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## Post-translational Regulation of Plant Immunity

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### Introduction

Plants are in constant association with microbial communities, and have evolved a multi-layered molecular defense strategy to protect themselves against pathogens. In the absence of specialized immune cells, plants rely on rapid alterations of signaling pathways within individual cells to achieve an appropriate defense response while balancing the trade-off with normal growth and metabolism. Much of our knowledge about the regulation of plant immune responses has been obtained using genetic and genomic approaches for identification of genes and gene expression patterns underlying defense pathways. However, genetic approaches are limited to examining the effects of losing or gaining gene functions, rather than dynamic regulation of cellular processes. Consequently, our current understanding of plant immune networks is far from complete. In recent years, there has been a significant shift from studying the underlying transcriptional networks to studying protein post-translational modification (PTM), which is a versatile regulatory process that rapidly alters the functional diversity of the proteome [1]. Current evidence shows that PTMs are critical for the rapid reprogramming of cells for defense signaling and for attenuating the response to achieve cellular homeostasis in all layers of the plant immune responses.

In this review, we seek to highlight discoveries that have demonstrated the power and versatility of PTMs as regulatory mechanisms for host cells to rapidly respond to pathogens and for pathogens to deploy effectors as a part of their virulence strategy.

### Phosphorylation dynamics during pathogen perception and pattern-triggered immunity

At the front line of molecular defense strategies, plants deploy a molecular surveillance system through the cell surface-anchored pattern-recognition receptors (PRRs) to detect microbe-associated and damage-associated molecular patterns (MAMPs and DAMPs) and initiate pattern-triggered immunity (PTI) (Fig. 1) [2]. PTI is effective against a broad range

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of microbes and is characterized by rapid calcium influx, production of reactive oxygen species (ROS), mitogen activated protein kinase (MAPK) phosphorylation cascades, callose deposition, and defense gene expression [3]. The activation of PTI also leads to plant growth inhibition, highlighting the trade-off between growth and defense physiology. Therefore, understanding how this immune response is dynamically regulated by PTM to achieve rapid and transient induction has been a major focus in deciphering the mechanisms of PTI.

Activation of the PRRs, FLAGELLIN-SENSITIVE 2 (FLS2) and EF-TU RECEPTOR (EFR), by peptides derived from bacterial flagellin (flg22) and elongation factor Tu (elf18), stimulates the recruitment of the leucine-rich receptor-like kinase (LRR-RLK) BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) and the receptor-like cytoplasmic kinase (RLCK) BOTRYTIS-INDUCED KINASE1 (BIK1). These plasma membrane (PM)-associated kinases are in turn activated by phosphorylation to initiate defense signal transduction [3,4]. BAK1 and BIK1 are shared by many PRR signaling pathways, including the growth hormone brassinosteroid (BR) signal mediated by the receptor BRASSINOSTEROID INSENSITIVE 1 (BRI1) [5]. Recent studies using targeted phosphoproteomics have revealed differential phosphorylation patterns for BAK1 in complex with distinct RLKs (i.e., FLS2, EFR, BIK1 and BRI1). For example, phosphorylation of BAK1 by BIK1 at four amino acids in the kinase domain and two in the activation loop is inhibited through phosphorylation mediated by FLS2 at Ser-286 [6••]. A similar inhibitory effect was observed for EFR through phosphorylation of Thr-455 in the BAK1 activation domain [6••]. The opposing activities of FLS2 and EFR against BIK1-mediated phosphorylation suggest that once BAK1 and BIK1 have become phosphorylated in response to MAMP perception, the PRRs can attenuate this immune response through inhibition of BAK1 phosphorylation mediated by BIK1.

Besides the opposing activities between BIK1 and FLS2/EFR on BAK1, phosphatases, such as SERINE/THREONINE PROTEIN PHOSPHATASE 2A (PP2A), can also counteract the kinase activity to suppress PTI [7]. Upon flg22 treatment, PP2A activity has to be reduced to allow induction of BAK1 activity and PTI signaling [7]. Additionally, the PM-localized PP2C38 interacts with FLS2-BIK1 as well as EFR-BIK1 and dephosphorylates BIK1 to inhibit PAMP-induced ROS production and stomatal closure [8••]. Co-immunoprecipitation (Co-IP) experiments demonstrated that PP2C38 and BIK1 directly interact with each other. A combined approach using genetics and phospho-proteomic analysis indicated that MAMP treatment induces BIK1-dependent phosphorylation of PP2C38 at Ser-77, which significantly diminishes the PP2C38 activity and its interaction with the receptor complexes [8••]. This interplay between immune regulatory kinases and phosphatases is a major mechanism by which plant cells achieve homeostasis during infection.

