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Transcriptional regulation by complex interplay between post-translational modifications

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Transcriptional reprogramming in response to developmental changes or environmental inputs is regulated by a wide variety of transcription factors and cofactors. In plants, the stability of many transcriptional regulators is mediated by the ubiquitin-mediated proteasome. Recent reports suggest that additional post-translational modifications modulate the ubiquitination and thus stability of transcriptional regulators. In addition to well-recognized phosphorylative control, particularly conjugation to the ubiquitin-like protein SUMO as well as thiol modification by nitric oxide to yield *S*-nitrosothiols, are emerging as key regulatory steps for governing protein ubiquitination in the nucleus. Complex interplay between these different post-translational modifications may provide robust control mechanisms to fine tune developmental and stress-responsive transcriptional programs.

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Introduction

To survive plants must efficiently respond to wide-ranging environmental cues and stresses by rapidly yet precisely reprogramming their transcriptomes. Transcription is regulated by a vast array of transcription factors and associated cofactors that are often subject to diverse post-translational modifications (PTMs). PTMs add a tremendous amount of complexity to cellular proteomes and their large variety and concurrent appearance in transcriptional regulators is thought to dramatically increase the nuclear proteome size from mere thousands to the order of millions of possible protein forms.

Post-translational control of transcriptional regulators by ubiquitination is especially prevalent in plants, with genomic analysis revealing that core components of the ubiquitination machinery may account for up to 6% of the Arabidopsis proteome [1]. Ubiquitination is facilitated by the sequential actions of three enzyme types; E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases. Polyubiquitination of proteins often targets them for degradation by the 26S proteasome, a large multi-protein complex harbouring the major proteolytic activity in all eukaryotic cells. This ubiquitin-proteasome system (UPS) is essential to the regulation of hormone-responsive genes. In some cases hormones are even perceived by co-receptors consisting of both a transcriptional regulator and an E3 ligase component. Hormone binding acts as a molecular glue, promoting recruitment of the transcriptional regulator to the E3 ligase and eventually resulting in its degradation. For example, the hormones auxin and jasmonate promote recruitment of transcriptional repressors to the Skp1/Cullin/F-box (SCF) E3 ligases, SCF^{TIR1} and SCF^{COI1}, respectively, leading to their proteasome-mediated degradation and activation of hormone-responsive gene expression [2,3]. More recently, salicylic acid (SA) was also identified to be perceived by a co-receptor consisting of both the Cullin3-RING E3 ligase (CRL), $CRL3^{NPR3/4}$, and its substrate NPR1, an indispensable master coactivator of plant immune genes [4–6].

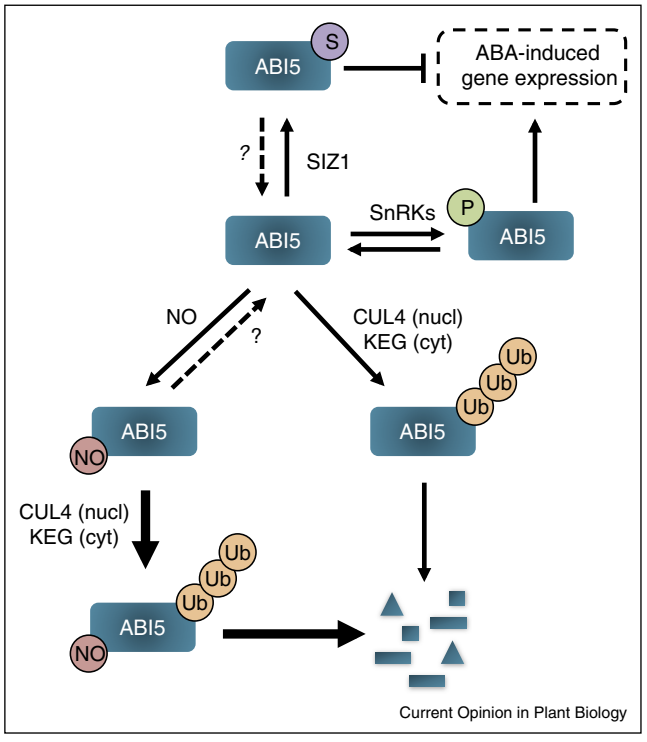
While cross talk between ubiquitination and phosphorylation has been well established [7], interplay between ubiquitination and other PTMs is only just emerging. Here we will highlight emerging evidence of combinatorial regulation of several developmental and stress-responsive transcription (co)factors in plants by ubiquitination and the additional PTMs. The interplay between ubiquitination and other PTMs adds additional layers of complexity to allow plants to fine-tune the nuclear levels and activity of transcriptional regulators.

