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Posttranslational Modifications of the Master Transcriptional Regulator NPR1 Enable Dynamic but Tight Control of Plant Immune Responses

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Summary

NPR1, a master regulator of basal and systemic acquired resistance in plants, confers immunity through a transcriptional cascade, which includes transcription activators (e.g., TGA3) and repressors (e.g., WRKY70), leading to the massive induction of antimicrobial genes. How this single protein orchestrates genome-wide transcriptional reprogramming in response to immune stimulus remains a major question. Paradoxically, while NPR1 is essential for defense gene induction, its turnover appears to be required for this function, suggesting that NPR1 activity and degradation are dynamically regulated. Here we show that sumoylation of NPR1 by SUMO3 activates defense gene expression by switching NPR1's association with the WRKY transcription repressors to TGA transcription activators. Sumoylation also triggers NPR1 degradation, rendering the immune induction transient. SUMO modification of NPR1 is inhibited by phosphorylation at Ser55/Ser59, which keeps NPR1 stable and quiescent. Thus, posttranslational modifications enable dynamic but tight and precise control of plant immune responses.

Introduction

In plants, pathogen-triggered increases in cellular levels of salicylic acid (SA) and exogenous application of SA both lead to transcription reprogramming and a broad-

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spectrum defense response known as systemic acquired resistance (SAR) (Fu and Dong, 2013). SAR is predominantly dependent on the activity of NPR1 (nonexpressor of pathogenesis-related (*PR*) genes 1), which was first identified through screens for mutants blocked in SA-mediated *PR* gene expression and resistance (Cao et al., 1994; Delaney et al., 1995; Wang et al., 2006). NPR1's central role in plant immunity has been firmly established (Pieterse et al., 2012). Therefore, elucidating its regulatory mechanism is critical for our understanding of plant immunity.

Like the mammalian immune regulator NF- κ B, the activity of NPR1 is tightly regulated to ensure proper immune induction with minimal detrimental effects on plant growth. Since NPR1 functions in the nucleus (Kinkema et al., 2000), its activity is regulated in part at the nuclear translocation step controlled by the cellular redox changes triggered by SA (Mou et al., 2003; Tada et al., 2008). In the nucleus, NPR1 confers immunity through a transcriptional cascade, including transcription activators (e.g., TGA3) and repressors (e.g., WRKY70), leading to the massive induction of antimicrobial *PR* genes (Despres et al., 2000; Lebel et al., 1998; Spoel et al., 2009; Wang et al., 2006; Zhang et al., 1999; Zhou et al., 2000). However, how NPR1 regulates transcription is poorly understood. It has been shown that NPR1 could provide the transactivation activity to the associated TGA transcription factors (TFs) when transiently expressed in plants (Johnson et al., 2003; Rochon et al., 2006). The structure of the protein suggests that, like other BTB (bric-a-brac, tramtrack, broad-complex) domain-containing proteins, NPR1 may serve as an adaptor for the CULLIN3 ubiquitin E3 ligase (Luke-Glaser et al., 2007; Petroski and Deshaies, 2005; Pintard et al., 2004) and be involved in the ubiquitination and possibly the degradation of a transcription repressor. In both scenarios, it is not known whether and how NPR1 interactions with TFs are regulated in plants. In yeast two-hybrid analysis, however, NPR1 has been shown to interact with TGA and NIMIN (NIM1-INTERACTING) TFs constitutively (Despres et al., 2000; Weigel et al., 2001; Zhang et al., 1999; Zhou et al., 2000), with the exception of TGA1 and TGA4 (Despres et al., 2003).

Both posttranslational modifications (PTMs) and protein stability may play a role in controlling NPR1 transcriptional activity. NPR1, while having the structure of an adaptor for the CUL3 E3 ligase complex, is itself regulated by the 26S proteasome in the nucleus (Spoel et al., 2009). Normally, NPR1 is constantly degraded via interaction with the NPR4-CUL3 E3 ligase to reduce the basal level of NPR1. Upon pathogen challenge, NPR1 is phosphorylated at the first I κ B-like phosphodegron (Ser11/Ser15), ubiquitinated by the NPR3-CUL3 E3 ligase and degraded (Fu et al., 2012; Spoel et al., 2009). Paradoxically, NPR1 turnover appears to be required for its full transcriptional activity in SAR even though it is a positive regulator of defense genes (Spoel et al., 2009). Proteasome-mediated recycling of the transcriptional complexes has been proposed as the underlying mechanism. Alternatively, a PTM that causes NPR1 instability may also be required for its transcriptional activity. However, phosphorylation of NPR1 at Ser11/Ser15 has not been shown to significantly alter its interaction with TGA TFs (Spoel et al., 2009). Therefore, how NPR1 transcriptional activity and degradation are dynamically regulated remains an outstanding question.

Sumoylation is a dynamic and reversible PTM that has not been examined for NPR1 regulation. The SUMO system is conserved in all eukaryotic organisms (Johnson, 2004; Mazur and van den Burg, 2012). The process begins with proteolytic cleavage of SUMO at the C-terminal di-glycine motif (GG), activation by SUMO E1 and then transfer to the SUMO E2 conjugating enzyme. Conjugation of SUMO to the lysine residue(s) in the target protein requires either a SUMO E3 ligase or a noncovalent interaction with a SUMO-interaction motif (SIM) (Johnson, 2004; Kerscher, 2007; Wang and Dasso, 2009). In *Arabidopsis*, overexpressing the small ubiquitin-like modifier 3 (SUMO3) enhances defense gene expression and disease resistance (van den Burg et al., 2010), but the cellular targets for SUMO3 have yet to be identified. Here we report that NPR1 is sumoylated upon immune induction and this PTM differentially affects NPR1 interactions with positive and negative TFs and its stability. Our study also uncovered an intricate interplay between sumoylation and phosphorylation in controlling NPR1 activity and turnover.

Results

NPR1 is modified by SUMO3

Mutations that cause constitutive NPR1 nuclear accumulation lead to elevation in *PR* gene expression (Mou et al., 2003). However, SA treatment of these mutants can further induce gene expression, suggesting that additional regulation occurs after NPR1 enters the nucleus. To understand these events, we tested different PTMs of NPR1. Since *SUMO3* is induced upon SA treatment, and *SUMO3* overexpression causes constitutive defense gene expression and elevated disease resistance (van den Burg et al., 2010; Wang et al., 2006), we first tested NPR1 interaction with SUMO3 using Y2H. The *Arabidopsis* genome encodes four functional SUMO proteins (Kurepa et al., 2003), and NPR1 interacted with only SUMO3 (Figure 1A).

NPR1 has six lysines that are potential sites for SUMO conjugation (Figure S1A). To examine whether any of these lysines are responsible for the NPR1-SUMO3 interaction, we replaced each lysine (K) with an arginine (R) or in combinations. Surprisingly, none of these mutations blocked or reduced the interaction in Y2H (Figures S1B and S1C), suggesting that NPR1 interacts with SUMO3 through a SIM, independent of an E3 ligase. Based on this hypothesis, we searched for the SIM sequence [VIL]-x-[VIL]-[VIL] or [VIL]-[VIL]-x-[VIL] (Kerscher, 2007; Wang et al., 2011) in NPR1 and found three putative SIMs (Figure 1B and S1A). Mutagenesis showed that only the *sim3* mutant was blocked in the NPR1-SUMO3 interaction (Figure 1C), indicating that SIM3 is likely the only functional SUMO-interaction motif in NPR1.

To validate the NPR1-SUMO3 interaction in plants, we performed a split luciferase assay by co-expressing NPR1 or *npr1^{sim3}* and SUMO3 fusion proteins in *Nicotiana benthamiana*. We found that only NPR1, not the *npr1^{sim3}* mutant, could interact with SUMO3 (Figure 1D). We next conducted a pull down assay using protein extracts from *35S:NPR1-GFP* or *35S:npr1^{sim3}-GFP* plants and purified recombinant GST-SUMO3. NPR1-GFP could be pulled down by GST-SUMO3, but not the *npr1^{sim3}-GFP* mutant (Figure S1D). Interestingly, the NPR1-GFP protein detected in the SUMO3 pull down assay (Figure S1D) was not the conjugated form according to its molecular weight (MW). This was not unexpected because

sumoylation is a reversible modification, which normally occurs in less than 5% of the total protein pool, and it can be rapidly removed by SUMO-specific proteases (Creton and Jentsch, 2010) which are likely to be present in the protein extracts.