Phosphorylation dynamics are essential for regulation of immune responses; therefore, they are also prime targets for the activity of bacterial type III effector proteins (TTEs). *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) delivers a protein tyrosine phosphatase (PTP), HopAO1, into host cells as part of its virulence strategy. When HopAO1 is ectopically expressed in *Arabidopsis*, it inhibits elf18- and flg22-induced ROS burst, MAPK activation, and resistance to *Pst* DC3000 [9]. HopAO1 interacts with the kinase domains of FLS2 and EFR in yeast and plant cells, and *in vitro* kinases assays demonstrated

that the presence of HopAO1 results in a significant decrease in elf18-induced Tyr phosphorylation on EFR [9]. The ability of *Pst* DC3000 to colonize host tissue is compromised in strains harboring a HopAO1 deletion, and this defect is absent when inoculated into *Arabidopsis fls2/efr* double mutants, confirming that a major virulence function of HopAO1 is the targeted dephosphorylation of PRRs to inhibit MAMP-induced immune responses [9].

## Ubiquitination and N-glycosylation contribute to PTI through degradation and cellular targeting of PRRs

PRR signaling dynamics are fine-tuned by ubiquitination, which facilitates their endocytosis and degradation by the 26S proteasome [10,11]. Yeast two-hybrid (Y2H) and Co-IP experiments using the BAK1 kinase domain as a bait demonstrated that flg22 treatment stimulates interaction between BAK1 and the plant U box (PUB) E3 ubiquitin ligases PUB12 and PUB13, and that the BAK1-PUB12/13 complex associates with FLS2 within 30 seconds of flg22 treatment [12]. Kinase assays using protoplasts expressing BAK1 or a kinase-inactive mutant indicated that BAK1 phosphorylates the C-terminal Armadillo (ARM) repeat domain of PUB13 in response to flg22 treatment, and chemical inhibition of kinase activity confirmed that BAK1-dependent phosphorylation is required for flg22-induced FLS2-PUB12/PUB13 interaction [12,13]. In vitro ubiquitination assays confirmed that PUB12 and PUB13 polyubiquitinate the cytosolic domain of FLS2, but not BAK1 or BIK1 [12]. Three additional, functionally redundant U-box E3 ubiquitin ligases, PUB22/23/24 have also been shown to negatively regulate PTI responses [14]. Upon flg22 perception, PUB22 ubiquitinates Exo70B2, a member of a vesicle tethering complex, and targets it for degradation; Exo70B2 is required for both early and late PTI responses [15,16]. Taken together, studies of ubiquitin-mediated regulation of PRRs indicate that their internalization and degradation likely serves to desensitize cells to continual MAMP stimulus, as well as to reset cells for new rounds of signaling [17].

Asparagine-linked protein glycosylation (N-glycosylation) contributes to protein folding and stability, secretion, and interactions with ligands and other proteins [18]. Treatment of *Arabidopsis* protoplasts with tunicamycin, which blocks N-glycosylation, and subsequent biochemical analyses showed that the extracellular LRR domains of both FLS2 and EFR are extensively N-glycosylated and that these modifications are required for their maturation and transport to the PM [19]. Site-directed mutagenesis has also been used to generate point mutations in EFR that disrupt individual N-X-(S/T) glycosylation motifs and affect discrete N-glycosylation events. When transiently expressed in *Nicotiana benthamiana*, which is deficient in elf18 perception, the point mutant of EFR, Asn-143-Gln, fails to elicit MAMP-induced ROS production [19], indicating that individual N-glycosylation sites also play a role in PRR stability and ligand recognition.

