Unveiling a multilayered regulatory network of NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 — A central node of salicylic acid-mediated plant defense

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Running title: Story of NPR1 and its regulatory network.

Abstract

Salicylic acid (SA) plays pivotal roles in plant defense against biotrophic and hemibiotrophic pathogens. Tremendous progress has been made in the field of the SA biosynthesis pathways and SA-mediated plant defense signaling networks in the past three decades. As one of the SA receptors, NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) functions as a master regulator of SA-mediated plant defense. The function of NPR1 is tightly regulated by transcriptional and post-translational regulation. This review discusses recent advances in SA and NPR1 biology, including SA perception, SA signaling, the function of SA and NPR1 in plant immunity, and the transcriptional and post-translational regulation of NPR1.

Keywords: *Arabidopsis thaliana*, *Pseudomonas syringae*, plant immunity, NPR1, salicylic acid

1 INTRODUCTION

In the natural environment, plants are constantly facing the challenge of various kinds of pathogenic infection, such as fungi, oomycetes, viruses, bacteria, and nematodes (Chen et al., 2020). Unlike animal counterparts, plants do not possess circulatory systems and specialized immune cells. In addition, as sessile organisms, plants cannot just move away from these pathogens. In order to survive from pathogenic attacks, plants have developed a sophisticated and multifaceted immune system to combat pathogens (Sun, Zhu, Balint-Kurti, & Wang, 2020).

The first line of defense is the physical barriers to infection, such as the cuticle and the cell wall. If pathogens were able to overcome the physical barriers, then plants have evolved a multilayer system of immune responses to fight against those pathogens (Jones & Dangl, 2006). The plant innate immune response depends on two main recognition systems to detect invaders. One system is initiated by the recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs), leading to PAMP-triggered immunity (PTI) (Dodds & Rathjen, 2010). One typical elicitor of PTI is the bacterial flagellin, which triggers defense responses in various plants (Gomez-Gomez & Boller, 2002). Flg22, a 22-amino acid sequence of the conserved N-terminal part of flagellin, is sufficient to induce full defense in plants. Flg22 is recognized by the receptor-like kinase (RLK) FLAGELLIN INSENSITIVE 2 (FLS2), which acts together with another RLK, BRASSINOSTEROID-INSENSITIVE1 (BRI1)-associated receptor kinase 1 (BAK1), to activate downstream immune responses (Chinchilla, Bauer, Regenass, Boller, & Felix, 2006; Chinchilla et al., 2007; Zipfel et al., 2004).

In order to avoid recognition by the PTI system of the host, pathogens have evolved a set of proteins called effectors to suppress the immune system of the plants. In this competition, plants have also evolved the second layer of the plant immune system. The second layer involves intracellular host receptors encoded by major resistance (*R*) genes to detect pathogen-derived effector molecules within the host cell, resulting in effector-triggered immunity (ETI). ETI is qualitatively stronger and faster than PTI and culminates in hypersensitive response. For instance, bacterial effectors from *Pseudomonas syringae,* AvrRpm1 and AvrB, are recognized by RESISTANCE TO PSEUDOMONAS SYRINGAE PV. MACULICOLA 1 (RPM1) protein in *Arabidopsis thaliana* resulting in accelerated defense responses, cessation of pathogen growth, and hypersensitive host cell death at the infection site (Desveaux et al., 2007; Mackey, Holt, Wiig, & Dangl, 2002). Another well-known bacterial effector AvrRpt2 is recognized by the R protein RESISTANT TO PSEUDOMONAS SYRINGAE2 (RPS2) in *Arabidopsis thaliana* to trigger ETI (Kunkel, Bent, Dahlbeck, Innes, & Staskawicz, 1993). Most *R* genes encode nucleotide-binding leucine-rich repeat (NB-LRR) proteins (Collier & Moffett, 2009). In general, most plants carry a repertoire of 50-1500 different *NLR* genes that mediate resistance to various viruses, bacteria, fungi, oomycetes, and nematodes (van Wersch, Tian, Hoy, & Li, 2020).