Combinatorial control by ubiquitination and SUMOylation

In addition to ubiquitin, various ubiquitin-like proteins exist with distinct and diverse functions, including small ubiquitin-like modifier (SUMO). Ubiquitin-like proteins are characterized as such by sharing a similar structure and enzymatic mechanism of conjugation with ubiquitin. Proteomic analyses of SUMO-modified proteins in Arabidopsis have identified hundreds of targets, many of which are involved in transcription regulation [8–10].

The bZIP transcription factor ABI5 has a central role in abscisic acid (ABA) signalling and is regulated by a multitude of PTMs [11]. ABI5 is phosphorylated by SNF1-related protein kinases (SnRK2.2, SnRK2.3 and SnRK2.6) that promote its transcriptional activity [12] (Figure 1). In absence of ABA, however, ABI5 is maintained at low levels due to ubiquitination by the cytoplasmic E3 ligase KEEP ON GOING (KEG) and subsequently degraded by the 26S proteasome [13•,14]. Interestingly, increased levels of ABA promote KEG self-ubiquitination and degradation, leading to stabilization of ABI5 and activation of ABA responses [15]. In contrast to ubiquitination, SUMOylation of ABI5 at Lys391 by the SUMO E3 ligase, SIZ1 (SAP and Miz 1), prevents its degradation [16]. Accordingly, *siz1* mutant plants displayed lower ABI5 protein levels but were curiously hypersensitive to ABA, suggesting that SUMOylation negatively regulates ABA signalling. Furthermore, blocking SUMOylation of ABI5 by expression of a mutant K391R transgene in *abi5* plants resulted in ABA hypersensitivity. Thus, SUMOylation of ABI5 not only prevents its degradation but also negatively regulates its intrinsic transcriptional activity by an unknown mechanism. Modification of ABI5 by ubiquitin and SUMO appears to occur at different Lys residues (Lys344 and Lys391, respectively) [13•,16], suggesting that these two related PTMs do not simply compete for the same site but rather act combinatorially (Figure 1; for effects of *S*-nitrosylation see discussion below).

