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SUMO-, MAPK- and resistance protein-signaling converge at transcription complexes that regulate plant innate immunity

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Abbreviations: DND1, defense-no death 1 or cyclic nucleotide gated channel 2/CNGC2 that conducts Ca²⁺ into cells; EIN3, ethylene-insensitive 3; ERF104, ethylene-response factor 104; ETI, effector-triggered immunity; HDA19, histone deacetylase 19/HDI; MAPK, mitogen-activated protein kinase; PRR, pattern-recognition receptor that triggers PTI; PTI, PAMP (pathogen-associated molecular pattern)-triggered innate immunity; SID2(ICS1/EDS16), isochorismate synthase 1/key enzyme for salicylic acid synthesis; SIN3, transcriptional co-repressor that exists in different complexes with histone deacetylases; SNC1, suppressor of npr1-1/resistance protein analogue belonging to TIR-NB-LRR class; SUMO, small ubiquitin-like modifier; TPR1, topless-related 1/co-repressor that exists in complex with histone deacetylases; WRKY, transcription factor carrying a short conserved “WRKY” motif

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Upon pathogen perception plant innate immune receptors activate various signaling pathways that trigger host defenses. PAMP-triggered defense signaling requires mitogen-activated protein kinase (MAPK) pathways, which modulate the activity of transcription factors through phosphorylation. Here, we highlight that the same transcription factors are also targets for conjugation by SUMO (Small ubiquitin-like modifier). SUMO conjugation determines recruitment and activity of chromatin-modifying enzymes, and thereby indirectly controls gene expression. SUMO conjugation is essential to suppress defense signaling in non-infected plants. Resistance protein signaling and SUMO conjugation also converge at transcription complexes. For example, the TIR-NB-LRR protein SNC1 interacts with histone deacetylase HDA19 and the transcriptional co-repressor Topless-related 1; both are SUMO targets. We present a model in which SUMO conjugation can transform transcription activators into repressors, thereby preventing defense induction in the absence of a pathogen.

MAPK Signaling and SUMO Conjugation Converge at Transcription Complexes

To resist pathogens plants primarily rely on their innate immune system that enables them to recognize invaders and mount defense responses. Two layers can be distinguished in this immune system. The first is activated by perception of conserved pathogen-associated molecular

patterns (PAMPs) by pattern-recognition receptors (PRRs). This defense response is called PAMP-triggered immunity or PTI (recently reviewed in ref. 1). Pathogens generally breach this line of defense by production of virulence factors (effector proteins). Some of these effectors can be recognized by “resistance” or R proteins, which trigger the activation of the second layer of defense, called effector-triggered immunity or ETI. Although quantitatively different, both ETI and PTI share many physiological responses, such as an oxidative burst, hormonal changes and transcriptional reprogramming.^{2,3} Furthermore, genetic studies and proteomics analyses revealed that many signaling components are shared between PTI and ETI signaling (Fig. 1), which means that plants employ a limited set of signaling pathways to regulate their defense responses. As depicted in Figure 1, signaling from either ETI or PTI receptors activates mitogen-activated protein kinase (MAPK) cascades that phosphorylate downstream transcription regulators controlling expression of ‘early’ defense genes. PTI/ETI-activated MAPK signaling primarily involves MAPK signaling via the kinases MPK4 and MPK3/MPK6 (recently reviewed in ref. 4). Known nuclear targets of these three kinases include many pivotal transcriptional regulators of the plant defense response such as WRKY transcription factors, EIN3 and ERF104.⁵⁻⁸

Recent studies revealed that in non-infected plants, expression of early defense genes is actively suppressed by various mechanisms (exemplified in