Besides the induction of defense at the site of infection, a common feature of both PTI and ETI is the activation of systemic defense response, which is often triggered in the distal parts of the infected plants. The activation thereby protects uninfected tissues against subsequent infections by a wide range of pathogens. This long-lasting and broad-spectrum induced disease resistance is referred to as systemic acquired resistance (SAR) (Pieterse, Leon-Reyes, Van der Ent, & Van Wees, 2009). SAR is associated with

increased levels of plant hormone salicylic acid (SA), a beta hydroxy phenolic acid, at the site of infection and in systemic tissues. SA is widely produced in prokaryotes and plants. Accumulation of SA leads to induction of of *pathogenesis-related (PR)* genes, some of which encode for proteins with antimicrobial activity (van Loon, Rep, & Pieterse, 2006).

In the last three decades, significant progress has been made in deciphering plant immune signaling governed by plant hormone SA. This review summarizes our current understanding concerning the function of SA and its receptor NPR1 in plant immunity. In addition, we highlight recent breakthroughs in the perception of SA because of its critical roles in the potentiation of PRRs (pattern recognition receptors)- and NLRs (leucine-rich repeat receptors)-mediated signaling. Finally, we focus on recent breakthroughs that have substantially advanced our understanding of how NPR1 is regulated at different levels.

2 ROLES OF SA AND ITS RECEPTOR NPR1 IN PLANT IMMUNITY

SA, better known as the active ingredient in aspirin (acetyl-SA), is a plant hormone that plays an important role in plant defense against biotrophic and semi-biotrophic pathogens (Fu & Dong, 2013; Qi et al., 2018). The first observation of SA's involvement in plant immunity was reported by Raymond F. White in 1979. He discovered that the application of aspirin in tobacco conferred resistance against tobacco mosaic virus (TMV) (White, 1979). Similarly, SA was shown with an increase in the phloem sap of cucumber before the induced resistance was detected in the systemic tissue (Métraux et al., 1990). Both studies indicate that endogenous SA plays a role as an internal defense signal for plant immunity.

2.1 Transportation and function of SA in SAR

The best-characterized role for SA in plant immunity is its role in systemic acquired resistance (SAR) (Gaffney et al., 1993). SA was initially considered as a mobile signal for SAR because the concentration of SA increases in both the primary infected and systemic uninfected tissue (Malamy, Carr, Klessig, & Raskin, 1990). Grafting experiments suggested that methyl salicylate (MeSA) is a critical, phloem-mobile SAR long-distance signal in tobacco (Park, Kaimoyo, Kumar, Mosher, & Klessig, 2007); however, the subsequent study concluded that MeSA is not the generic mobile signal for SAR (Attaran, Zeier, Griebel, & Zeier, 2009). Later, it was suggested that pathogen-induced SA moves via the extracytosolic apoplast compartment (Lim et al., 2016). Indeed, it was shown that the SA level was increased in the apoplastic collected from *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 carrying *avrRpt2* inoculated plants when compared with that in mock-inoculated plants (Lim et al., 2016). In contrast, two other SAR-associated chemical signals, glycerol-3-phosphate (G3P) and azelaic acid (AzA), are transported preferentially via plasmodesmata (Lim et al., 2016). More recently, Lim et al. showed that transport of SA from local to distal tissues is indeed essential for SAR and that this transport is governed by water potential in the infected tissue (Lim et al., 2020). Cuticle regulates the active transport of the SA. In cuticle-defective mutants, reduced water potential preferentially routes SA to cuticle wax rather than to the apoplast (Lim et al., 2020). N-hydroxyl pipecolic acid (NHP), which is catalyzed from pipecolic acid (Pip) by flavin-containing monooxygenases 1 (FMO1), was recently identified as a mobile signal for SAR (Chen et al., 2018; Hartmann et al., 2018). SA contributes to the induction of Pip biosynthesis. Pip biosynthetic genes *AGD2-LIKE DEFENSE RESPONSE PROTEIN 1*

(*ALD1*) and *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 4* (*SARD4*) are upregulated upon SA treatment (Ding et al., 2018). In the *npr1-1* mutant, the accumulation of Pip in the primarily infected leaves is delayed, suggesting that NPR1 positively regulates Pip biosynthesis (Návarová, Bernsdorff, Döring, & Zeier, 2012). Interestingly, SA biosynthetic genes *ISOCHORISMATE SYNTHASE 1* (*ICS1*), *ENHANCED DISEASE SUSCEPTIBILITY 5* (*EDS5*) and *AVRPPHB SUSCEPTIBLE 3* (*PBS3*) are induced by Pip, suggesting that there are amplification loops between SA and Pip (Hartmann et al., 2018).