Figure 1

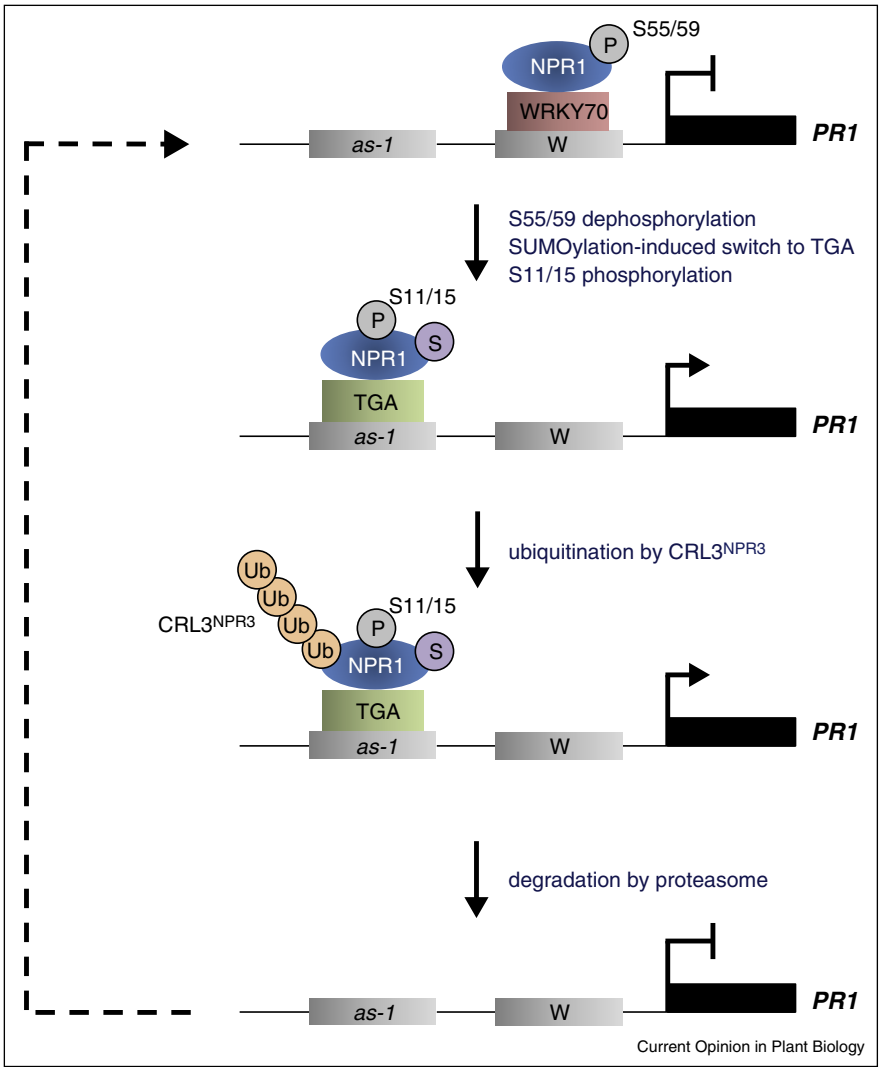


Multiple post-translational modifications regulate accumulation and activity of the ABA-responsive transcription factor ABI5. The ABA-responsive transcription factor ABI5 is subject to a variety of post-translational modifications that mediate seed germination and plant growth. Phosphorylation (P) by SnRK2 protein kinases (SnRKs) promotes transcriptional activity of ABI5. SUMOylation (S) by the SUMO E3 ligase SIZ1 at K391 stabilizes ABI5 and suppresses its transcriptional activity. Additionally, ABI5 is polyubiquitinated by nuclear (nucl) CUL4 and cytoplasmic (cyt) KEG ubiquitin ligases, and consequently degraded by the proteasome. Nitric oxide strongly promotes ABI5 recruitment to CUL4 and KEG ubiquitin ligases by *S*-nitrosylating (NO) Cys153 of ABI5. Thus, *S*-nitrosylation directly or indirectly opposes the stabilizing effect of ABI5 SUMOylation. The shown post-translational modifications are presumably reversible (indicated by dashed arrows), but the underlying mechanisms have not yet been uncovered.

In addition to ABI5, the R2R3 MYB-type transcription factor MYB30 mediates ABA signalling and is also SUMOylated by SIZ1 [17•]. Similar to ABI5, SUMOylation of MYB30 at Lys283 prevented its degradation and also appeared to be required for its transcriptional activity, as expression of a mutant K283R transgene did not fully restore ABA sensitivity in *myb30* plants. More recently, MYB30-interacting E3 ligase 1 (MIEL1) was identified as a RING-type E3 enzyme responsible for MYB30 ubiquitination and proteasomal degradation [18•]. The site(s) of MYB30 ubiquitination are yet to be determined and once revealed may provide further insight into how the UPS and SUMO compete for this substrate.

