomanov *et al.*, 2018).

In contrast, the phosphorylation of protein kinases is an established process and will therefore not be discussed here in detail. Recently, the protein kinase SnRK1, which can be activated by phosphorylation and has a pivotal role in stress and energy signalling (Emanuelle *et al.*, 2016), was shown to

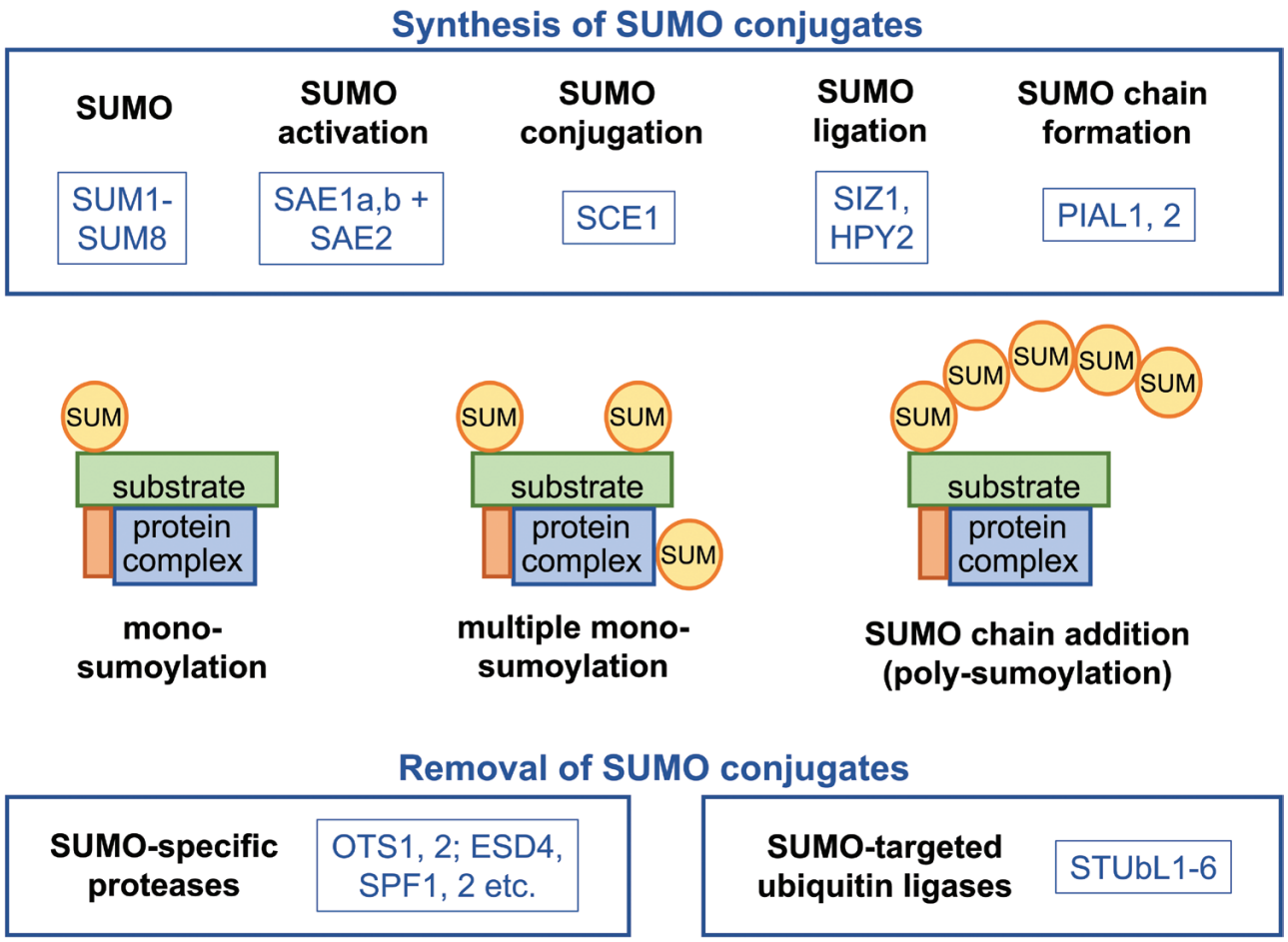

Fig. 1. Steps involved in synthesis and removal of SUMO conjugates, listing genes from Arabidopsis. SUMO conjugation starts by activation of SUMO (genes *SUM1* to *SUM8* in Arabidopsis) by the heterodimeric SUMO activating enzyme (SAE). Transfer of SUMO to SUMO conjugating enzyme (SCE) is followed by transfer to a substrate. SUMO ligases assist in this transfer. In addition, a dedicated function promotes SUMO chain formation. SUMO conjugate removal can occur by desumoylating enzymes, or by recognition of multiple SUMO moieties by SUMO-targeted ubiquitin ligases (STUbLs), which results in ubiquitin- and proteasome-mediated degradation. Schemes in the middle show structures of sumoylated substrates, which are frequently protein complexes.