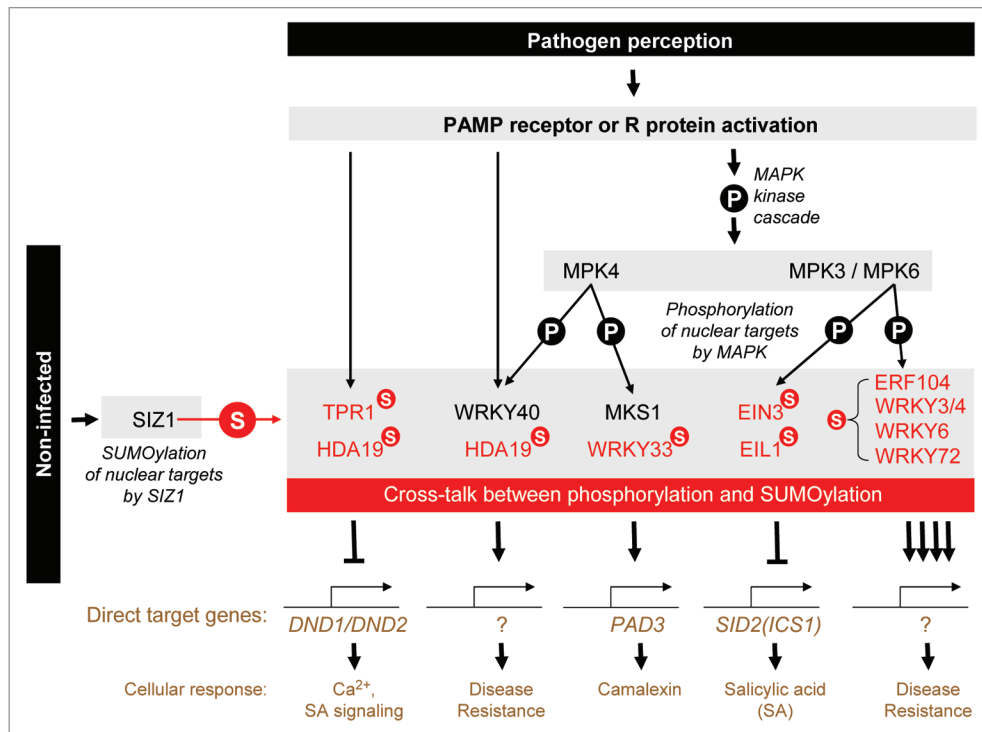


Figure 1. Pathogen perception triggers MAPK-dependent phosphorylation of nuclear targets. These targets are key regulators of plant defense gene expression, and are themselves also SUMO conjugation targets. In addition, certain effector receptors (R proteins) can interact with the chromatin-modifying enzyme HDA19 and its corepressor TPR1. The latter two proteins are SUMO targets and their SUMOylation likely promotes gene repression. P = phosphorylation, S = SUMOylation.

ref. 6, 9 and 10). An important negative regulator of gene expression is the protein Small Ubiquitin-like Modifier (SUMO). SUMO is a post-translational modification that can be reversibly conjugated to target proteins, similar to its well-known cousin Ubiquitin.¹¹ In contrast to ubiquitylation, reversible SUMO conjugation only requires the actions of the SUMO activating enzymes SAE1/SAE2, the SUMO conjugating enzyme SCE1 and SUMO proteases. However, SUMO conjugation can be promoted by SUMO E3 ligases, such as SIZ1 or HPY2 (NSE1).^{11,12} Plants with disturbed SUMOylation levels (like a *siz1* mutant) exhibit (i) constitutive expression of early and late defense genes, (ii) increased accumulation of the defense hormone salicylic acid (SA) and (iii) increased disease resistance.^{13,14} To explain this phenotype, we proposed that SUMOylation of specific transcription factors suppresses the expression of early defense genes.¹⁵ This model is based on the notion that SUMOylation determines the recruitment and activity of chromatin-modifying enzymes in