## Downstream of the PRRs – MAPK cascades and production of reactive oxygen species

Upon pattern recognition and activation of PRR-RLK complexes, immune signaling is propagated by MAPK phosphorylation cascades leading to transcriptional reprogramming for defense [2]. Recent studies of the FLS2 signaling complexes have revealed a connection between PRR/RLK signals and the MAPK phosphorylation cascade, which has been a missing link in PTI signaling against bacterial pathogens. MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 7 (MKKK7) was first identified in a large-scale proteomics screen for differential protein phosphorylation upon flg22 treatment [20]. MKKK7 interacts directly with FLS2, and quantitative phospho-proteomic analyses indicated that upon flg22 treatment, MKKK7 is phosphorylated at two different serine residues in a temporally distinct manner [21••]. MKKK7 appears to be a negative regulator of PTI because *mkkk7* mutants enhanced MPK6 phosphorylation, increased expression of flg22-induced *WRKY29* and *FRK1*, and enhanced resistance to *Pst* DC3000 [21••]. The upstream kinases that act on MKKK7 have yet to be firmly established.

Phosphatases that regulate the MAPK cascade include PROTEIN TYROSINE PHOSPHATASE 1 (PTP1) and the dual-specificity phosphatase (DSP) MKP1, which target MPK3 and MPK6, and down-regulate defense responses against *Pst* DC3000 [22–24]. Members of the PROTEIN PHOSPHATASE 2C (PP2C) family, AP2C1 and AP2C2, were recently shown to be negative regulators of PTI against *P. syringae*. The *ap2c1* mutants exhibited enhanced activation of MPK3, 4, and 6 in response to flg22 and elf18 treatment, as well as to *Pst* DC3000 infection, resulting in upregulation of genes encoding SA biosynthetic enzymes, WRKY TFs, MAPKK/MAPK kinases, and ultimately enhanced callose deposition and bacterial resistance [25].

Another hallmark of PTI is the production of reactive oxygen species (ROS), which have antimicrobial activities and contribute to defense signaling, cell-wall reinforcement, and stomatal closure to limit pathogen entry into host tissues [26,27]. The respiratory burst oxidase homolog (RBOH) proteins, which are PM-localized NADPH oxidases, are required for PTI-triggered ROS production and are activated through phosphorylation by calcium-dependent protein kinases (CDPKs) and the RLCK BIK1 (Fig. 1) [26,28–30]. ROS signaling is linked to metabolic pathways important for defense against pathogens. GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD), a key enzyme of the oxidative pentose phosphate pathway, is regulated by cellular redox status and phosphorylation. G6PD generates NADPH and metabolic intermediates for numerous biosynthetic pathways and exhibits pathogen-inducible activity that is required for the ROS burst [31,32]. The *Arabidopsis* GLYCOGEN SYNTHASE KINASE3 (GSK3)/Shaggy-like kinase ASK $\alpha$ , well-known for its role in regulating abiotic stress and brassinosteroid signaling responses, also promotes PTI [31,33••]. Treatment of seedlings with multiple MAMPs including flg22, chitin, and the DAMP PEPTIDE 1 (PEP1), rapidly induces ASK $\alpha$  protein levels and enzymatic activity. Plants over-expressing ASK $\alpha$  display increased ROS production and transcription of PTI marker genes, while these responses are attenuated in *aska* mutants [33••]. In response to abiotic stress, ASK $\alpha$  phosphorylates G6PD6 at Thr-467 [31], and

expression of phospho-mimetic (T467E)/phospho-null (T467A) G6PD6 variants in *Arabidopsis* revealed that phosphorylation at this residue is also critical for enhanced ROS production. Furthermore, infection by *Pst* DC3000 stimulates ASK $\alpha$  expression levels and kinase activity, and both *aska* and *g6pd6* result in increased susceptibility to the bacterial infection while expression of wild type G6PD6 or T467E restores resistance [33••]. The results from this work suggest that upon MAMP treatment, ASK $\alpha$  phosphorylates G6PD6 at Thr-467 leading to increased ROS production and enhanced PTI [31,33••].