Table 1. Consequences of sumoylation and phosphorylation for proteins discussed in the text

	Consequence of modification by:		
Protein	**Sumoylation**	**Phosphorylation**	**References**
SUMO1, SUMO2	Substrate for ubiquitylation	Unknown	Tomanov *et al.* (2014), Nukarinen *et al.* (2017), PhosPhat database Heazlewood *et al.* (2008), phosphat.uni-hohenheim.de
SUMO activating enzyme subunit SAE2	Unknown	Unknown	Miller *et al.* (2013), PhosPhat database
SUMO ligase SIZ1	Unknown	Unknown	Miller *et al.* (2013), PhosPhat database
SUMO chain forming ligase PIAL2	Unknown	Unknown	Tomanov *et al.* (2014), PhosPhat database
Protein kinase SnRK1	Activation (but reduced half-life)	Activation	Crozet *et al.* (2016), Emanuelle *et al.* (2016)
Nitrate reductase	Activation	Down-regluation	Huber *et al.* (1992), Park *et al.* (2011)
Phytochrome B	Down-regulation	Down-regulation	Medzihradszky *et al.* (2013), Sadanandom *et al.* (2015)
Transcription factor WRKY33	Unknown	Activation	Mao *et al.* (2011), Miller *et al.* (2013)
Transcription factor ABI5	Down-regulation	Activation	Kobayashi *et al.* (2005), Miura *et al.* (2009)
Transcription factor CESTA	Localization to subnuclear microdomains	Localization to nucleoplasm	Khan *et al.* (2014)
Transcriptional regulator NPR1	Modification by SUMO3: induction of phosphorylation at Ser 11/15	Decreased half-life, increased ability to activate defence genes	Spoel *et al.* (2009), Saleh *et al.* (2015)

be modified by sumoylation (Crozet *et al.*, 2016). The process is SIZ1-dependent and shortens the half-life of SnRK1. One possible scenario is that after activation, or as part of activation, SnRK1 invites sumoylation, which is followed by ubiquitylation and proteasome-dependent degradation. The effect of this sumoylation–ubiquitylation cascade (which might involve SUMO chains) may be a timing mechanism to ensure that turnover follows activation with a certain time delay, restricting kinase activity to a limited, but not too narrow, time window.

Common substrates: case studies

Nitrate reductase

As a catalyst of the first and rate-limiting step in production of ammonium ions from nitrate (its reduction to nitrite), nitrate reductase (NR) activity is regulated in accordance with the need for ammonium ions and with nitrate availability. Part of this regulation occurs at the post-transcriptional level. Phosphorylation was identified as a regulatory step that decreases NR activity (Huber *et al.*, 1992; Hey *et al.*, 2010). More recently, sumoylation was shown to be an activating modification (Park *et al.*, 2011). Thus, sumoylation and phosphorylation have antagonistic effects on NR activity.