2.2 SA amplifies PTI signal

SA also plays a critical role in PTI. The PTI elicitors flg22- and elf18-induced resistance against *Pst* DC3000 was compromised in SA biosynthesis mutant *sid2-2* (Tsuda, Sato, Stoddard, Glazebrook, & Katagiri, 2009). In addition, *sid2-2* and *npr4-4D* (carries a gain-of-function mutation in *NPR4*, which constitutively represses SA signal) mutants are more susceptible to *Pst* DC3000 *hrcC*, a type III secretion system-deficient bacterial strain (Ding et al., 2018; Tsuda, Sato, Glazebrook, Cohen, & Katagiri, 2008). In agreement with these reports, a recent study showed that NPR1 plays a prominent role in MAMP signaling (Chen et al., 2017). NPR1 positively regulates cell wall-associated plant defense in response to the *Pst* DC3000 *hrcC* (Chen et al., 2017). Activation of the early MAMP marker genes was also significantly impaired in *npr1-2* mutant after pathogen challenge (Chen et al., 2017). SA rapidly induces genes encoding PAMP receptors such as *FLS2*, *EF-Tu receptor* (*EFR*), *CHITIN ELICITOR RECEPTOR KINASE 1* (*CERK1*), and co-receptor *BAK1-LIKE 1* (*BKK1*) (Ding et al., 2018; Tateda et al., 2014). In addition, SA also induces a large number of genes encoding signaling components that act downstream of PAMP receptors, such as constituents of mitogen-activated protein (MAP) kinase

cascades, including *MAPKKK5*, *MKK1*, *MKK2*, *MKK4*, and *MPK11*, and subunits of heterotrimeric G proteins, for instance, *EXTRA-LARGE G-PROTEIN 2* (*XLG2*) and *ARABIDOPSIS G-PROTEIN GAMMA-SUBUNIT 1* (*AGG1*) (Ding et al., 2018; Zhang & Li, 2019). Thus, SA may play important roles in the amplification of PTI signals and NPR1 plays a prominent role in PTI signaling.

2.3 SA plays a dual role in ETI

SA plays dual roles in effector-triggered immunity. Firstly, SA is required for ETI. Initial evidence came from the finding that *Arabidopsis thaliana* expressing the bacterial enzyme salicylate hydroxylase cannot accumulate SA and is, therefore, more susceptible to the ETI elicitor *Pst avrRpt2* (Delaney et al., 1994). Early studies revealed that SA accumulation is associated with the onset of hypersensitive response during *R* gene-mediated defense responses (Nawrath & Metraux, 1999). Activation of ETI by Pseudomonas effectors AvrRpm1 and AvrRpt2 in Arabidopsis results in dramatic increases in local SA levels, in a ICS1- and EDS5-dependent manner (Nawrath & Metraux, 1999). SA amplifies ETI signal through positive regulation of several sensor NLR genes, such as *RPM1*, *RPS6*, *HOPZ-ACTIVATED RESISTANCE 1* (*ZAR1*), and *RESISTANCE TO LEPTOSPHAERIA MACULANS 3* (*RLM3*) (Ding et al., 2018). Interestingly, *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*), *PHYTOALEXIN DEFICIENT4* (*PAD4*), *SENESCENCE-ASSOCIATED GENE 101* (*SAG101*), and *NON RACE-SPECIFIC DISEASE RESISTANCE 1* (*NDR1*), which are required for TNL (Toll-like/Interleukin 1 receptor-type NLR)- and CNL (coiled-coil-type NLR)-mediated plant immunity, are also induced by SA (Ding et al., 2018; Falk et al., 1999; Feys, Moisan, Newman, & Parker, 2001). On the other hand, the activation of SA signaling also plays