ROS can also influence growth-defense balance through crosstalk among hormones such as indole-3-acetic acid (IAA) for growth and salicylic acid (SA) and jasmonic acid (JA) for defense against biotrophic and necrotrophic pathogens, respectively. The H<sub>2</sub>O<sub>2</sub> scavenging enzyme CATALASE 2 (CAT2) was recently shown to participate in SA-mediated repression of IAA biosynthesis during defense responses [34,35••]. SA binds to CAT2 and inhibits its activity resulting in H<sub>2</sub>O<sub>2</sub> accumulation [35••]. The increase in H<sub>2</sub>O<sub>2</sub> promotes sulfenylation of Tryptophan (Trp) Synthetase  $\beta$  subunit 1 (TSB1) to inhibit its activity leading to reduction of Trp levels and ultimately decreased auxin production [35•]. This PTM of TSB1 likely occurs on Cys-308 as indicated by inhibited enzymatic activity and the insensitivity of Cys-308-Ser to H<sub>2</sub>O<sub>2</sub>-mediated sulfenylation [35•]. These conclusions were supported by the finding that over-expression of TSB1 could rescue the reduced Trp and IAA concentrations in the *cat2-1* mutant [35•]. CAT2 also interacts with and promotes the activity of ACYL CO-ENZYME A OXIDASE (ACX2 and ACX3), peroxisomal enzymes in the JA biosynthetic pathway. Treatment with SA, or induction of SA biosynthesis in response to biotrophic pathogens significantly inhibits the interaction among these enzymes leading to reduction in JA biosynthesis [35••]. Since some pathogens have the ability to stimulate IAA biosynthesis in plant tissues to facilitate infection [34] and/or take advantage of inhibitory SA/JA crosstalk in their favor, this work has revealed a mechanism by which host cells can counter these virulence strategies through SA-mediated inhibition of IAA and JA biosynthesis during defense signaling.

## Post-translational dynamics during effector-triggered immunity and systemic acquired resistance

PTI can be overcome by pathogens through delivery of TTEs into host cells. The virulence function of effectors is often achieved through PTM of host proteins to manipulate immune signaling cascades [36]. In response, plants have evolved resistance (R) proteins, which are intracellular nucleotide-binding leucine-rich repeat (NB-LRR) receptors that either directly or indirectly detect the activity of effector proteins to initiate effector-triggered immunity (ETI). ETI commonly leads to calcium and ROS accumulation, massive transcriptional reprogramming, and induction of programmed cell death (PCD) at the site of infection that limits the spread of the pathogen [37–40]. ETI also triggers the biosynthesis of the defense hormone SA and expression of antimicrobial pathogenesis related (PR) proteins in infected (local) and systemic tissue leading to establishment of systemic acquired resistance (SAR), which protects against additional infection [41].

### (1) PTMs at the host-pathogen interface

The *Arabidopsis* RPM-1 INTERACTING PROTEIN 4 (RIN4) is a multi-functional immune response regulator at the host-pathogen interface that is targeted by at least five *P. syringae* effectors and guarded by the host NB-LRRs RPM1 and RPS2 [42–45]. RPM1 could detect phosphorylation of RIN4-Thr166 by the effector (AvrB and AvrRpm1)-mediated expression and activation of a host RLCK, RIPK [44,46]. In this case, the perturbation caused by the effectors are detected by the cognate host NB-LRRs to trigger ETI. However, effectors normally evolved to help the pathogens. For example, a bacterial cysteine protease, AvrPphB, contributes to the dynamics by cleaving RIPK, thereby suppressing RPM1-mediated ETI, resulting in increased susceptibility to *Pst* DC3000 expressing AvrB [47•].

The host RIN4 also serves as a regulatory link between ETI and PTI. Upon flg22 perception, RIN4 is rapidly phosphorylated at Ser-141 leading to enhanced PTI [48]. This phosphorylation event is upstream of and independent from phosphorylation of RIN4 at Thr-166 that activates ETI in response to AvrB/AvrRpm1 and does not affect the cleavage of RIN4 by AvrRpt2 [48]. Phosphorylation of RIN4-Thr-166, which represses PTI signaling, is reduced in response to flg22 treatment but enhanced in the presence of AvrB offering insight into the mechanism by which *P. syringae* subverts the host's initial defense responses [49••]. Additionally, triple phospho-mimic RIN4 mutants (T21D, S160D, or T166D) exhibit enhanced interaction with, and activation of the plasma membrane H<sup>+</sup>-ATPase 1 (AHA1), and consequently wider stomatal apertures and enhanced colonization of spray inoculated *P. syringae* [49••]. Although the evidence demonstrates that RIN4 pSer-141 requires interaction with FLS2, the kinase responsible for this modification remains elusive.