To determine whether the NPR1-SUMO3 interaction could lead to covalent conjugation of SUMO3 to NPR1, we first performed a sumoylation reconstitution assay using *E. coli*-produced proteins (Elrouby and Coupland, 2010; Okada et al., 2009). SUMO E1 and E2 ligases, SUMO3 and the candidate substrate NPR1 or *npr1^{sim3}* protein were expressed together in *E. coli* and co-purified. Since NPR1 sumoylation is independent of an E3 ligase, it is not included in the E3 ligase reaction. We detected multiple sumoylated NPR1 bands when we expressed the mature SUMO3 (GG) with NPR1, but not with *npr1^{sim3}* (Figure 1E). The SUMO modifications were absent in the *sumo3-AA* mutant, in which the C-terminal cleavage di-glycine motif (GG) required for SUMO conjugation was replaced by alanines (Johnson, 2004). This result indicated that NPR1 can be poly-sumoylated or multi-sumoylated independent of an E3 ligase. Additionally, the assay showed that SIM3 is required for NPR1 sumoylation.

We also performed co-immunoprecipitation (Co-IP) of cMyc-NPR1 and HA-SUMO3 in yeast. Surprisingly, we found that not only was NPR1 modified by SUMO3, its stability was also regulated by this PTM as the intensity of the sumoylated NPR1 bands was higher in samples treated with the proteasome inhibitor MG115 (Figure 1F). These data indicate that NPR1 can be modified by the yeast sumoylation machinery and degraded by the proteasome, making it a convenient system for studying NPR1-partner interactions, PTMs, and turnover.

Finally, we investigated NPR1 sumoylation *in planta*. After enrichment by IP, sumoylation of NPR1 was detected in NPR1-GFP upon SA treatment, but not in samples from *npr1^{sim3}*-GFP regardless of the treatment (Figure 1G and S1E). We also detected lower molecular weight signals in the *npr1^{sim3}*-GFP samples. We suspect that these signals are artifacts or other SUMO3-modified proteins interacting with NPR1 and *npr1^{sim3}* that are captured by the IP. Taken together, our results show that NPR1 specifically interacts with SUMO3 and is sumoylated in response to SA induction.

Sumoylation is required for NPR1 protein degradation

Sumoylation affects protein stability, subcellular localization and functional activity (Geiss-Friedlander and Melchior, 2007). The apparent conservation of the sumoylation and proteasome machineries between yeast and plants allowed us to study the effect of sumoylation on NPR1 stability using the yeast system. We found that NPR1, *npr1^{sim1}* and *npr1^{sim2}* proteins were unstable in the presence of SUMO3, while mutation of SIM3 stabilized the *npr1^{sim3}* protein (Figure 2A). This was clearly due to sumoylation because NPR1 could neither interact with nor be destabilized by the *sumo3-AA* mutant (Figures 2B and 2C).

We then examined SUMO3-mediated NPR1 degradation over time in extracts from *35S:NPR1-GFP* and *35S:npr1^{sim3}-GFP* plants. We found that transcribed at similar levels (Figure S2), the *npr1^{sim3}* protein was more resistant to degradation mediated by the

proteasome as addition of MG115 resulted in stabilization of both NPR1 and $npr1^{sim3}$ proteins (Figure 2D). This result is consistent with the previous finding that NPR1 degradation requires proteasome activity (Spoel et al., 2009) and indicates that sumoylation is a regulatory step involved in this process.

To determine if sumoylation of NPR1 is necessary for its nuclear localization, we transiently expressed NPR1-GFP and $NPR1^{sim3}$ -GFP in *N. benthamiana*. Our microscopic analysis showed that NPR1-GFP was localized to the nucleus, in nuclear bodies, while $npr1^{sim3}$ -GFP was visible in the cytoplasm in addition to the nucleus and did not form nuclear bodies (Figure 2E and 2F). These data suggest a role for sumoylation in NPR1 nuclear body formation. Whether NPR1-containing nuclear bodies are involved in protein degradation or other sub-nuclear processes remains to be investigated.

NPR1 interaction with SUMO3 is regulated by phosphorylation

NPR1 phosphorylation at Ser11/Ser15 (Figure 3A) is required for its ubiquitination and degradation (Spoel et al., 2009). Since sumoylation of NPR1 is also involved in its degradation (Figure 2D), we examined the effect of Ser11/Ser15 phosphorylation on NPR1-SUMO3 interaction. We replaced Ser11 and Ser15 with alanines (S11/15A) or aspartic acids (S11/15D), and examined their interactions with SUMO3. We found that $npr1^{S11/15D}$ enhanced the interaction with SUMO3 while the $npr1^{S11/15A}$ had no detectable effect (Figure 3B). Besides Ser11/Ser15, NPR1 contains a second I κ B-like motif at Ser55/Ser59 (Figure 3A), which we also replaced with alanines (S55/59A) or aspartic acids (S55/59D). In contrast to $npr1^{S11/15D}$, $npr1^{S55/59D}$ completely blocked interaction with SUMO3 in yeast and *in planta* (Figure 3B and 3C). Taken together, these results imply different roles for NPR1 phosphorylation at these I κ B-like sites regarding the NPR1-SUMO3 interaction.

To determine if the enhancement of NPR1-SUMO3 interaction caused by S11/15D mutation leads to an enhancement of NPR1 sumoylation, we used the *E. coli* sumoylation system to compare S11/15D and S55/59D mutants to the wild type NPR1. Sumoylation of NPR1 was enhanced by the S11/15D mutation while S55/59D blocked its sumoylation (Figure 3D). We then investigated whether the phosphorylation status of NPR1 is involved in SUMO3-mediated NPR1 degradation in yeast and *in planta*. We found that S11/15D enhanced NPR1 degradation while S55/59D blocked it (Figures 3E and 3F). These effects were clearly due to sumoylation because they were absent when SUMO3 was replaced by the sumo3-AA mutant, which cannot be conjugated to the substrates (Figure 3G). These results point to opposing roles for NPR1 phosphorylation at the two I κ B-like sites on NPR1 stability. Similar to the $npr1^{sim3}$ mutant (Figure 2E), the $npr1^{S55/59D}$ -GFP protein was detected in both the nucleus and the cytoplasm (Figure 3H) and did not form nuclear bodies (Figure 3I), in contrast to the sumoylation-competent mutants, $npr1^{S11/15A}$, $npr1^{S11/15D}$, and $npr1^{S55/59A}$ (Figure S3).

NPR1 interaction with SUMO3 is required for Ser11/Ser15 phosphorylation

Since phosphorylation at Ser11/Ser15 and interaction with SUMO3 both facilitate NPR1 degradation, we further investigated the interplay between these two PTMs. While S11/15D enhanced NPR1-SUMO3 interaction and NPR1 sumoylation (Figures 3B-D), the interaction

with SUMO3 appeared to be required for phosphorylation at Ser11/Ser15 (Figure 4A). The *npr1^{sim3}* mutant, which cannot interact with SUMO3 or be sumoylated, was completely blocked in phosphorylation at Ser11/Ser15 similar to *npr1^{S11/15A}* (Figure 4A). Additionally, the *npr1^{S55/59D}* mutant inhibited in sumoylation (Figure 3D) was also blocked in S11/15 phosphorylation (Figure 4A). These data suggest that sumoylation of NPR1 occurs before Ser11/Ser15 phosphorylation and the latter PTM in turn facilitates further sumoylation of NPR1 by forming a signal amplification loop. Consistent with this hypothesis, Ser11/Ser15 phosphorylation is not required for sumoylation, because the combined *npr1^{S11/15D_sim3}* mutant showed the *npr1^{sim3}* phenotype rather than the *npr1^{S11/15D}* phenotype with regard to interaction with SUMO3 and protein stability (Figures 4B-F). Moreover, the non-phosphorylatable *npr1^{S11/15A}* mutant could still interact with SUMO3 (Figure 3B and 3C) and be degraded at a similar rate as that of the wild-type NPR1 (Figures 3E-G).