The transcription coactivator NPR1 is a master regulator of SA-responsive genes and associated immunity against biotrophic pathogens. In the absence of SA, nuclear NPR1 is thought to be ubiquitinated by $CRL3^{NPR4}$ and undergoes proteasomal degradation to prevent activation of immune genes [5,19,20]. Immune activation increases SA levels and results in phosphorylation of NPR1 at Ser11/15, probably promoting the switching of NPR1 to the alternate E3 ligase $CRL3^{NPR3}$. This leads to the ubiquitination and turnover of NPR1 that paradoxically is necessary for full induction of its target genes [19,20]. Recent work has revealed that regulation of NPR1 activity by PTMs is even more complex with the finding that SUMOylation also modulates this coactivator [21••]. Modification of NPR1 by SUMO3 appeared to be a prerequisite for phosphorylation at Ser11/15 and was shown to promote its proteasomal degradation. SUMOylation in turn was found to be controlled by a dephosphorylation event at Ser55/59 of NPR1 (Figure 2). Importantly, SUMOylation of NPR1 coactivator was proposed to modulate its association with different transcription factors. While unmodified NPR1 associated with the WRKY70 transcriptional repressor, SUMOylated NPR1 preferentially interacted with the TGA3 transcription activator. Chromatin immunoprecipitation studies of the well-defined SA-responsive *PR1* promoter further showed that mutant NPR1 that cannot be modified by SUMO3 was constitutively localised to a WRKY binding motif known as a W-box motif. By contrast, SA-induced wild-type NPR1 switched its localisation to an *as-1* element that is known to

Figure 2



Interplay between phosphorylation, SUMOylation and ubiquitination regulates NPR1 function and activity at the *PR1* gene promoter. In the absence of SA signalling, NPR1 is thought to bind the transcriptional repressor WRKY70 at the W-box element of the *PR1* promoter. Phosphorylation (P) of NPR1 at S55/59 appears to promote this state by preventing NPR1 SUMOylation. Activation of SA signalling leads to NPR1 dephosphorylation at S55/59, triggering SUMOylation of NPR1 by SUMO3 (S). This SUMOylation allows the switching of NPR1 association from WRKY70 to TGA transcription activators and induction of *PR1* gene expression. Furthermore, SUMOylation of NPR1 promotes its phosphorylation at S11/15, leading to NPR1 ubiquitination (Ub) by the $CUL3^{NPR3}$ E3 ligase and subsequent proteasome-mediated degradation.

be occupied by TGA factors [21••]. Thus, activation of immune genes may require a SUMOylation-induced switch in NPR1 interaction partners (Figure 2). Considering the importance of SUMOylation to ABA signalling, it is interesting to note that CRL3-mediated NPR1 degradation appears to be promoted by ABA, suggesting that hormone cross talk between SA and ABA may be established at the post-translational level by modulation of NPR1 SUMOylation and ubiquitination [22••].

Perception of the hormone gibberellin (GA) also triggers a UPS-mediated signalling pathway. In this case, binding of GA to its receptor GIBBERELLIN INSENSITIVE DWARF 1 (GID1), promotes association with DELLA transcriptional repressors resulting in recruitment of an SCF^{SLY} E3 ligase complex that targets DELLAs for degradation [23]. In a recent study, DELLAs were shown to be SUMOylated, which not only protects them from degradation, but appears to act as a GID1-sequestering mechanism to allow accumulation of non-SUMOylated DELLAs, thereby limiting plant growth during stress [24]. Indeed, a SUMO-interacting motif (SIM) was identified in GID1 that facilitates this process. Proteins containing SIMs can interact non-covalently with SUMO and

thus SUMOylation can facilitate protein-protein interactions between SIM-containing proteins and SUMO conjugates. Recently, a class of SIM-containing E3 ubiquitin ligases were reported in plants [25] related to the SUMO-targeted ubiquitin ligases (STUbLs) found in yeast and mammals [26,27]. These E3 ligases specifically ubiquitinate SUMOylated proteins. Consequently, SUMO modification of a protein can also result in its ubiquitination and proteasomal degradation. Accordingly, Arabidopsis STUbL4 was shown to reduce protein levels of the transcriptional repressor CYCLING DOF FACTOR *2* (CDF2) to promote flowering, presumably through proteasomal degradation [25]. It is expected that plant STUbLs play various important roles in transcription regulation during hormone signalling due to the prevalence of ubiquitin and SUMO modifications in these pathways.