In addition to targeting upstream signaling components, such as RIN4, some effectors have evolved to help pathogens evade immune responses through PTM of downstream components, such as TFs. Co-IP experiments demonstrated that PopP2 from *Ralstonia solanacearum* acetylates WRKY41,70, and 33, which are positive regulators of defense gene expression, and reduces their ability to bind to gene promoters resulting in suppression of host immune responses [50••,51••]. To counteract this pathogen virulence strategy the NB-LRR RESISTANCE TO *RALSTONIA SOLANACEARUM* 1 (RRS1) is activated directly through effector-mediated PTMs of its extra C-terminal WRKY DNA binding domain [50–52]. Biochemical and proteomics analyses revealed that PopP2 also acetylates RRS1 on four lysine residues in its WRKY domain with Lys-1221 being a key residue for this modification [50••,51••]. The WRKY domain of RRS1-R from *Arabidopsis* accessions Ws-2 and Nd-1 has an extended C-terminus that is hypothesized to interact with negative regulatory *cis*-elements in the promoters of genes that initiate ETI [51•]. Therefore, PopP2-dependent acetylation of RRS1 would attenuate its ability to bind to W-box motifs present in defense gene promoters and consequently trigger ETI [50•,51•]. Taken together, the results of these two studies reveal that acetylation of WRKY TFs is a virulence function of PopP2, and that the integrated WRKY domain of RRS1 has evolved as a decoy for recognition of the effector activity and induction of ETI.



## (2) NPRs are regulators of two opposing SA-mediated immune responses

During ETI, the plant defense hormones against biotrophic and necrotrophic pathogens, SA and JA respectively, both accumulate in the infected tissue [53]. Although many studies support an antagonistic SA-JA relationship that depends on the transcription cofactor NPR1 [54,55], there is also considerable evidence indicating that these hormones have a cooperative role during ETI [56,57]. A recent study has now shown that JA plays a positive role in establishment ETI through a mechanism that does not require NPR1 or the canonical JA receptor, CORONATINE INSENSITIVE 1 (COI1), but requires the SA receptors NPR3 and NPR4. NPR3 and NPR4 interact with JAZ transcriptional repressors in yeast and *in planta*, and promote JAZ1 degradation during early stages of ETI to activate expression of JA biosynthesis and signaling genes (Fig. 2) [58••]. Given that NPR3/NPR4 are substrate adapters for an E3 ubiquitin ligase complex, the evidence suggests that ubiquitination is the PTM leading to JAZ degradation by the proteasome during early ETI responses.

In contrast to ETI, which is a signal-specific local response, SAR confers broad-spectrum resistance in uninfected systemic tissues. NPR1 is both a positive regulator of SAR and a negative regulator of ETI and associated PCD (Fig. 2) [41]. In addition to SA-induced, redox-sensitive localization dynamics, the activity of NPR1 in the nucleus is tightly regulated by PTMs [59]. The discoveries of two opposing phosphorylation events (Ser55/Ser59 inhibits and Ser11/Ser15 activates NPR1) and their interplay with sumoylation, a PTM affecting NPR1 differential associations with TFs, TGAs and WRKYs, have revealed complex posttranslational regulation of NPR1-dependent transcription during immune responses [60••]. Sumoylation of NPR1, which is phosphorylated at Ser11/Ser15 but dephosphorylated at Ser55/Ser59, is also required for interaction with NPR3 and NPR4 leading to its degradation by the proteasome [60••,61]. Differences in the SA binding affinities for NPR3 (low) and NPR4 (high) and the opposing effects of SA binding to their interactions with NPR1 (i.e., SA facilitates NPR3-NPR1 interaction but disrupts NPR4-NPR1 interaction) [61], allows NPR1 degradation to occur in cells with high SA concentration (infection site) to remove inhibition on ETI and PCD. NPR1 is stabilized in systemic tissue where SA concentration is too low for NPR3-NPR1 interaction but high enough to disrupt NPR4-NPR1 interaction [61]. Thus, post-translational control of the NPR1 protein level allows PCD in local tissue and establishment of SAR in systemic tissues [61].