Sumoylation of NPR1 determines its interactions with different partner proteins

Since sumoylation of NPR1 affects its degradation, we tested the effect of sumoylation on NPR1 interactions with its CUL3 E3 ligase adaptors, NPR3 and NPR4. SA binding to NPR3 promotes interaction with NPR1, while SA binding to NPR4 disrupts binding to NPR1 (Fu et al., 2012). We found that *sim3* and *S55/59D* mutations diminish the SA-dependent interactions between NPR1-NPR3 and NPR1-NPR4 (Figures 5A, 5B, and S4). Conversely, facilitating NPR1 sumoylation by the *S11/15D* mutation enhanced interactions with NPR3 and NPR4. However, the combined *S11/15D_sim3* mutant blocked NPR1-NPR3 and NPR1-NPR4 interactions. These data indicate that SIM3 is required for NPR1 sumoylation and interactions with NPR3 and NPR4, consistent with the positive effect of this site on NPR1 degradation.

NPR1 is a coactivator of the TGA TFs which are required for *PR* gene expression (Despres et al., 2000; Wang et al., 2006). Even though the NPR1-TGA interaction was detected using Y2H (Despres et al., 2000; Zhang et al., 1999; Zhou et al., 2000), evidence for their *in vitro* interaction is still lacking. Since NPR1 can be sumoylated in yeast, we hypothesized that sumoylation might be required for its interaction with TGAs. Our data show that the sumoylation-deficient *npr1^{sim3}* mutant could no longer interact with TGA3 in the Y2H assay (Figure 5C). Besides the TGA activators, *PRI* was predicted to be repressed by the WRKY TFs (Lebel et al., 1998). However, WRKYs were never identified as NPR1 interactors in previous Y2H screens. Surprisingly, the *npr1^{sim3}* mutation, while blocking NPR1 interaction with TGA3, promoted its interaction with WRKY70 (Figure 5C), which was reported to be a direct transcriptional target of NPR1 and serves as a negative regulator of the SA biosynthesis gene *ICS1* (Wang et al., 2006).

In contrast to TGA3 and WRKY70, the *npr1^{sim3}* mutation did not influence known NPR1 interactions with the NIMIN TFs (Figure S5A). Since NIMIN2 interacts with the N-terminal half of NPR1 while NIMIN1 and NIMIN3 bind to the C-terminal of NPR1 (Weigel et al., 2001), the sustained binding activities of *npr1^{sim3}* to the NIMINs suggest that the *sim3* mutation does not significantly perturb protein structure. In parallel, we found that *npr1^{sim3}* has a dominant-negative effect on resistance when crossed into Col-0 (Figure S5B), indicating that the mutant has maintained the right structure to compete with endogenous

NPR1. Furthermore, the *npr1*^{S55/59D} mutant, in which sumoylation is blocked, had similar effects on interactions with TGA3 and WRKY70 as the *npr1*^{sim3} mutant (Figure 5D).

To study the effect of sumoylation on NPR1 differential binding to TFs, we performed Co-IP experiments using NPR1 or *npr1*^{sim3} produced using the *E. coli* sumoylation assay introduced in Figure 1E. We found that TGA3 could pull down only the sumoylated NPR1, as demonstrated by the higher MWs of the bands, whereas WRKY70 pulled down the unmodified NPR1 and the *npr1*^{sim3} mutant (Figure 5E upper panel). We also performed the Co-IP experiment in the reverse order and found that sumoylated NPR1 could pull down more TGA3 than the *npr1*^{sim3} mutant (Figure 5E lower panel). In contrast, *npr1*^{sim3} pulled down more WRKY70 than NPR1. Together, these results clearly showed that the sumoylation of NPR1 determines its interaction with different TFs.

Sumoylation of NPR1 alters its association with different *cis*-elements in the *PR1* gene promoter

To investigate the effect of sumoylation on NPR1 transcriptional activity, we performed a ChIP assay on the *PR1* gene promoter which has both positive *cis*-elements (*as-1*) for TGA and negative *cis*-elements (W-box) for WRKY TFs (Lebel et al., 1998; Pape et al., 2010). We found that promoter region containing the W-box was significantly enriched in the DNA fraction precipitated from *NPR1-GFP* (Figure 5F). This association was released after treatment with SA. In the *npr1*^{sim3}-*GFP* plants, however, binding of *npr1*^{sim3} to the W-box was constant and insensitive to SA induction. Since these W-boxes have been shown to negatively affect *PR1* gene expression (Lebel et al., 1998), we hypothesize that the non-sumoylated NPR1 normally interacts with WRKY70 to inhibit *PR1* gene expression. Upon SA induction, sumoylation of NPR1 causes dissociation from WRKY and relieves its repression through an unknown mechanism. In contrast, the association of NPR1-GFP with the *as-1* element, required for *PR1* gene activation (Lebel et al., 1998), was dramatically enhanced upon SA induction. This increase was not observed in the *npr1*^{sim3}-*GFP* mutant consistent with our finding that only the sumoylated NPR1 interacts with TGA3. Sumoylation of NPR1 switches the protein from binding to a WRKY TF at the W-box to association with a TGA TF at the *as-1* element. Since the *npr1*^{sim3} mutant is insensitive to this SA-induced switch, we propose that sumoylation is the ultimate modification required for NPR1 transcriptional activity.

The interplay between sumoylation and phosphorylation at Ser55/Ser59 is essential for transient activation and safe storage of NPR1

To test our hypothesis that sumoylation is the ultimate modification required for NPR1 activity, we tested the basal levels of resistance in *35S:NPR1-GFP* and *35S:npr1*^{sim3}-*GFP* plants using a suboptimal inoculum of *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm* ES4326). In the *npr1*^{sim3}-*GFP* plants, *Psm* ES4326 growth was significantly higher than in *NPR1-GFP*, although not as high as in the *npr1-2* mutant (Figure 6A). This suggests that in the absence of pathogen challenge there is a residual level of NPR1 sumoylation that provides basal resistance to the plants. This hypothesis was further confirmed by the sumoylation-inhibited *npr1*^{S55/59D}-*GFP* mutant, which also displayed enhanced susceptibility to the pathogen (Figure 6B).

Our results also suggest that phosphorylation of NPR1 at the Ser55/Ser59 site plays a role in keeping the protein stable and inactive. We found that phosphorylation at Ser55/Ser59 occurred in mock-treated plants and was reduced upon SA treatment (Figure 6C). Moreover, blocking Ser55/Ser59 phosphorylation (S55/59A) resulted in elevated levels of *PR1* and *PR2* expression (Figure 6D) and severely retarded plant growth compared to the other mutants used in this study (Figures 6E and S6).

To test our hypothesis that sumoylation is the ultimate PTM required for NPR1 transcriptional activity, we monitored *PR* gene expression after spraying *NPR1-GFP* and *npr1^{sim3}-GFP* plants with SA. We found that *PR1* and *PR2* expression was significantly blocked in *npr1^{sim3}-GFP* plants compared to *NPR1-GFP* (Figure 6F). Consequently, the *npr1^{sim3}-GFP* mutant was insensitive to SA-induced resistance against *Psm* ES4326 (Figure 6G). The *npr1^{S55/59D}-GFP* plants were similarly insensitive to SA induction in *PR* genes and in resistance against *Psm* ES4326 (Figures 6H and 6I). These data strongly demonstrated that sumoylation is an essential molecular switch for NPR1 transcriptional activity in response to SA.

Discussion

Sumoylation has diverse regulatory functions in eukaryotes. For the mammalian immune regulator NF- κ B inhibitor (I κ B), sumoylation prevents its degradation required for NF- κ B activation (Desterro et al., 1998). In *Arabidopsis*, sumoylation plays a role in DELLA protein stability by sequestering GID1, which is required for degradation of DELLA (Conti et al., 2014). In contrast, sumoylation of human promyelocytic leukemia (PML) facilitates its interactions with many proteins and promotes its degradation (Lallemand-Breitenbach et al., 2008). In this study, we identified SUMO3 as a modifier of NPR1 and showed that NPR1 sumoylation is the final regulatory step required for its transcriptional activity and degradation (Figures 1, 2 and 4-6).