Recent analyses (Gibbs *et al.*, 2014*b*; Vicente *et al.*, 2017) show that in addition to its function as a key enzyme in nitrogen metabolism, NR is also a hub that integrates metabolic and other inputs with the transcriptional stress response. NR not only generates NO_2^- from NO_3^-, it can also reduce NO_2^- to NO, which is a signalling molecule. NR may either produce NO in its own active centre, or donate reduction equivalents to another molybdenum cofactor-containing protein, NO-forming nitrite reductase (NOFNiR; Chamizo-Ampudia *et al.*, 2017). NR appears to provide the major route for NO synthesis in plants. In darkness, and under a broad range of stress conditions (including carbon or nitrogen shortage), NR activity is down-regulated. At the post-translational level, down-regulation includes increased phosphorylation at a hinge region (Huber *et al.*, 1992; Lambeck *et al.*, 2012). Stress-responsive protein kinase SnRK1 is a major kinase involved, and was shown to bind to NR (Polge *et al.*, 2008). However, other kinases, in particular Ca^{2+}-dependent protein kinases, may also be involved (Lambeck *et al.*, 2010). 14-3-3 proteins with affinity to phosphorylated NR mediate down-regulation by inhibiting electron transfer to the molybdenum cofactor (Bachmann *et al.*, 1996; Lambeck *et al.*, 2010, 2012). As a consequence, intracellular NO levels decrease. This decrease impacts on the abundance of Group VII ETHYLENE RESPONSE FACTOR (ERFVII) transcription factors. Activity of these stress regulators is controlled by modulation of protein abundance via the Cys-Arg/N-end rule pathway of ubiquitin-mediated proteolysis (Gibbs *et al.*, 2014*a*). Oxidation of the amino-terminal Cys residue requires both oxygen and NO (Gibbs *et al.*, 2014*a*; Vicente *et al.*, 2017), and the recently discovered PLANT CYSTEINE OXIDASEs (Weits *et al.*, 2014). The ERFVIIs are short-lived if oxidation occurs, due to amino-terminal arginylation of the oxidized Cys, subsequent ubiquitylation and proteasome-dependent degradation (Gibbs *et al.*, 2011; Licausi *et al.*, 2011). Low NO levels (or hypoxic conditions) prevent Cys oxidation, resulting in accumulation of ERFVIIs, which induce stress response genes. Several studies have shown that abiotic stresses, including drought and salinity, reduce NR activity (Foyer *et al.*, 1998; Debouba *et al.*, 2007; Fresneau *et al.*, 2007). Reduced NO levels, resulting from reduced NR activity, were shown to enhance ERFVII action, bolstering plant stress tolerance (Vicente *et al.*, 2017).

In contrast to the inhibitory function of phosphorylation, sumoylation activates NR (Park *et al.*, 2011). Unfortunately, data on the *in vivo* dynamics of NR sumoylation are not available. In particular, it is not clear whether *in vivo* decoration of NR with SUMO is transient, or longer lasting. One hypothesis is that (transient) sumoylation helps NR to switch from an inactive to the active conformation. Independent of the detailed mechanism, sumoylation of NR can be expected to modulate stress responses, as an antagonist to phosphorylation. It has been shown that, whereas many substrates show increased sumoylation under stress conditions, a minor fraction of substrates is less sumoylated (Miller *et al.*, 2013). If NR belongs to this fraction of sumoylation substrates, then SUMO conjugation co-regulates the shift from stress response to growth resumption. In sum, the impact of SUMO conjugation on stress responses may also occur via its influence on nitrate reductase activity.

Figure 2 shows schematically the known post-translational modifications that impact on NR, and their consequences. While phosphorylation of NR was first described in the context of light–dark regulation, the scheme capitalizes on stress-induced down-regulation of NR. It remains to be seen whether dark-induced down-regulation of NR differs from stress-induced down-regulation.

Phytochrome B

In contrast to other examples, phosphorylation and sumoylation do not have an antagonistic effect on phytochrome B. Rather, both modifications decrease the biological response to red light, althought by different mechanisms. Whereas phosphorylation results in faster dark reversion (Medzihradszky *et al.*, 2013), sumoylation interferes with binding of phytochrome B to (at least one of) the phytochrome interacting factor transcriptional regulators (Sadanandom *et al.*, 2015).

Transcription factors

Proteomic analysis by Miller *et al.* (2013; so far the most extensive dataset for plants), as well as other publications (Budhiraja *et al.*, 2009; Elrouby and Coupland, 2010; Miller *et al.*, 2010; López-Torrejón *et al.*, 2013) list a significant number of transcription factors as sumoylation substrates. Likewise, phosphorylation of transcription factors and other chromatin proteins is widespread. However, direct experimental cross-reference of the two modifications on the same transcription factor is still rare. We selected three proteins as examples, WRKY33, ABI5, and CESTA.