## Conclusions

Several PTMs stand out as major players of immune regulation; notably, phosphorylation, ubiquitination, and sumoylation. Our understanding of other modifications such as S-nitrosylation, acetylation, and sulfenylation, and their importance to plant immune responses and pathogen virulence mechanisms is also expanding. Although phosphorylation is a prominent posttranslational mechanism for propagating rapid and transient immune responses, identifying the kinases upstream of a known substrate remains a major challenge. The use of targeted, quantitative proteomics and the application of proximity-dependent affinity labeling of protein complexes *in vivo* show promise for the discovery of unknown kinases [62,63••,64••]. Additionally, advances in the sensitivity of proteomics instrumentation is rapidly increasing our ability to detect PTMs that occur in low

stoichiometry, and therefore will open the door to understanding how these modifications contribute to immune responses. Another exciting avenue for future studies, which will be paramount to understanding the dynamic immune system of plants, will be to connect protein kinases to their counteracting protein phosphatases and understand both the quantitative and the physiological significance of these opposing modifications. Gaining an in-depth understanding of how PTMs facilitate efficient immune responses and link PTI, ETI, and SAR will provide a dynamic and holistic view of plant immunity, and lead to new strategies for improving plant performance in the face of pathogen challenge.

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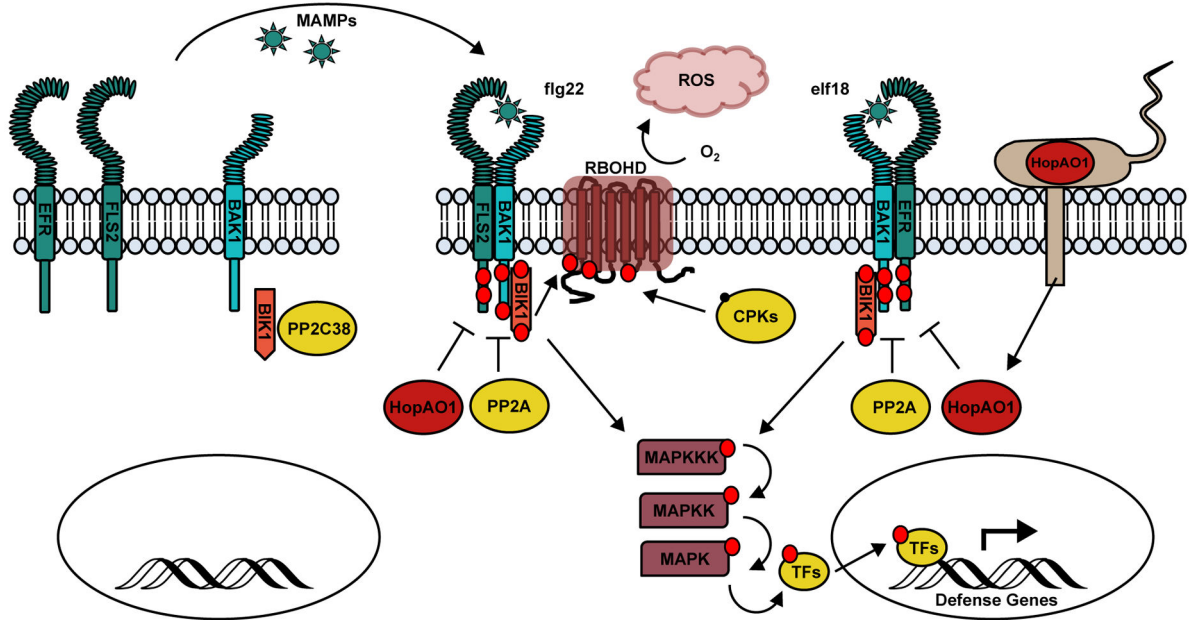
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**Highlights**