SUMO3 targets NPR1 through binding to SIM3, instead of an E3 ligase (Figure 1). This SIM-dependent sumoylation allows a specific SUMO paralog (e.g., SUMO3 for NPR1) to recognize its substrate (Wilkinson and Henley, 2010). For E3 ligase-independent sumoylation, conjugation may occur at a non-consensus lysine residue as is the case for the human USP25 (ubiquitin specific peptidase 25) and DNA helicase BLM (Bloom syndrome, RecQ helicase-like) proteins (Wilkinson and Henley, 2010). Both of these proteins contain a SIM similar to SIM3 found in NPR1 (Figure 1B) (Meulmeester et al., 2008; Zhu et al., 2008). We hypothesize that NPR1 is sumoylated at a non-consensus lysine because mutating lysines in the six potential SUMO consensus sites did not affect NPR1 interaction with SUMO3 (Figures S1B and S1C).

The regulation of NPR1 sumoylation requires further investigation. It is known that the *SUMO3* gene expression is induced by SA treatment and pathogen challenge (van den Burg et al., 2010). Moreover, mimicking phosphorylation at Ser55/Ser59 can block NPR1 sumoylation indicating that protein conformation also plays a regulatory role. If sumoylation occurs in the nucleus, NPR1 nuclear translocation may be another rate limiting step for this PTM.

The SIM3 in NPR1 is located in the ankyrin-repeat domain responsible for protein-protein interactions (Figures 1, 5 and S1A). NPR1 modification by SUMO3 may change the conformation of the domain to allow binding to NPR3, NPR4 and TGA3, but block interaction with WRKY70. For NPR3 and NPR4, binding to NPR1 may occur at the SUMO3 moiety via their own SIMs or vice versa. A well-studied example for sumoylation serving as an interaction platform for the recruitment of other SIM-containing proteins is the mammalian PML, which organizes the formation of PML nuclear bodies containing more than 100 proteins (Shen et al., 2006).

The interplay between sumoylation and phosphorylation of NPR1 discovered in this study shed light on the mystery of why degradation appeared to be required for the full activity of NPR1 (Spoel et al., 2009). We found that sumoylation of NPR1 not only facilitates its degradation but is also required for differential interactions with WRKY and TGA TFs. At the *PR1* gene promoter, sumoylation of NPR1 is required for SA-triggered dissociation from the inhibitory W-box and for the SA-induced association with the active *cis*-element *as-1* (Figure 5F). Which of these two regulatory steps is essential for SA-mediated induction of *PR1* will require future investigation. If the function of NPR1 is to provide the transactivation activity to TGA TFs as previously proposed (Despres et al., 2003; Despres et al., 2000; Fan and Dong, 2002; Johnson et al., 2003; Rochon et al., 2006), then sumoylation-mediated interaction with TGA is the essential step. However, this does not exclude the possibility that sumoylation of NPR1 is required for the removal of transcription repression by a WRKY TF at the W-box. These two mechanisms are not mutually exclusive.

As summarized in Figure 7, we hypothesize that at resting state, NPR1 is phosphorylated at Ser55/Ser59, which inhibits sumoylation and promotes interaction with WRKY70 to repress *PR1* transcription. At the same time, there must be a residual level of sumoylation responsible for conferring basal defense. SA accumulation promotes dephosphorylation of Ser55/Ser59 through an unknown mechanism and induces sumoylation of NPR1, resulting in dissociation from WRKY70. Modification of NPR1 by SUMO3 is required for phosphorylation at Ser11/Ser15 to form a signal amplification loop to generate more activated NPR1. The activated NPR1 interacts with the TGA3 to induce *PR1* gene expression. Subsequently, the modified NPR1 is ubiquitinated and targeted for proteasomal degradation by NPR3. This coupling of protein activation and turnover through sumoylation determines the transient nature of NPR1 activation, which is essential for balancing defense and growth. Our study illustrates how dynamic PTMs enable a single protein to perform its multiple functions through engagements with different partners. The mechanism uncovered in this study may well be extended to other systems.

Experimental Procedures

Plant Material

The CDSs of *NPR1* and the *npr1* mutants were recombined into the plant binary vector pK7FWG2 to generate C-terminal GFP fusions and transformed into the *npr1-2* plants by floral dipping (Clough and Bent, 1998). The *npr1* mutants (*npr1*^{S11/15A} and *npr1*^{S11/15D}) transgenic lines used here were previously described (Spoel et al., 2009).

Yeast Two-Hybrid Analysis

The yeast strains AH109 and Y187 were transformed with the desired combinations of pGADT7 and pGBKT7 constructs, respectively. All protocols were carried out according to Clontech Yeast Protocols Handbook. NPR1-NPR3/NPR4 interactions were tested with or without 100 μ M sodium salicylate added to the media.

Split Luciferase Assay

Split luciferase assay was carried out as described (Chen et al., 2008).

Co-Immunoprecipitation (co-IP)

Co-IP from yeast diploid cells carrying the desired combination of pGADT7 and pGBKT7 constructs was conducted following the manufacturer's instructions for anti-c-Myc magnetic beads (Pierce, Rockford, IL USA). Western blots were probed with α -SUMO3 (Agrisera, Sweden) and α -NPR1 antibodies.

Sumoylation Reaction in *E. coli*

The reconstituted sumoylation system in *E. coli* was established as previously described (Elrouby and Coupland, 2010; Okada et al., 2009). Proteins were purified and analyzed by western blotting using α -GST (GenScript, Piscataway, NJ, USA) and α -SUMO3 antibodies.

Protein Degradation Analysis

The cell free degradation assay was performed as described (Spoel et al., 2009). The levels of the GFP-tagged proteins were analyzed by western blotting using α -GFP antibody (Clontech, Mountain View, CA USA).

Confocal Microscopy of GFP Fusion Proteins

Microscopic analysis of transgenic *Arabidopsis* plants and *N. benthamiana* transiently expressing GFP fusions of *NPR1* and *npr1* mutants was performed with a LSM510 inverted confocal laser scanning microscope (Zeiss, Thornwood, NY, USA) using a 40 \times oil-immersion objective.

Western Blot with Phospho-Specific Antibodies

Protein extraction was performed as described by Spoel et al. (2009). Western blots were probed with α -GFP, α -pS11/15 (Spoel et al., 2009), and α -pS55/59 antibodies. The antigenic peptides (CSNSFES(phos)VFDS(phos)PDDFY) and anti-serum for pS55/59 antibody were generated by ProteinTech Group (Chicago, IL).

Immunoprecipitation (IP) and detection of sumoylated NPR1-GFP

Three-week-old *35S:NPR1-GFP* and *35:npr1^{sim3}-GFP* plants were treated with water or 0.5 mM SA for six hours. Proteins were extracted in binding buffer supplemented with 50 μ M MG115, and 50 μ M N-Ethylmaleimide (Sigma-Aldrich, St. Louis, MO). IPs were conducted using GFPTrapA beads. Samples were probed with α -GFP and α -SUMO3 antibodies.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (Saleh et al., 2008). The purified ChIP samples were subject to qPCR analysis. Fold of enrichment was calculated according to the comparative Ct method (Schmittgen and Livak, 2008) using the input samples as normalizers.

Pathogen Infection

Plant infection with *Pseudomonas syringae maculicola* (*Psm*) ES4326 was performed as previously described (Durrant et al., 2007). To induce SAR, plants were sprayed with 1 mM SA and 24 hr later pressure-infiltrated with *Psm* ES4326 ($OD_{600nm} = 0.001$). *In planta* growth of *Psm* ES4326 was determined three days after infection as described previously (Wang et al., 2006). All experiments were repeated three times with similar results.

Quantitative PCR

Gene expression was analyzed by quantitative real-time PCR with gene specific primers for *NPR1*, *PR1*, *PR2* and *UBQ5* (Table S1) as described by (Yan et al., 2013).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- The plant immune regulator NPR1 is sumoylated upon immune induction by salicylic acid
- Sumoylation switches NPR1 's interaction from transcription repressors to activators
- Sumoylation is required for NPR1 immune activity as well as degradation
- Sumoylation is inhibited by phosphorylation, which keeps NPR1 stable and quiescent

(F) Co-IP from yeast cells expressing BD-cMyc and AD-HA-SUMO3, BD-cMyc-NPR1 and AD-HA, or BD-cMyc-NPR1 and AD-HA-SUMO3. Equal amounts of cell culture were untreated (-) or treated (+) with 100 μ M MG115 and incubated for 3 hr. Proteins were purified with cMyc-magnetic beads and analyzed by western blot using α -SUMO3 and α -NPR1 antibodies.