an essential role in the negative regulation of cell death during ETI. It was shown that SA pre-treatment in Arabidopsis Col-0 plants blocks HR activated by *Pseudomonas syringae* pv. *maculicola* ES4326 carrying *avrRpm1* (Devadas & Raina, 2002). In addition, increased ion leakage was observed in *eds5-3* compared to wild type infected with *Pst* DC3000 *avrRpt2* (Radojicic, Li, & Zhang, 2018). Finally, it was revealed that SA-mediated suppression of cell death is dependent on NPR1. Fu et al. found that NPR1 functions as a negative regulator of programmed cell death (PCD), because the *npr3 npr4* double mutant can no longer undergo PCD in response to pathogen effectors (Fu et al., 2012). In line with this, a previous report also suggested that NPR1 suppresses hypersensitive response (Rate & Greenberg, 2001). Very recently, a breakthrough study unveiled a detailed mechanism by which NPR1 promotes defense and restricts cell death (Figure 1) (Zavaliev, Mohan, Chen, & Dong, 2020). Zavaliev et al. showed that NPR1 promotes cell survival by targeting substrates for ubiquitination and degradation through formation of salicylic acid-induced NPR1 condensates (SINCs) (Figure 1) (Zavaliev, Mohan, Chen, & Dong, 2020). SINC induction facilitates formation of the NPR1-Cullin 3 E3 ubiquitin ligase complex to ubiquitinate SINC-localized substrates, such as EDS1 and specific WRKY transcription factors, and promote cell survival during ETI (Figure 1) (Zavaliev, Mohan, Chen, & Dong, 2020). In conclusion, SA plays a dual role in the regulation of PCD in plants.

3 NPR1, NPR3, AND NPR4 ARE *BONA FIDE* SA RECEPTORS

3.1 SA perception by NPR1/3/4

It is widely believed that plant and animal hormones transduce their signals by binding to one or more receptors. Despite the discovery of dozens of SA binding proteins (Chen,

Ricigliano, & Klessig, 1993; Ding et al., 2018; Du & Klessig, 1997; Fu et al., 2012; Kumar & Klessig, 2003; Manohar et al., 2015; Slaymaker et al., 2002; Yuan, Liu, & Lu, 2017), only NPR1, NPR3, and NPR4 are considered as *bona fide* SA receptors (Ding et al., 2018; Fu et al., 2012; Wu et al., 2012). NPR3 and NPR4 display high affinity with SA, while the SA binding activity of NPR1 was controversial (Ding et al., 2018; Fu et al., 2012; Wu et al., 2012). Until very recently, Wang et al. re-evaluated SA binding by NPR1 and found that less than 0.02% of the total MBP–NPR1 in the sample was able to bind SA (Wang et al., 2020). By contrast, about 8% of NPR4 were able to bind to SA at the same saturating concentration (Wang et al., 2020). This explains why the SA binding activity of NPR1 was barely detected, which is reported by Fu et al., 2012. Furthermore, Wang et al. identified amino acids 373-516 within the NPR4 C-terminal domain as the SA-binding core (SBC) (Wang et al., 2020). Wang et al. also characterized SBC surface residues that affect SA binding and found that three mutants NPR4(F426L), NPR4(E469I), and NPR4(K505Q), showed reduced SA binding activity (Wang et al., 2020). By contrast, mutations of T459 to G increased SA binding to NPR4 by 50% (Wang et al., 2020). Interestingly, when the T459G substitution was combined with F426L, the ability of NPR4 to bind SA was substantially enhanced. NPR1 and NPR4 share nearly identical hormone-binding residues. NPR1 is also equipped with an SBC module (amino acids 386-525) that is capable of sensing SA, despite the absence of Cys529 that was previously reported to be required for SA binding (Wang et al., 2020; Wu et al., 2012).