Protein *S*-nitrosylation versus ubiquitination

Developmental processes and environmental stress responses often reprogram the transcriptome via alterations in cellular redox potential. Fluctuations in redox potential may be sensed by reactive thiol groups of Cys residues [28]. The diversity of possible thiol redox states offers a molecular framework to use a single residue for a wide range of molecular switches, such as alterations in protein stability, activity, conformation, and localisation. Amongst these different thiol redox states, *S*-nitrosylation, the addition of a nitric oxide (NO) moiety to a reactive thiol group to form a protein-SNO, has been consolidated as a ubiquitous PTM in plant biology. The past few years have seen many efforts to identify the *S*-nitrosylated plant proteome and suggest that this PTM plays a key role in many aspects of plant biology [29–33]. While the utilized methodologies often fail to identify specific subcellular or low abundance proteins, several independent reports describe important roles for *S*-nitrosylation of transcriptional regulators that are also modulated by ubiquitination.

Recently, an intriguing interplay between *S*-nitrosylation and ubiquitination was demonstrated in the transcriptional control of seed germination by ABA signalling. The ABA-responsive ABI5 transcription factor is a master regulator of seed germination and seedling arrest [34–37]. As described above, the stability of ABI5 is controlled by both SUMOylation and ubiquitination. A new study now suggests that upon seed imbibition a transient burst in NO production leads to *S*-nitrosylation of ABI5 at Cys153 [38••]. *S*-nitrosylation of ABI5 did not impact its ability to homo-dimerize nor to bind to its DNA-binding motif, suggesting this modification does not markedly change ABI5 conformation. Instead, *S*-nitrosylation recruited ABI5 for ubiquitination by both nuclear Cullin4 (CUL4) and cytoplasmic Keep on Going (KEG) E3 ligases, resulting in its degradation by the proteasome (Figure 1). This scenario suggests that SNO modifications evolved to not only regulate protein activity directly, but also by selectively priming proteins for ubiquitin-mediated degradation. Indeed, *S*-nitrosylation has been reported to influence a variety of PTMs, including ubiquitination, SUMOylation, phosphorylation, palmitoylation, and acetylation [39]. In this respect it will be important to uncover if and how SNO-induced ubiquitination of ABI5 directly counteracts protective SUMOylation (Figure 1).