(G) *In planta* sumoylation of NPR1. IP of NPR1-GFP and *npr1*^{sim3}-GFP (sim3-GFP) was conducted using total protein extract (Input) from leaves treated with water (-) or (+) 0.5 mM SA for 6 hr. Samples were adjusted for equal amounts of total protein and IP samples were analyzed by western blotting (WB) using α -GFP and α -SUMO3 antibodies. See also Figure S1.

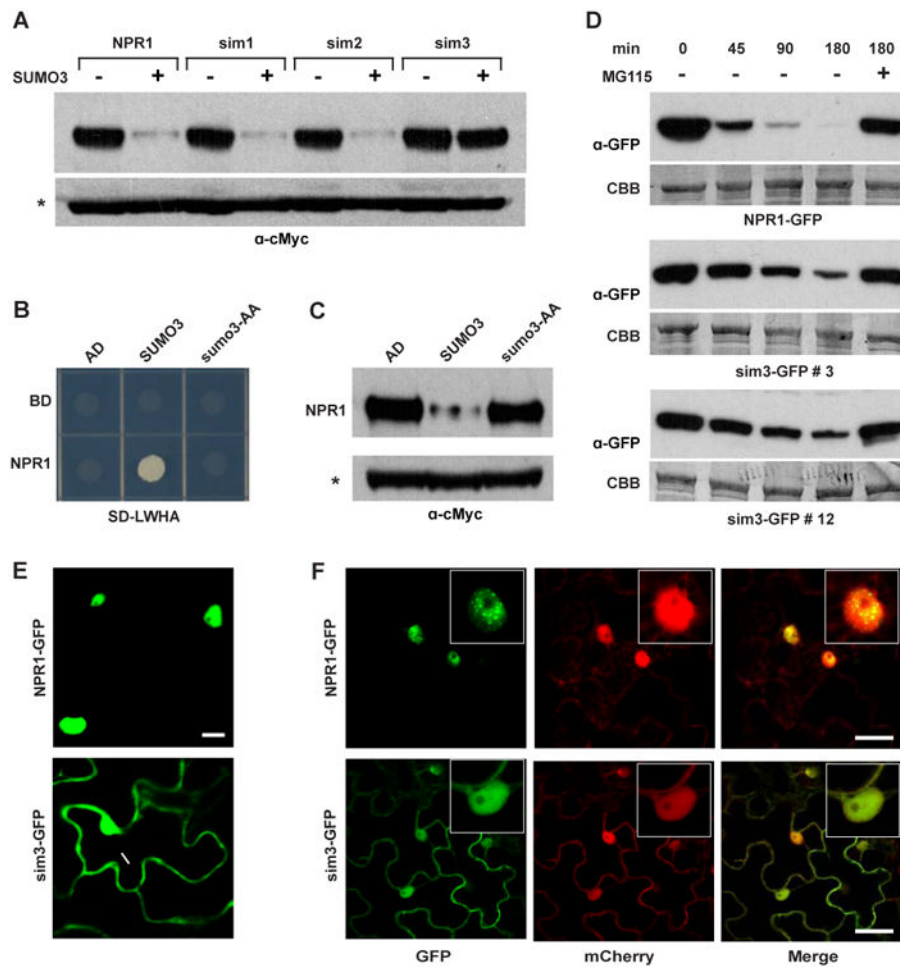


Figure 2. Sumoylation Facilitates Proteasome-Mediated Degradation of NPR1

(A) Protein degradation assay in yeast. Cells were expressing BD-cMyc-NPR1 (NPR1) or BD-cMyc-npr1^{sim} mutants (sim1, sim2, and sim3) and AD-HA-SUMO3 (SUMO3). Cells were treated with Cycloheximide (100μg/ml) for 1.5 hr. Proteins were extracted and analyzed by western blotting using an α-cMyc antibody. Star indicates a loading control. (B) Interaction of BD-cMyc-NPR1 (NPR1) and AD-HA-SUMO3 (SUMO3) or AD-HA-sumo3-AA (sumo3-AA) mutant in yeast.

(C) Degradation of NPR1 in yeast. Yeast cells were expressing BD-cMyc-NPR1 (NPR1) and AD-HA-SUMO3 (SUMO3) or the AD-HA-sumo3-AA (sumo3-AA) mutant. Total protein was analyzed as in (A).

(D) *In vitro* degradation of NPR1-GFP and npr1^{sim3}-GFP (sim3-GFP). Protein extracts from *35S:NPR1-GFP* (in *npr1-2*) (NPR1-GFP), and two different lines of *35S:npr1^{sim3}-GFP* (sim3-GFP #3 and sim3-GFP #12; in *npr1-2*) plants were incubated at room temperature for the time points indicated. The proteasome inhibitor MG115 (50 μM) was used. Proteins were analyzed by western blotting using an α-GFP antibody. CBB, Coomassie Brilliant Blue protein stain.

(E) Confocal micrographs of transgenic *Arabidopsis* expressing *35S:NPR1-GFP* (NPR1-GFP) or *35S:npr1^{sim3}-GFP* (sim3-GFP). Arrow indicates nucleus. Bars = 10 μm.

(F) Confocal micrographs of *N. benthamiana* co-expressing free mCherry and *35S:NPR1-GFP* (NPR1-GFP) or *35S:npr1^{sim3}-GFP* (sim3-GFP) 24 hr after *Agrobacterium* infiltration. Close-up images show nuclear bodies. Bars = 25 μ m. See also Figure S2.

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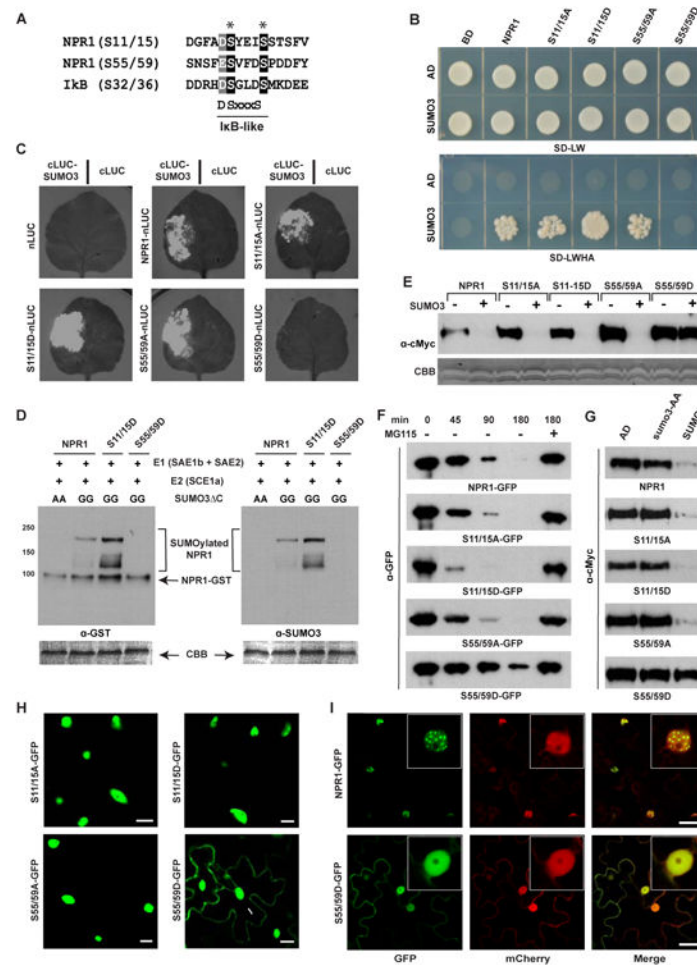


Figure 3. Phosphorylation of IκB-like Sequences Ser11/Ser15 and Ser55/Ser59 Differentially Influences NPR1 Sumoylation and Protein Stability

(A) Sequence alignments of IκB-like sequences. The identical amino acids in the IκB-like sites are shaded black, conserved amino acids are shaded in grey and phosphorylatable serines are marked by *.