3.2 SA signaling by NPR1/3/4

As a SA receptor, NPR1 functions as a transcriptional activator that promotes SA-induced defense gene expression. The *npr1* mutant was first identified during a screening of

Arabidopsis mutants that do not respond to SA or its active analogs (Cao, Bowling, Gordon, & Dong, 1994; Delaney, Friedrich, & Ryals, 1995). Loss of NPR1 results in reduced *PR* gene expression and increased susceptibility to pathogens (Cao et al., 1994; Delaney et al., 1995). NPR1 consists of an N-terminal bric-a-brac, tramtrack, and broad-complex (BTB) domain, ankyrin repeats, and a C-terminal domain containing a nuclear localization signal (NLS) and a putative transactivation domain (Kinkema, Fan, & Dong, 2000; Rochon, Boyle, Wignes, Fobert, & Despres, 2006). The NLS is required for SA-induced NPR1 nuclear translocation and function in SAR (Kinkema et al., 2000). In the cytosol, NPR1 mainly exists as oligomers. Upon pathogen infection or SA treatment, NPR1 is reduced from an oligomeric state to a monomeric state, and it is translocated to the nucleus to activate downstream transcription cascades (Mou, Fan, & Dong, 2003). NPR1 itself does not have a DNA binding domain; thus, NPR1-mediated signaling requires interaction with other transcription factors. Yeast two-hybrid screening has revealed that NPR1 interacts with seven members of the TGA transcription factor family (Boyle et al., 2009; Despres, DeLong, Glaze, Liu, & Fobert, 2000; Kim & Delaney, 2002; Zhou et al., 2000). Histone acetyltransferases (HATs or HACs) are well-known transcriptional coactivators that facilitate transcription through relaxing specific region of chromatin by histone acetylation, which makes DNA more accessible to transcription factors (Barlev et al., 2001; Ogryzko, Schiltz, Russanova, Howard, & Nakatani, 1996). Interestingly, HAC1 and HAC5 interact with NPR1 forming a coactivator complex with TGAs, which are recruited to the *PR* chromatin to activate the transcription of *PR* genes (Jin et al., 2018).

NPR1 positively regulates SA-mediated plant immunity, whereas NPR3 and NPR4 function as negative regulators of plant defense (Fu et al., 2012). NPR3 and NPR4 function as adaptors of the Cullin3 ubiquitin 3 E3 ligase (CUL3) to mediate NPR1 degradation (Fu et al., 2012). In support of this finding, it was shown that *npr3 npr4* double mutant accumulates a higher level of NPR1 protein (Fu et al., 2012). Also, NPR3 and NPR4 have been shown to facilitate the degradation of JAZ proteins to promote ETI (Liu et al., 2016). In addition to regulating NPR1 protein level, NPR3 and NPR4 serve as redundant transcriptional co-repressors that prevent activation of defense gene expression when the SA level is low. NPR3/NPR4 negatively regulates the expression of *SARD1*, *CBP60g*, and *WRKY70* through interaction with transcription factors TGA2/TGA5/TGA6 (Ding et al., 2018). In contrast, NPR1 promotes the expression of *SARD1* and *WRKY70* in response to SA (Ding et al., 2018). In the presence of SA, the transcription repressor activity of NPR3/4 was inhibited (Ding et al., 2018). The SA insensitive *npr4-4D* mutant not only displays enhanced disease susceptibility but also blocks INA-induced disease resistance (Ding et al., 2018). Thus, NPR1 and NPR3/4 play opposite roles in transcriptional regulation of SA-induced gene expression, although both are considered as *bona fide* SA receptors.

3.3 Structural basis of NPR proteins

Despite extensive efforts, the structure of NPR proteins has not been resolved until very recently. Wang et al. identified amino acids 373 to 516 within the NPR4 C-terminal domain as the SA-binding core (SBC) (Wang et al., 2020). By using hydrogen–deuterium-exchange mass spectrometry (HDX-MS), Wang et al. confirmed that the SBC of NPR4 has a deuterium uptake profile that is sensitive to SA (Wang et al., 2020). SBC of NPR4