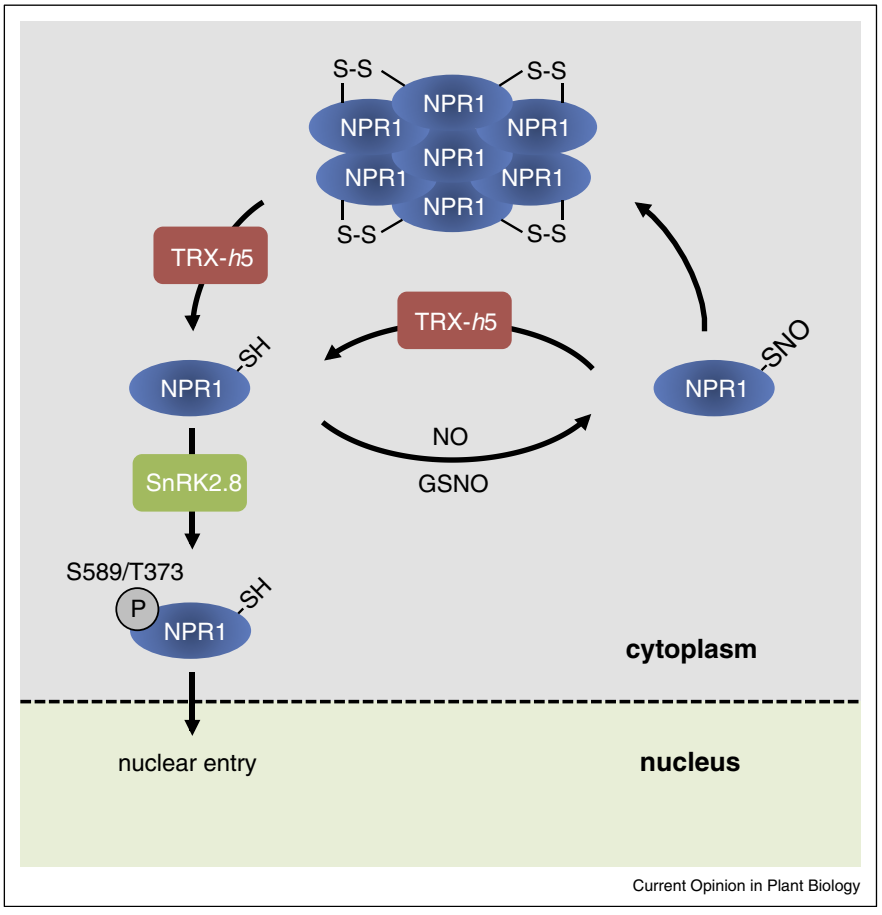
S-nitrosylation has also been reported to regulate the unstable MYB30 transcription factor during the hypersensitive cell death response, an effective strategy to hinder pathogen invasion. MYB30 positively regulates cell death by promoting gene expression and synthesis of very long fatty acids [40]. In unchallenged plants, nuclear levels of MYB30 are kept low by activity of the ubiquitin ligase MIEL1 [18•]. Upon infection, expression of MIEL1 is downregulated, thereby raising MYB30 levels and programming cell death. Curiously, MYB30 is also targeted by inhibitory *S*-nitrosylation at two Cys residues located in its DNA-binding domain [41••]. It is plausible that SNO-induced rejection from the DNA renders MYB30 more susceptible to MIEL1-mediated ubiquitination and degradation. In partial analogy to ABI5, such a mechanism would again put *S*-nitrosylation at odds with the stabilizing effect of SUMO modifications on MYB30.

The unstable, SA-responsive transcription coactivator NPR1 is subject to several different redox-based modifications. In resting cells, NPR1 is stabilized in the cytoplasm by intermolecular disulphide bonds that generate a large oligomer [42]. Upon activation of immunity, SA induces cellular redox changes that together with the activity of Thioredoxin-*h*5 (TRX*h*5) result in transient reduction of these disulphide bonds, allowing NPR1 monomer to translocate into the nucleus where it activates immune genes. Interestingly, SA is also thought to induce transient *S*-nitrosylation of NPR1, which facilitates NPR1 re-oligomerization (Figure 3). Because NPR1 exhibits profound instability in the nucleus [19], its transient *S*-nitrosylation prevents nuclear entry and stabilizes the protein, a process that was shown to be necessary for maintaining NPR1 protein homeostasis during immune responses [43]. Moreover, nuclear entry of NPR1 in sites distal to infection was shown to be mediated by NPR1 phosphorylation at Ser589 and possibly Thr373 by SNF1-RELATED PROTEIN KINASE 2.8 (SnRK2.8) (Figure 3) [44•]. Taken together with the fact that the related kinases SnRK2.2 and SnRK2.6 are targeted by inhibitory *S*-nitrosylation at a Cys residue highly conserved among all members of the SnRK family [45,46•], it seems likely that SNOs regulate NPR1 nuclear entry, and thus its stability, at multiple post-translational control points.