transcription complexes and thereby controls transcription.¹⁶⁻¹⁸

Recent support for our working model comes from a large-scale proteomics analysis aimed at identifying *Arabidopsis thaliana* proteins that are differentially SUMOylated upon abiotic stress treatment.¹⁹ As we predicted, many mainly nuclear proteins were retrieved that are involved in processes such as transcription, RNA metabolism, chromatin remodeling and DNA repair. Among the 350 candidates, especially relevant are histone H2B and proteins that control histone acetylation (e.g., GCN5, HDA19 and the corepressors SIN3 and TOPLESS), histone methylation and DNA methylation; all these proteins affect transcription via chromatin remodeling. Moreover, we noted that the list of SUMO targets considerably overlapped with proteins that are phosphorylation targets of MPK4 and MPK3/6 (Fig. 1). The set includes five defense-related WRKY transcription factors, EIN3, EIL1 and ERF104. This overlap implies crosstalk in defense signaling as it shows that MAPK signaling

and SUMOylation converge to regulate the same targets present in transcription complexes involved in defense signaling. Although this is a novel concept for plant signaling, it is mechanistically similar to the mammalian system where MAPK cascades also converge with SUMO signaling to control gene expression.²⁰

WRKY Transcription Factors as Targets of SUMOylation

Many WRKY transcription factors are associated with plant defense. We previously identified two plant genes in which a WRKY domain was fused to a SUMO protease domain. According to the Rosetta stone principle, this implies that SUMO and WRKYs might act together in the same pathway.¹⁵ Miller et al.¹⁹ revealed that indeed five WRKYs are SUMO1 targets (WRKY3, WRKY4, WRKY6, WRKY33, WRKY72). Besides being SUMO substrates each of them can also be phosphorylated *in vitro* by MAPK kinases, linking SUMOylation and MAPK signaling.⁵ Notably, many

WRKYs act both as *activator* and as *repressor* of defense-gene expression, examples of which are the SUMO targets WRKY6 and WRKY72.²¹⁻²³ Also, overexpression of the SUMO target WRKY4 was found to suppress pathogen-induced *PR1* expression.²⁴ To explain the apparent paradox that the same WRKY exhibits a dual activity, we here propose that the SUMOylated variants might act as transcriptional repressors, whereas the non-SUMOylated variants are activators.

SUMOylation of WRKY4 and the closely related WRKY3 occurred on the lysine residue in the SUMOylation consensus motif in the C-terminus of both proteins.¹⁹ The presence of a SUMOylation consensus motif in many WRKYs,¹⁵ besides those in four of the five identified SUMO targets (WRKY3, WRKY4, WRKY6 and WRKY72),¹⁹ implies that SUMOylation might be a more generic control mechanism for this class of transcriptional regulators.

As outlined above SUMO and MAPK signaling appear to be coupled, but the question is now how do these modification affect each others function on the same target? An interesting observation is that the SUMO targets WRKY33 and ERF104 are both sequestered by inactive MAPK kinases. WRKY33 is, together with MKS1, sequestered by MPK4. Upon activation of MPK4 by PTI signaling, WRKY33 is released from this complex.⁶ Likewise, ERF104 is sequestered by MPK6, and released upon MPK6 activation in combination with ethylene signaling.⁷ It is unknown whether SUMOylation of WRKY33 and ERF104 has a role in sequestering them in their respective MAPK complexes or whether SUMOylation controls WRKY33/ERF104-dependent gene repression/activation downstream of these MAPK kinases. In addition, WRKY33 appears to be SUMOylated in response to H₂O₂ treatment. Reactive-oxygen species (ROS) are well known signals in the plant defense response, suggesting that ROS signaling potentially induces SUMOylation of WRKY33. Stress conditions such as H₂O₂ treatment or heat shock are known to trigger massive SUMO conjugation to a large set of proteins.²⁵ The biological function of this mass SUMOylation remains to be

determined. Complementation studies with SCE1- or SUMO-fusion constructs could resolve this issue and reveal whether these SUMOylated proteins are indeed sequestered with MAPKs.

SUMO conjugation might also indirectly influence the activity of WRKYs. For example, the *A. thaliana* WRKY38 and WRKY62 interact with histone deacetylase HDA19.²⁶ These WRKYs themselves act as transcriptional activators, but together with HDA19 they act as negative regulators of gene expression and defense. *AtHDA19* is a SUMO target and SUMOylation of the highly conserved mammalian homolog promotes its histone deacetylase activity, which results in gene repression.²⁷ It will be interesting to investigate whether SUMO conjugation of HDA19 influences its interaction with WRKY38 and WRKY62.