was then crystallized and determined its structure at 2.3 Å resolution (Wang et al., 2020). The structure of the NPR4 SBC consists of five tightly packed α-helices and the C-terminal four-helix-bundle-like fold, while the SA-binding site is located at the tapered end of the four-helix bundle of the SBC of NPR4 (Wang et al., 2020). The SA-binding pocket is characterized by its hydrophobicity and its central location within the receptor SBC domain. It completely buries the SA inside an internal cavity at the tapered end of the four-helix-bundle-like fold, leaving no gap for the ligand to enter or escape (Wang et al., 2020). The lack of a ligand-entry pathway suggests that SA binding involves a major conformational remodeling of the NPR4 SBC (Wang et al., 2020). By revealing the structural mechanisms of SA perception by NPR4 SBC, Wang et al. provides initial insights into the structure-function relationships of NPR proteins, which in turn sheds light on the interplay between NPR proteins in SA signaling, and provides a new direction for engineering plant immunity.

4 TRANSCRIPTIONAL REGULATION OF *NPR1* GENE

Despite its pivotal role in plant immunity, the transcriptional regulation of *NPR1* is not extensively studied. As of now, there are only two transcription factors that have been found to bind to *NPR1* promoter (Chai, Liu, Zhou, & Xing, 2014; Yu, Chen, & Chen, 2001). WRKY18 was the first transcription factor that was reported to specifically recognize the W-box motif in the *NPR1* promoter (Yu et al., 2001). The W-box motif in the *NPR1* promoter is essential for its gene expression (Yu et al., 2001). Mutations in the W-box sequences abolish their recognition by WRKY DNA binding proteins, rendering the promoter unable to activate a downstream reporter gene (Yu et al., 2001). The *npr1* mutants containing an *NPR1* gene with a mutated W-box are unable to induce SA-

dependent gene expression or resistance (Yu et al., 2001). SA induces a number of other *WRKY* genes, suggesting that additional WRKY family proteins are involved in regulation of *NPR1* gene expression. Indeed, ChIP assay showed that WRKY6 binds to the W-box of the *NPR1* promoter (Chai et al., 2014). Further analyses showed that the mRNA level of *NPR1* is reduced in *wrky6* mutants and enhanced in *WRKY6* overexpressing lines. *WRKY6*-induced *NPR1* gene expression is required for SA-induced leaf senescence, but it is not clear if *WRKY6* is involved in SA-mediated plant immunity.

Interestingly, NPR1 protein also regulates its own gene expression. It was long found that *NPR1* transcript accumulation in the *npr1* mutants was not induced by INA (Kinkema et al., 2000). Later, Zhang et al. showed that *Pst* DC3000-induced *NPR1* transcript accumulation in *npr1-3* mutant was significantly lower than that in wild type (Zhang, Wang, Zhang, Sun, & Mou, 2012). These studies indicate that a functional NPR1 protein is required for the full expression of *NPR1.* Recently, Chen et al. demonstrated that a functional NPR1 protein promotes *NPR1* gene expression by binding to its promoter (Chen et al., 2019). The finding that functional NPR1-GFP, but not npr1-2, is able to induce *npr1-2* gene expression demonstrates that NPR1 protein promotes its own gene expression. Since NPR1 does not have a DNA binding domain, the binding of NPR1 to its own promoter must be mediated by transcription factors. Indeed, it has been shown that WRKY18 interacts with NPR1, an interaction that is enhanced by SA (Chen et al., 2019). Despite these discoveries, there remain several gaps in our understanding of how *NPR1* expression is regulated. Finally, CYCLIN-DEPENDENT KINASE 8 (CDK8) filled this gap in knowledge (Figure 2). NPR1 interacts with CDK8, which recruits RNA polymerase II to the promoter of NPR1 to facilitate its gene expression. Interestingly,

CDK8 also interacts with WRKY18 and WRKY6, which are positive regulators of *NPR1*. It will be interesting to investigate how CDK8 regulates the transcription factor activity to understand further how *NPR1* gene is regulated. CDK8 kinase module also includes Mediator 12 and 13 (MED12/13), mutation of which causes the plants to accumulate a low amount of *NPR1* transcript, which results in a loss of systemic acquired resistance. Further study is required to fully understand the role of MED12 and MED13 in the regulation of *NPR1* expression.