(B) Interactions among BD-cMyc-NPR1 (NPR1) or BD-cMyc-npr1^{S11/15A} (S11/15A), BD-cMyc-npr1^{S11/15D} (S11/15D), BD-cMyc-npr1^{S55/59A} (S55/59A), BD-cMyc-npr1^{S55/59D} (S55/59D) mutants and AD-HA-SUMO3 in yeast. Equal numbers of cells were spotted on SD-LW and SD-LWHA plates.

(C) Split luciferase assays. The NPR1 or npr1-phosphomimic mutants and SUMO3 proteins were fused to the N- or C-terminal half of luciferase (nLUC or cLUC), and transiently expressed in *N. benthamiana*. The luciferase activities were monitored by a CCD camera two days after infiltration.

(D) *E. coli* sumoylation assay. Proteins were extracted from *E. coli* BL21 (C41) cells coexpressing E1 heterodimer (SAE1b and SAE2), E2 (SCE1a), the mature SUMO3 (SUMO3 C) and either NPR1-GST (NPR1), npr1^{S11/15D}-GST (S11/15D) or npr1^{S55/59D}-GST (S55/59D). Proteins were purified using GST-magnetic beads and analyzed by western blot using α-GST and α-SUMO3 antibodies. GG, wild-type SUMO3 C; AA, mutant

SUMO3 C with the di-glycine (GG) required for conjugation replaced by alanines. CBB, Coomassie Brilliant Blue protein stain.

(E) Degradation of NPR1 and *npr1* mutants in yeast. Total protein was extracted from equal amounts of cell culture and analyzed by western blotting using an α -cMyc antibody. CBB, Coomassie Brilliant Blue protein stain.

(F) *In vitro* degradation assay. Protein extracts from *35S:NPR1-GFP* (NPR1), *35S:npr1^{S11/15A}-GFP* (S11/15A), *35S:npr1^{S11/15D}-GFP* (S11/15D), *35S:npr1^{S55/59A}-GFP* (S55/59A), *35S:npr1^{S55/59D}-GFP* (S55/59D) were incubated at room temperature for the time points indicated. The proteasome inhibitor MG115 (50 μ M) was used. Proteins were analyzed by western blotting using an α -GFP antibody.

(G) Degradation assay. Yeast cells carrying BD-cMyc-NPR1 (NPR1) or BD-cMyc-*npr1^{S11/15A}* (S11/15A), BD-cMyc-*npr1^{S11/15D}* (S11/15D), BD-cMyc-*npr1^{S55/59A}* (S55/59A), BD-cMyc-*npr1^{S55/59D}* (S55/59D) mutants and AD-HA-SUMO3 (SUMO3) or AD-HA-sumo3-AA (sumo3-AA) were grown and total protein was analyzed as in (E).

(H) Confocal micrographs of transgenic *Arabidopsis 35S:npr1^{S11/15A}-GFP* (S11/15A-GFP), *35S:npr1^{S11/15D}-GFP* (S11/15D-GFP), *35S:npr1^{S55/59A}-GFP* (S55/59A-GFP), *35S:npr1^{S55/59D}-GFP* (S55/59D-GFP). Arrow indicates nucleus. Bars = 10 μ m.

(I) Confocal micrographs of *N. benthamiana* co-expressing free mCherry and *35S:NPR1-GFP* (NPR1-GFP) or *35S:npr1^{S55/59D}-GFP* (S55/59D-GFP) 24 hr after *Agrobacterium* infiltration. Close-up images show nuclear bodies. Bars = 25 μ m.

See also Figure S3.

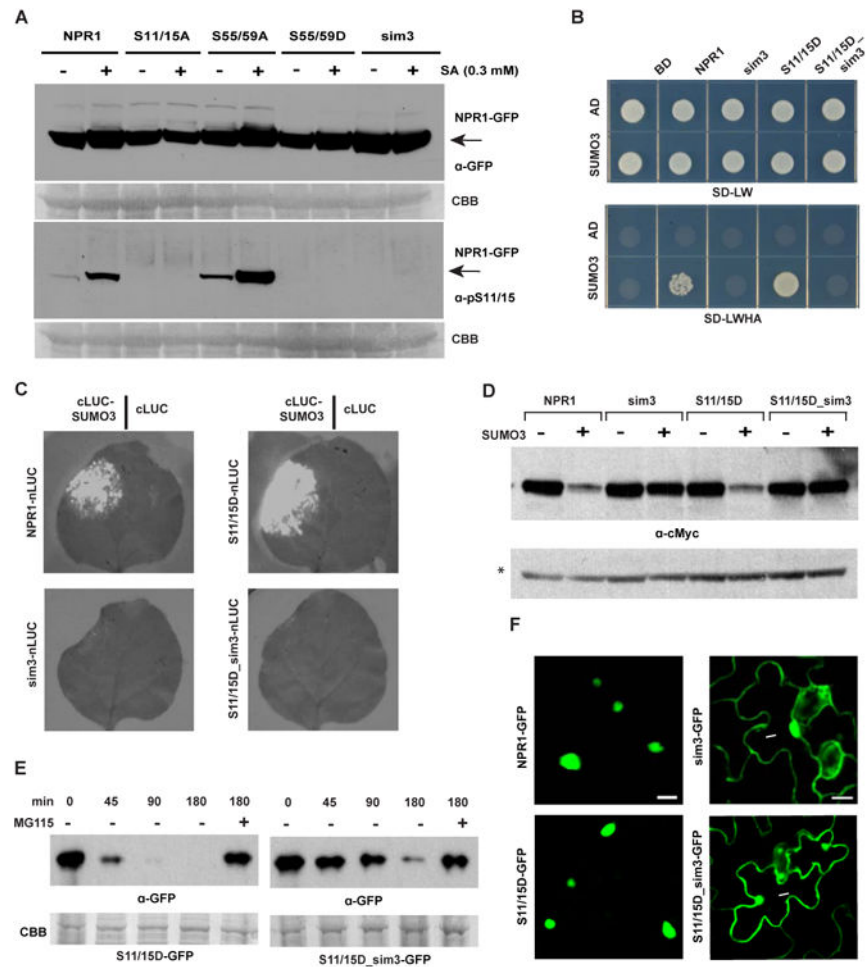


Figure 4. NPR1 Interaction with SUMO3 Is Required for and Dominant over Phosphorylation at Ser11/Ser15

(A) *In planta* phosphorylation of Ser11/Ser15. Total protein was extracted from seedlings expressing *35S:NPR1-GFP* (NPR1), *35S:npr1^{S11/15A}-GFP* (S11/15A), *35S:npr1^{S55/59A}-GFP* (S55/59A), *35S:npr1^{S55/59D}-GFP* (S55/59D), *35S:npr1^{sim3}-GFP* (sim3) treated with water (-) or (+) 0.3 mM SA for 8 hr. Proteins were analyzed by western blotting using α -GFP and α -pS11/15 phospho-specific antibodies. CBB, Coomassie Brilliant Blue protein stain.

(B) Interaction of BD-cMyc-NPR1 (NPR1), BD-cMyc-npr1^{sim3} (sim3), BD-cMyc-npr1^{S11/15D} (S11/15D) or BD-cMyc-npr1^{S11/15D_sim3} (S11/15D_sim3) and AD-HA-SUMO3 in yeast. Equal numbers of cells were spotted on SD-LW and SD-LWHA plates.

(C) Split luciferase assay. The NPR1, npr1^{sim3} (sim3), npr1^{S11/15D} (S11/15D), or npr1^{S11/15D_sim3} (S11/15D_sim3) and SUMO3 proteins were fused to the N- or C-terminal half of luciferase (nLUC or cLUC) and transiently expressed in *N. benthamiana*. The luciferase activities were monitored by a CCD camera two days after infiltration.

(D) Degradation assay. Yeast total protein was extracted from equal amounts of cell culture as described in (B), after growth overnight in SD-LW liquid media, analyzed by SDS-PAGE and western blotting using an α -cMyc antibody. Star indicates a nonspecific protein used as a loading control.