In conclusion, although many details remain to be elucidated, the emerging picture is that the activity of at least a subset of WRKYs is controlled by both MAPK signaling and SUMOylation and that their balanced action controls WRKY activity and thereby defense gene expression.

R Protein-mediated Transcriptional Reprogramming also Converges at SUMO Targets

The R protein MLA10 interacts directly with two barley homologs of WRKY40,⁹ which is a putative MPK4 substrate.⁵ A link between R proteins and WRKYs was implied before as some R proteins, such as RRS1-R, carry a WRKY domain.²⁸ Shen et al.⁹ showed that WRKY40 and its closest homologs WRKY18 and WRKY60 act as basal suppressors of defense in *A. thaliana*. They also reported that the WRKY homologs in barley carry an EAR-repressor motif in their N-termini. Although the functionality of this motif was not examined in this study, EAR-repressor motifs are linked to gene repression in that they recruit HDA19. In plants recruitment of HDA19 to EAR-repressor-containing proteins is mediated by two types of co-repressor complexes: SIN3/SAP18 and TOPLESS.^{29,30} Besides HDA19, both the SIN3 and the TOPLESS protein families (including TPR1) turned out to be SUMO targets as well.¹⁹ Together, these data link

SUMO conjugation and HDA19-related gene repression via the WRKYs to MLA10 function. A more direct link between an R protein and SUMOylation is exemplified by the *Arabidopsis thaliana* SNC1 protein. SNC1 was shown to interact directly with TPR1 and HDA19,¹⁰ which indicates that SNC1 and possibly related R proteins (i.e., TIR-NB-LRR proteins) interact in the nucleus with SUMO-modified transcriptional co-repressors. These repressors might prevent activation of the plant defense response in non-infected plants. The effect of SUMOylation of TPR1 on gene expression is unknown, but it likely influences recruitment of TPR1 to HDA19 or other chromatin-modifying enzymes present in transcription complexes. In line, the mammalian homolog of TPR1 (GROUCHO) interacts more tightly with HD1 (homolog of *A. thaliana* HDA19) upon SUMOylation.³¹ The observation that many of these co-repressors are SUMO targets provides a first clue on how SIZ1-dependent SUMO conjugation suppresses defense-gene expression in non-infected plants. Future studies should reveal whether the direct gene targets of the SNC1/TPR1/HDA19 complex, which include the defense-signaling genes *DND1*(*CNGC2*) and *DND2*, are upregulated in the *siz1* mutant or in a *sumo1/amiR-SUMO1/2'* double mutant.

EIN3/EIL1, Another Mutual Target of MAPK Signaling and SUMO Conjugation

Other joint targets for both MAPK signaling and SUMO conjugation are the transcription factors EIN3 and its closest homolog EIN3-like 1 (EIL1). Phosphorylation of EIN3 by MPK6 stabilizes EIN3, allowing transcriptional regulation of EIN3 targets.⁸ As part of the regulation of the ethylene response, EIN3 and its homologs are targeted for Ubiquitin/26S proteasome mediated protein degradation by the F-box proteins EBF1/EBF2. EBF1/EBF2 are themselves also regulated by proteolysis.^{32,33} Target genes of EIN3 and EIL1 include genes encoding the PAMP receptor FLS2 and the SID2(ICS1) protein. SID2 is the key enzyme for salicylic acid synthesis upon activation of a defense response. EIN3/