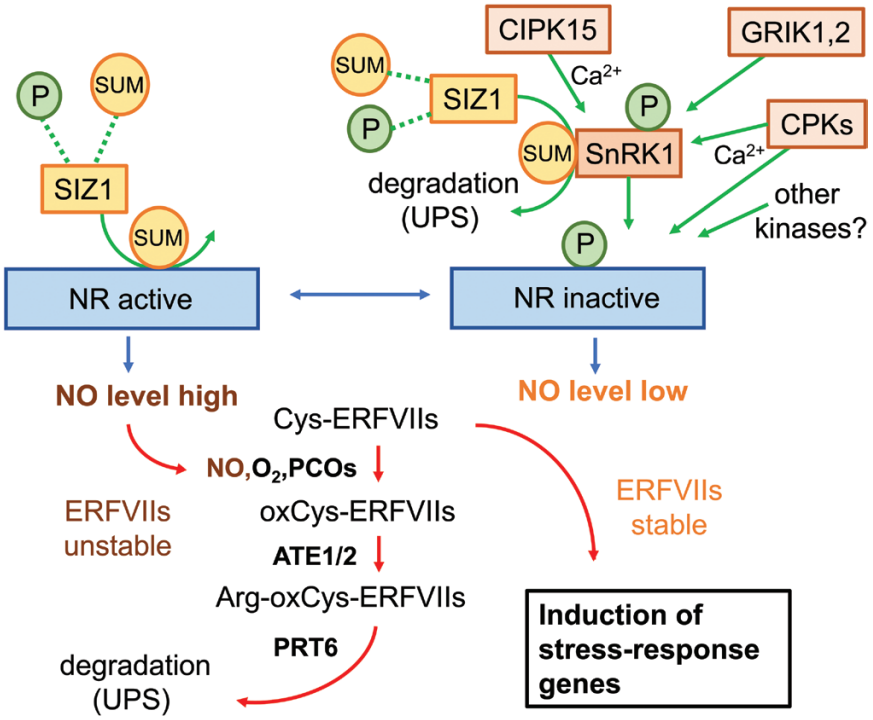

Fig. 2. Nitrate reductase (NR) as an example of a protein modified by both phosphorylation and sumoylation, emphasizing its role as a regulatory hub. Phosphorylation of NR can occur by the stress-responsive SNF1-like kinase SnRK1, which is itself regulated by sumoylation and phosphorylation. Phosphorylation of SnRK1 can be Ca^{2+}-dependent (via CIPK15), or -independent (via GRIK1 or 2, a.k.a. SnAK2 and 1, respectively). NR phosphorylation can also occur directly via Ca^{2+}-dependent kinases (CPKs), or presumably by other kinases. For NR, phosphorylation is an inactivating modification. Down-regulation occurs via binding of 14-3-3 proteins to the phospho-epitope (not shown). In contrast, sumoylation of NR is an activating modification, and normal activity of NR in wild-type cells depends on SIZ1. It is currently unclear whether SIZ1-dependent NR modification is a permanent or a transient mark. In parallel with NR's ability to reduce nitrate, NR-produced nitric oxide (NO) levels change *in vivo*. As a downstream event, NO regulates turnover of stress-responsive ERFVII transcription factors via the N-end rule pathway. Under low NO conditions, ERFVIIs cannot be oxidized at their amino-terminal Cys residue. This oxidation also requires oxygen and plant cysteine oxidase enzymes (PCOs). L-Arginyl tRNA:protein arginyltransferase (ATE1 or ATE2 of Arabidopsis) adds an Arg residue to oxidized amino-terminal Cys, but not to unmodified Cys. Ubiquitin ligase PRT6 has a binding site for amino-terminal Arg, which leads to ubiquitylation of ERFVIIs. As a consequence, modification of NR impacts on the induction of stress-responsive genes, shifting resources from growth to defence. Additional abbreviations used: CIPK, calcineurin B-like-interacting protein kinase; GRIK, geminivirus Rep-interacting kinase; SnAK, SnRK1 activating kinase; UPS, ubiquitin–proteasome system.

WRKY33 (At2g38470)

WRKY33 is listed as a phospho-protein in the PhosPhAt database, and in the dataset of Miller *et al.* (2013) as a sumoylation substrate. Its phosphorylation in response to a pathogen stimulus has been documented (Mao *et al.*, 2011) and leads to increased transcription of genes necessary for synthesis of the phytoalexin camalexin. Therefore, phosphorylation is an activating modification. In contrast, SUMO modification of chromatin proteins has repeatedly been associated with transcriptional repression. Unfortunately, the impact of sumoylation on WRKY33 has not been published so far.

ABI5 (At2g36270)

ABSCISIC ACID INSENSITIVE5 (ABI5) is a bZIP transcription factor discovered as an element of abscisic acid response. It functions in seed germination and more generally in stress signalling. Its phosphorylation by SnRK2 type kinases (Kobayashi *et al.*, 2005; Fujii *et al.*, 2007; Nakashima *et al.*, 2009) stabilizes the ABI5 protein and increases activity. Dephosphorylation was also studied, identifying its contribution to adjustment of ABI5 activity (Dai *et al.*, 2013). In contrast, sumoylation decreases ABI5 activity, and *siz1* mutants cause abscisic acid hypersensitivity (Miura *et al.*, 2009). It remains to be investigated whether phosphorylation and sumoylation of ABI5 are in any way interdependent.