(E) *In vitro* degradation assay. Protein extracts from *35S:npr1^{S11/15D}-GFP* (S11/15D-GFP) and *35S:npr1^{S11/15D_sim3}-GFP* (S11/15D_sim3-GFP) were incubated at room temperature for the time points indicated. The proteasome inhibitor MG115 (50 μ M) was used. Proteins were analyzed by western blotting using an α -GFP antibody.

(F) Confocal micrographs of transgenic *Arabidopsis* expressing *35S:npr1^{S11/15D}-GFP* (S11/15D-GFP) and *35S:npr1^{S11/15D_sim3}-GFP* (S11/15D_sim3-GFP). Arrow indicates nucleus. Bars = 10 μ m.

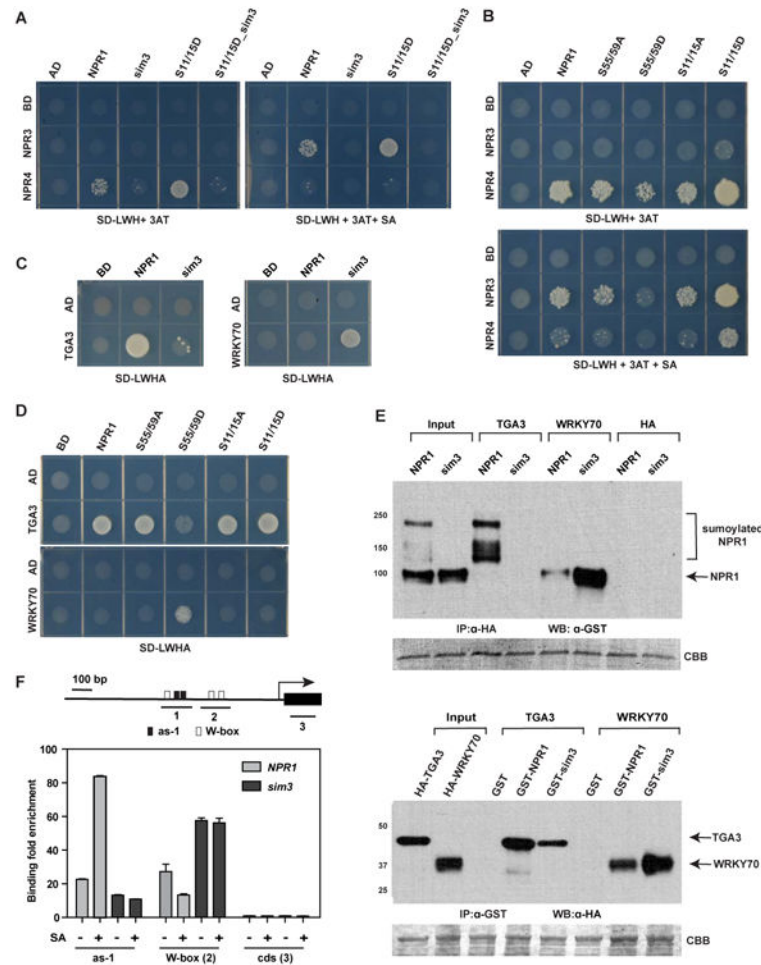


Figure 5. Sumoylation of NPR1 Determines Its Interactions with Partner Proteins

(A) Interaction of BD-NPR3 (NPR3) or BD-NPR4 (NPR4), and AD-NPR1 (NPR1), AD-npr1^{sim3} (sim3), AD-npr1^{S11/15D} (S11/15D) or AD-npr1^{S11/15D_sim3} (S11/15D_sim3) in yeast. Equal numbers of cells were spotted on SD-LW and SD-LWH plates containing 3 mM 3-AT with or without 100 μ M SA.

(B) Interaction of BD-NPR3 (NPR3) or BD-NPR4 (NPR4), and AD-NPR1 (NPR1) or AD-npr1^{S11/15A} (S11/15A), AD-npr1^{S11/15D} (S11/15D), AD-npr1^{S55/59A} (S55/59A), AD-npr1^{S55/59D} (S55/59D) in yeast. Cultures were grown and analyzed as in (A).

(C) Interaction of BD-NPR1 (NPR1) or BD-npr1^{sim3} (sim3) and AD-TGA3 (TGA3) or AD-WRKY70 (WRKY70) in yeast. Equal numbers of cells were spotted on SD-LW and SD-LWHA plates.

(D) Interaction of BD-NPR1 (NPR1), BD-npr1^{S11/15A} (S11/15A), BD-npr1^{S11/15D} (S11/15D), BD-npr1^{S55/59A} (S55/59A), BD-npr1^{S55/59D} (S55/59D), and AD-TGA3 (TGA3) or AD-WRKY70 (WRKY70) in yeast. Cultures were analyzed as in (C).

(E) *In vitro* pull down assay. Equal amounts of GST-tagged NPR1 and npr1^{sim3} proteins produced using the *E. coli* sumoylation system were mixed with *in vitro* synthesized HA-tagged TGA3 or WRKY70. The Co-IP was performed using HA-magnetic beads and the proteins were analyzed western blotting using an α -GST antibody (upper panel), or the Co-IP was performed using GST-magnetic beads and the proteins were analyzed by western

blotting using an α -HA antibody (lower panel). CBB, Coomassie Brilliant Blue protein stain.

(F) ChIP assay was performed using *35S:NPR1-GFP (NPR1)* and *35S:npr1^{sim3}-GFP (sim3)*. Plants were untreated (-) or treated (+) with 1 mM SA for 16 hr. Upper panel: diagram of the *PRI* gene with the analyzed *cis* elements (as-1 elements and W-boxes) and the coding region (cds) highlighted by short horizontal lines 1, 2, and 3, respectively. The fold enrichments of untreated and SA-treated samples after ChIP were determined through qPCR and calculated against input. The error bars represent \pm SEM (n = 3). The experiment was performed twice with similar results.

See also Figures S4 and S5.

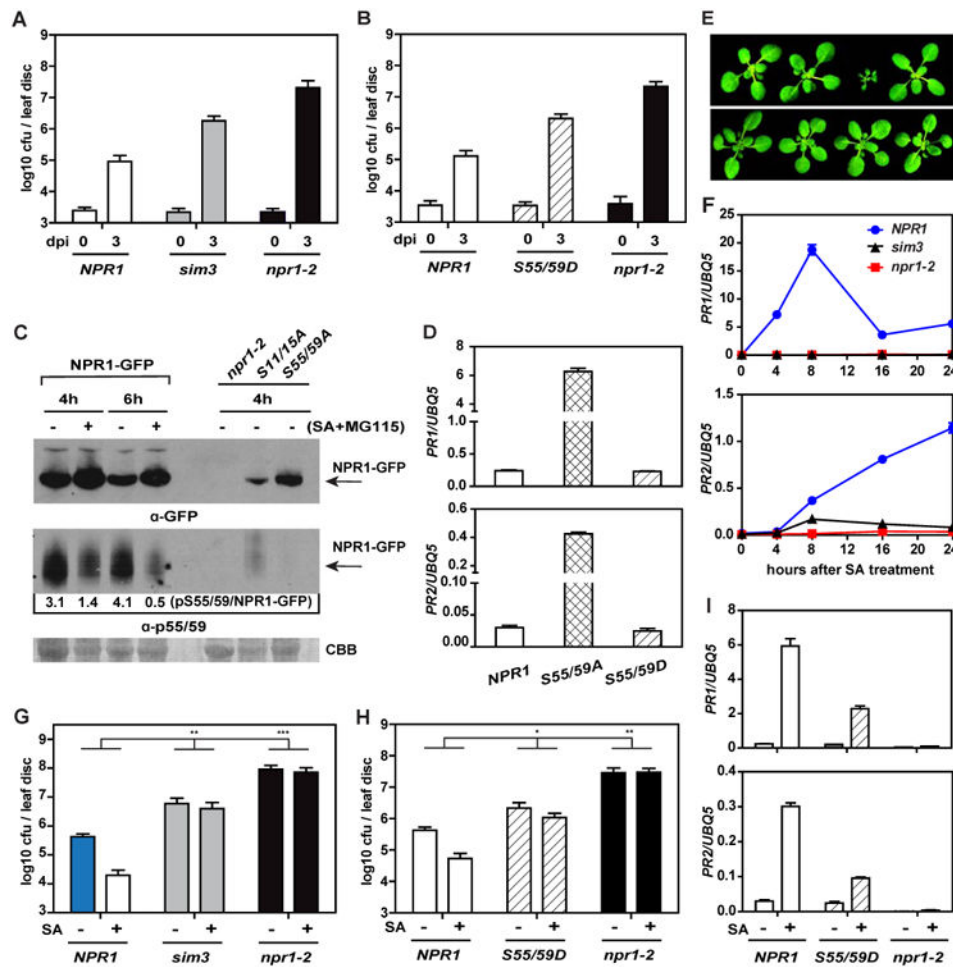


Figure 6. Phosphorylation at Ser55/Ser59 and Sumoylation of NPR1 Are Required for Safe Storage and Transient Activation of NPR1-Mediated Defense