EIL1 act as transcriptional activators of *FLS2* expression, while they are suppressors of *SID2* expression.^{34,35} EIN3 can indirectly be linked to SUMO by its putative EAR-repressor motif, linking it to HDA19-mediated gene repression.³⁶ A more direct link with SUMO is the presence of a SUMOylation-consensus motif in both EIN3 and EIL1. This motif is conserved between the two proteins (EIN3:VK³⁶⁶EE, EIL1:IK³⁷²EE). Experimental support for the functionality of this motif in EIL1 is the retrieval of EIL1 as an interactor of the SUMO conjugating enzyme SCE1 in a yeast 2-hybrid screen.³⁷ Furthermore, EIL1 could be SUMOylated in vitro implying functionality of the motif. Future studies should resolve how EIN3 SUMOylation influences EIN3 protein stability in response to ethylene signaling, and how SUMOylation affects expression of EIN3 target genes. This class of transcriptional regulators exemplifies how different signaling cascades merge, as EIN3 is now shown to be subject to phosphorylation, ubiquitylation and SUMOylation.

Different Roles for Different SUMOs?

The proteomics studies described above focused on SUMO1 targets. However, besides SUMO1 the genome of *A. thaliana* contains seven more genes encoding SUMO homologs. Four of these, *SUMO1*, *-2*, *-3* and *-5*, are expressed and their products are conjugated to other proteins.¹⁸ Previous data linked plant defense to the SUMO E3 ligase SIZ1.¹³ In addition, Cheong et al. demonstrated that the interaction of SIZ1 with SCE1 is needed for defense regulation by SIZ1.³⁸ We demonstrated that indeed SUMO1 or SUMO2 conjugation is needed to prevent defense activation. In addition, we extensively studied SUMO3 function.¹⁴ SUMO3 shares only 48% protein sequence identity with SUMO1 and SUMO2. In *A. thaliana* *SUMO1* and *SUMO2* are the most highly expressed SUMO genes, whereas *SUMO3* expression is low and restricted to specific tissues. Interestingly, *SUMO3* gene expression is induced nearly 100-fold during SA signaling, peaking 4–6 hrs post PTI activation. The different expression kinetics

imply specialized functions for the different SUMO homologs and indeed we found that they have distinct roles in plant disease resistance. SUMO1 and SUMO2 act upstream of SA signaling and are required as basal suppressors of plant defense. The kinetics of *SUMO3* expression place it downstream of SA and we found that it promotes rather than suppresses disease resistance. Next to our genetic studies,¹⁴ biochemical data indicated that SUMO3 has unique properties and potentially SUMOylates other targets than SUMO1 and SUMO2.¹⁸ A large-scale SUMO conjugation assay on putative SUMO targets from *Arabidopsis* identified a large group of proteins that was indeed only modified by SUMO3.³⁷ This emphasizes that in *Arabidopsis thaliana* SUMO3 function has diverged from that of SUMO1/SUMO2 and that SUMO3 can act as a distinct post-translation modification. Taken together, these observations reveal an additional regulatory layer in SUMO-regulated control of defense signaling. A Blast analysis of the available sequenced plant genomes indicates that SUMO3 is absent outside the Brassicaceae (van den Burg HA, Takken FLW, unpublished data). This suggests that the Brassicaceae have acquired a unique post-translational modification with a distinct role in defense signaling. It would be interesting to investigate whether in other plant families other SUMO homologs evolved that have a similar function.

Concluding Remarks and Future Perspectives

In the absence of attacking microbes, plants actively suppress PTI- and ETI-signaling at the level of gene expression. We noted that many key transcriptional regulators of these defense signaling pathways are subject to both MAPK-dependent phosphorylation as well as SUMO-dependent conjugation. We propose a model in which SUMO conjugation of these transcription factors transforms them into repressors, whereas their phosphorylation after defense activation reverts them into transcriptional activators. How SUMOylation transforms an activator into a repressor is unknown, but in both PTI and ETI signaling HDA19-containing transcriptional

repressor complexes seem to be involved. Notably, the latter complexes are also subject to SUMO conjugation, which could provide a self-supporting feedback mechanism. Future studies should investigate the significance and hierarchy of the different post-translational modifications on these transcriptional regulators. These studies should provide better insight in how SUMO conjugation determines their activity and how they regulate plant innate immune responses.

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