CESTA (At1g25330)

CESTA is a transcription factor of the brassinosteroid response. For this protein, biological data on both phosphorylation and sumoylation were published in a comparative study (Khan *et al.*, 2014). Brassinosteroid treatment led to localization of CESTA to subnuclear compartments (speckles). A CESTA variant that cannot be sumoylated did not show this behaviour. In contrast, preventing phosphorylation by Ser to Ala changes at phosphorylation sites resulted in constitutive localization to speckles. Most likely, phosphorylation prevents sumoylation, suggesting a direct antagonistic effect of phosphorylation on sumoylation.

Nonexpressor of pathogenesis-related genes1

Nonexpressor of pathogenesis-related genes1 (NPR1) is a central hub for induction of (biotic) defence genes. A rise in the level of salicylic acid (SA) triggers a series of modifications that activate NPR1, but at the same time decrease its half-life,

effectively limiting the duration of induction. SA decreases NPR1 phosphorylation at Ser 55/59. This allows conjugation to SUMO3. SUMO3 modification in turn triggers phosphorylation at Ser 11/15, which results in a more active, more short-lived protein (Spoel *et al.*, 2009; Saleh *et al.*, 2015).

Omics approaches can guide candidate searches

A phosphoproteomics analysis of mutants in SUMO conjugation allowed the analysis of relationships between phosphorylation and sumoylation in a more systematic manner (Nukarinen *et al.*, 2017). In plants grown under standard (non-stress) greenhouse conditions, 54 phosphoproteins were identified as having altered abundance in sumoylation mutants. Interestingly, these proteins had a high abundance of canonical sumoylation motifs (ψKxE; where ψ symbolizes a hydrophobic amino acid, K the lysine residue that is linked to the SUMO carboxyl terminus, x any amino acid, and E glutamic acid), and of SUMO interaction motifs (Hecker *et al.*, 2006). This suggests tight connections to SUMO modification and/or SUMO-modified proteins. The significance of these findings remains to be elucidated. The data are in line with trends based on continuously improving proteomics methods to identify the modified proteome *en masse*, as a basis for functional studies.

An elegant set of proteomics experiments with human tissue culture cells (Becker *et al.*, 2013; Hendriks *et al.*, 2014; 2017; Hendriks and Vertegaal, 2016) revealed a multitude of sumoylation substrates. In one of these studies, the extent of phosphorylation was specifically monitored in sumoylation substrates (Hendriks *et al.*, 2017). The authors came to the conclusion that the number of substrates co-modified by both processes is much larger than expected by chance. The authors suggest that this high coincidence is due to the fact that both modifications rely on short recognition motifs that reside in flexible (hence accessible) regions of proteins. Such regions are more frequent in larger proteins. Indeed, both modifications are more often found in large proteins (Hendriks *et al.*, 2017). It should be noted, however, that this characterization is unlikely to hold for plant transcription factors such as WRKY33, which are not large proteins. There is also a specific link between sumoylation and phosphorylation, provided by a defined sequence context. In animals (so far not yet identified in plants), phosphorylation-dependent sumoylation motifs require phosphorylation before the sumoylation machinery can attach SUMO (Hietakangas *et al.*, 2006). In terms of wiring logic, this implies an 'and' for the final substrate response in the terminology of formal logic, as both phosphate and SUMO modification must be present together for certain outputs. This contrasts with the listed examples from plants (Table 1), where the two modifications more often act antagonistically, probably wired in the form called 'and not' (corresponding to the colloquial either–or): a common substrate is modified either by SUMO, or by phosphorylation (and these modifications result in distinct substrate responses).

Summary and outlook

Emerging data allow a first view on signal integration between SUMO conjugation and phosphorylation. Both modifications are employed in a wide range of cellular processes, and substrate selection is incompletely understood. However, proteomics-based datasets of modified proteins should facilitate case-by-case studies. Selected cases, for which biological data are available, underscore the importance and attractiveness of such studies. Both sumoylation and phosphorylation impact on the balance between growth and stress response, against biotic as well as abiotic challenges. Their co-ordination offers additional modes of response to plant cells, with the added flexibility certainly contributing to plant survival and fitness.

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