Untangling complex PTM networks in transcriptional regulation

In this review we have outlined several recent findings that begin to reveal an increasingly important role for

Figure 3



Regulation of NPR1 nuclear import by redox-based modifications and phosphorylation. In resting cells NPR1 is stabilized in the cytoplasm by formation of large oligomers mediated by intermolecular disulphide bonding (S–S). Transient *S*-nitrosylation (–SNO) of NPR1 cysteine residues by NO and GSNO stimulates oligomer formation, while their direct reduction by the protein-SNO reductase, TRX-*h*5, promotes the monomeric state. Activation of immunity leads to changes in cellular redox status that together with TRX-*h*5 activity reduce NPR1 oligomers to monomers. Monomeric NPR1 is phosphorylated (P) at S589/T373 by SnRK2.8 leading to its nuclear import.

cross-communication between ubiquitination and other PTMs in a wide range of plant developmental and stress response programs. In addition to phosphorylation, particularly SUMOylation and *S*-nitrosylation are emerging as potent direct and indirect control mechanisms for transcription (co)factor ubiquitination and stability. Identification of enzymes involved in SUMO conjugation and protease pathways is already enabling further functional testing of the various roles SUMOylation may play in transcription-associated ubiquitination events. Research into the enzymatic control of *S*-nitrosylation is still in its infancy but recent developments indicate that this modification is controlled by at least two SNO scavenging pathways. *S*-nitrosoglutathione reductase (GSNOR1) controls levels of the physiological NO donor, *S*-nitrosoglutathione, thereby indirectly regulating the level cellular protein-SNO [47]. By contrast, TRX-*h*5 was recently found to act as a direct protein-SNO reductase during plant immunity and it is likely that other plant TRX enzymes display a similar enzymatic function [48]. GSNOR1 and TRX enzymes are thought to control overlapping but distinct protein-SNO branches and transcriptional programs, so their genetic manipulation has the potential to reveal specific effects of *S*-nitrosylation on the ubiquitination and SUMOylation of transcriptional regulators. Furthermore, enzymes that generate various post-translational modifications may be modified themselves, thereby introducing an added layer of complexity in the regulation of transcriptional regulators. For example, SnRK2 kinases that phosphorylate and activate ABI5 are themselves targets of inhibitory *S*-nitrosylation [45,46•].

The importance of ubiquitination to plant biology is well illustrated by the fact that many pathogen effectors modulate the activity of host E3 ligases and in some cases even mimic them to promote virulence [49,50].

Similarly, cases are now being uncovered in which effectors act on different PTMs that cross talk with ubiquitin. For example, the type III effector XopD from *Xanthomonas euvesicatoria* displays deSUMOylation activity towards the ethylene-responsive transcription factor SlERF4 from tomato. XopD-mediated deSUMOylation caused destabilization of SlERF4 and suppressed its transcriptional activity likely through ubiquitin-mediated degradation [51••,52]. Hence, SUMOylation, *S*-nitrosylation and most probably other PTMs are emerging as unexpected but integral regulators of ubiquitin signalling in the plant nucleus.

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Genetic screens for Arabidopsis seed germination upon pharmacological treatment with ABA and NO led to identification of the ABA-insensitive and NO-insensitive *abi5* mutant. By applying the biotin-switch technique the authors show that ABI5 is *S*-nitrosylated at Cys153. *S*-nitrosylation of ABI5 did not impact its DNA-binding capacity but promoted its recruitment to CUL4-based and KEG ubiquitin ligases, resulting in its proteasome-mediated degradation. The authors conclude that ABI5 plays a pivotal role in controlling seed germination by mediating cross talk between ABA and NO.

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Gene expression analysis revealed a strong induction of the *SnRK2.8* gene, encoding a SNF1-related kinase, in distal tissues of Arabidopsis plants after inoculation with avirulent *P. syringae*. Genetic evidence indicated that SnRK2.8 is required for induction of SA-dependent systemic acquired resistance. Notably, this kinase was shown to interact and phosphorylate the immune coactivator NPR1 at sites distal to attempted infection. SnRK2.8-mediated phosphorylation of NPR1 was necessary for its SA-induced translocation into the nucleus where it activates immune genes.

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