(A) Three-week-old *35S:NPR1-GFP* (in *npr1-2*) (NPR1), *35S:npr1^{sim3}-GFP* (in *npr1-2*) (*sim3*), and *npr1-2* plants were inoculated with *Psm ES4326* ($OD_{600nm} = 0.0001$). Growth of *Psm* was determined at 0 and 3 days after infection. Error bars represent 95% confidence intervals, $n = 8$. cfu, colony-forming units. dpi, days post inoculation. This experiment was repeated three times with similar results.

(B) Infection of *35S:NPR1-GFP* (in *npr1-2*) (NPR1), *35S:npr1^{S55/59D}-GFP* (in *npr1-2*) (*S55/59D*), and *npr1-2* mutant plants was carried out as in (A).

(C) *In planta* phosphorylation of Ser55/Ser59. Seedlings expressing *35S:NPR1-GFP*, *35S:npr1^{S11/15A}-GFP* (*S11/15A*), *35S:npr1^{S55/59A}-GFP* (*S55/59A*), and the *npr1-2* mutant were treated with water (-) or (+) 0.3 mM SA and 50 μ M MG115 for 4 or 6 hr. Protein extracts were analyzed by western blotting using α -GFP and α -pS55/59 phospho-specific antibodies. Signal intensities were measured using ImageJ software and the ratios of phospho-S55/59 to NPR1-GFP are indicated. Extracts from seedlings expressing GFP-tagged *npr1^{S11/15A}*, *npr1^{S55/59A}*, and *npr1-2* treated with water for 4 hr were included as control samples for antibody specificity. CBB, Coomassie Brilliant Blue protein stain.

(D) Gene expression analysis. RNA was extracted from *35S:NPR1-GFP* (NPR1), *35S:npr1^{S55/59A}-GFP* (*S55/59A*) and *npr1-2* plants. The expression of *PR1* and *PR2* was

analyzed using qPCR and normalized against *ubiquitin 5 (UBQ5)*. Error bars represent SD (n = 3).

(E) Photograph of three-week-old *npr1-2*, *35S:NPR1-GFP* (NPR1), *35S:npr1^{S55/59A}-GFP* (S55/59A), *35S:npr1^{S55/59D}-GFP* (S55/59D), *35S:npr1^{sim3}* (sim3), *35S:npr1^{S11/15A}* (S11/15A), *35S:npr1^{S11/15D}* (S11/15D) and *35S:npr1^{S11/15D-sim3}* (S11/15D_sim3) plants.

(F) Gene expression analysis. RNA was extracted from three-week-old *35S:NPR1-GFP* (NPR1), *35S:npr1^{sim3}-GFP* (sim3) and *npr1-2* plants treated with 1 mM SA. Tissue samples were collected during a time course after SA treatment. The expression of *PR1* and *PR2* was analyzed as in (D).

(G) Three-week-old *35S:NPR1-GFP* (NPR1), *35S:npr1^{sim3}-GFP* (sim3) and *npr1-2* plants were treated without (-) or with (+) 1 mM SA and inoculated 24 hr later with *Psm* ES4326 (OD_{600nm} = 0.001). Growth of *Psm* was determined three days after infection. Error bars represent 95% confidence intervals, n = 8. cfu, colony-forming units. dpi, days post inoculation. Statistical analysis was performed using two-way ANOVA, ** *p*-value < 0.005, *** *p*-value < 0.0005. This experiment was carried out three times with similar results.

(H) Three-week-old *35S:NPR1-GFP* (NPR1), *35S:npr1^{S55/59D}-GFP* (S55/59D) and *npr1-2* plants were treated without (-) or with (+) 1 mM SA and inoculated 24 hr later with *Psm* ES4326 (OD_{600nm} = 0.001). Growth of *Psm* and statistical analysis were conducted as in (G). * *p*-value < 0.05, ** *p*-value < 0.005.

(I) Gene expression analysis. RNA was extracted from three-week-old *35S:NPR1-GFP* (in *npr1-2*) (NPR1), *35S:npr1^{S55/59D}-GFP* (in *npr1-2*) (S55/59D) and *npr1-2* plants treated with 1 mM SA for 24 hr. The expression of *PR1* and *PR2* was analyzed as in (F).

See also Figure S6.

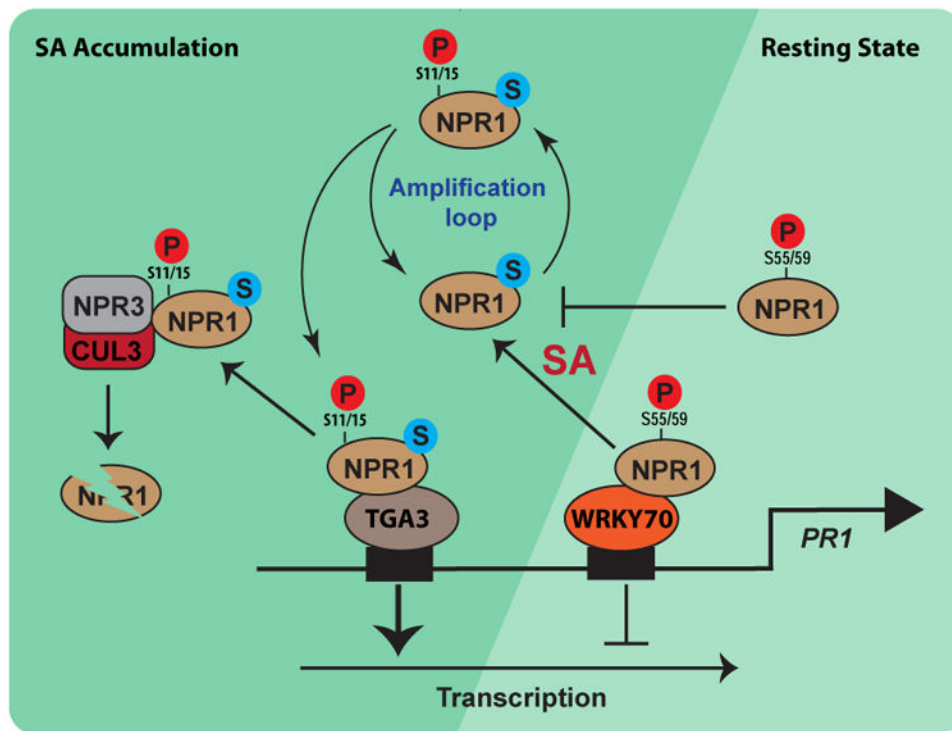


Figure 7. The Interplay between Sumoylation and Phosphorylation of NPR1 Controls Its Differential Transcription Cofactor Activity as a Master Immune Switch

SA accumulation promotes dephosphorylation of Ser55/Ser59 through an unknown mechanism and induces sumoylation of NPR1, resulting in dissociation from WRKY70 and inactivation of this repressor. Modification of NPR1 by SUMO3 is required for its phosphorylation at Ser11/Ser15 to form a signal amplification loop to generate more activated NPR1. This activated form of NPR1 interacts with the TGA3 transcription activator to induce *PR1* gene expression. Subsequently, the modified NPR1 is ubiquitinated and targeted for degradation by the 26S proteasome mediated by interaction with NPR3 to ensure the transient nature of the immune induction. P: phosphorylation, S: sumoylation, as1: as-1 element, W: W-box