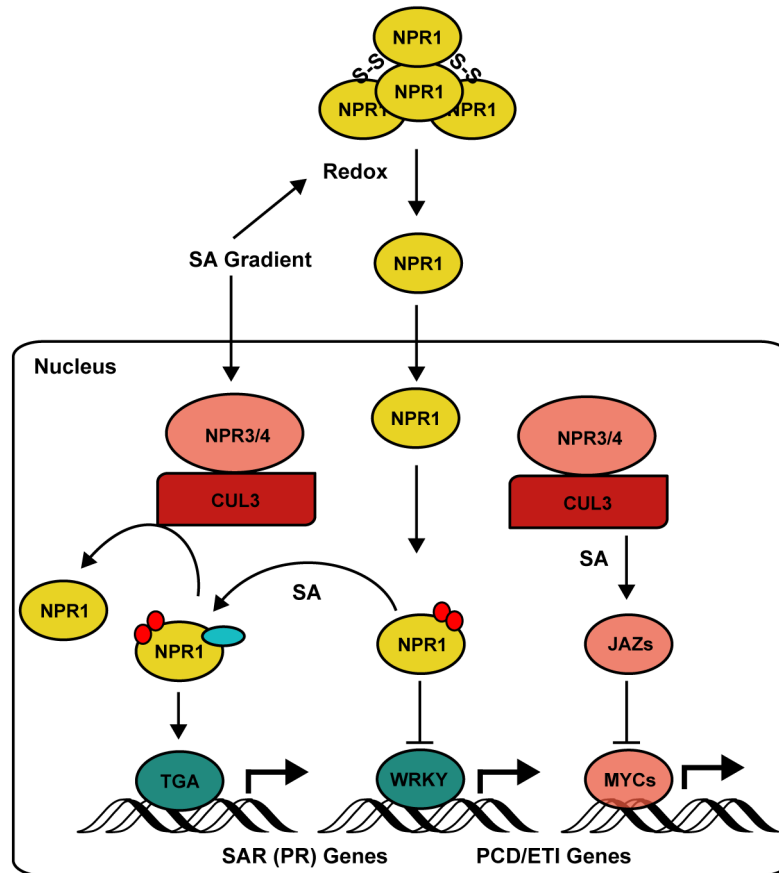
- Phosphorylation is a rapid and transient switch for PTI.
- PTM links metabolic pathways important for defense.
- PTM of TSB1 counters pathogen virulence strategies.
- PTM at the host-pathogen interface during ETI.
- NPRs are regulators of both ETI and SAR.



### Figure 1. Post-translational signaling mechanisms for pattern-triggered immunity

Cell surface-anchored pattern-recognition receptors (PRRs: FLS2, EFR) detect microbe-associated molecular patterns (MAMPs) and initiate pattern-triggered immunity (PTI). Activation of FLS2 and EFR by bacterial-derived peptides flagellin (flg22) and elongation factor Tu (elf18), stimulates the recruitment of the leucine-rich receptor-like kinase (LRR-RLK) BAK1 and the receptor-like cytoplasmic kinase (RLCK) BIK1, and induces auto- and trans-phosphorylation (red circles) of their cytoplasmic kinase domains. Phosphorylation of PRR-RLK-RLCK complex is attenuated by protein phosphatases (PP2A, PP2C38). MAMP perception triggers the production of reactive oxygen species (ROS) by the NADPH oxidase RBOHD at the plasma membrane, which is activated through phosphorylation by calcium-dependent protein kinases (CPKs) and BIK1. PTI signaling is propagated through mitogen-activated protein kinase cascades (MAPKKK, MAPKK, MAPK) resulting in phosphorylation and activation of transcription factors (TFs) that induce transcriptional reprogramming for defense. Pathogens deliver effector proteins into the host cell to suppress PTI. For example, the *Pseudomonas syringae* effector HopAO1 is a protein tyrosine phosphatase that dephosphorylates PRRs to and inhibits the host immune response.





**Figure 2. NPRs are regulators of two opposing hormone-mediated immune responses**  
 NPR1 normally exists as a high molecular weight oligomer in the cytoplasm. In response to salicylic acid (SA) - induced changes in cellular redox, NPR1 monomers are released into the nucleus. At resting state, NPR1, which is phosphorylated (red circles) at Ser55/Ser59, interacts with WRKY, a transcriptional repressor of *PR* genes. Sumoylated (light blue oval) NPR1, which is phosphorylated at Ser11/Ser15 but dephosphorylated at Ser55/Ser59, interacts with the transcriptional activator TGA3 to promote *PR* gene expression and establishment of systemic acquired resistance (SAR). NPR3 and NPR4 mediate SA-dependent degradation of NPR1 by the proteasome. NPR3 and NPR4 also promote the SA-enhanced degradation of JAZ transcriptional repressors to induce JA gene expression, resulting in activation of JA synthesis and signaling during early effector-triggered immune (ETI) responses.