5 POST-TRANSLATIONAL REGULATION OF NPR1 PROTEIN

5.1 Conformational changes of NPR1

SA affects NPR1 function in two stages: first, it induces *NPR1* gene expression; second, SA promotes the conformational change of NPR1 and facilitates the translocation of NPR1 into the nucleus. The function of NPR1 is tightly regulated by its conformational change. In an uninduced state, NPR1 is present as an oligomer formed through intermolecular disulfide bonds (Mou et al., 2003). There are 17 cysteine residues in NPR1, ten of which are highly conserved. Site-directed mutagenesis showed that this oligomer contains intermolecular disulfide bonds between cysteine residues positioned within the BTB domain (Cys^{82}) and the region between the BTB and Ankyrin domains (Cys^{150}, Cys^{155}, Cys^{156}, Cys^{160}, and Cys^{216}) (Mou et al., 2003). Mutations at residues Cys^{82} and Cys^{216} in NPR1 result in increased monomer accumulation, constitutive nuclear localization, and NPR1-mediated gene expression in the absence of pathogen challenge (Mou et al., 2003). NPR1 is sensitive to redox changes. Upon SAR induction, a biphasic change in cellular reduction potential occurs, resulting in a reduction of NPR1 from oligomeric form to monomeric form (Mou et al., 2003). Monomeric NPR1 accumulates in

the nucleus and activates defense genes expression. NPR1 conformational changes are regulated by S-nitrosylation and thioredoxins (Figure 3). S-nitrosylation of Cys^{156} by S-nitrosoglutathione (GSNO) facilitates the assembly of NPR1 oligomer (Tada et al., 2008). Upon pathogen infection or accumulation of SA, changes in cellular redox potential lead to the reduction of cysteines through the activity of thioredoxins (TRX-h3 and TRX-h5) and release of NPR1 monomers to localize to the nucleus (Tada et al., 2008). Both NPR1 and TGA1 are well-described redox-regulated signaling compounds (Despres et al., 2003). Interestingly, not only NPR1, but also TGA1 are S-nitrosylated after treatment with GSNO (Lindermayr, Sell, Muller, Leister, & Durner, 2010). Mass spectrometry analyses revealed that the Cys residues 260 and 266 of TGA1 are S-nitrosylated and S-glutathionylated (Lindermayr et al., 2010). GSNO protects TGA1 from oxygen-mediated modifications and enhances the DNA binding activity of TGA1 to the *as-1* element at *PR1* promoter in presence of NPR1 (Lindermayr et al., 2010).

5.2 Phosphorylation of NPR1

Besides modifications of the cysteine residues that affect the NPR1 oligomer–monomer switch, phosphorylation of NPR1 was also found to be required for its nuclear import and establishment of SAR. SnRK2.8 interacts with and phosphorylates NPR1; however, SnRK2.8 does not affect the NPR1 monomerization reaction (Lee et al., 2015). Phosphorylation within the C terminal NLS (Ser^{589}) by SnRK2.8 was found to be required for nuclear import and the establishment of SAR (Figure 3) (Lee et al., 2015). Furthermore, genetic evidence indicates that an additional threonine (Thr^{373}), which is identified by phosphoproteomic analysis of *in vitro* phosphorylated NPR1, might also be modified by SnRK2.8 as the npr1 (T373A) mutant fails to enter the nucleus (Lee et al., 2015). Another

kinase that was found to interact with and phosphorylate NPR1 was PROTEIN KINASE SOS2-LIKE5 (PKS5), a pathogen-responsive member of the sucrose non-fermenting 1 (SNF1)-related kinase 3 (SnRK3) subgroup (Xie, Zhou, Deng, & Guo, 2010). PKS5 phosphorylates the C-terminal region of NPR1. In *pks5* mutants, the expression level of two NPR1 target genes, *WRKY38* and *WRKY62*, is reduced and/or delayed (Xie et al., 2010). Despite this discovery, the phosphorylation site of NPR1 is still unknown. More recently, it was shown that mitogen-activated protein kinase MPK1 directly interacts with and phosphorylates NPR1 (Zhang et al., 2020). Meanwhile, MPK1 also mediates NPR1 monomerization (Zhang et al., 2020). Further research is required to understand the mechanism by which PKS5 and MPK1 phosphorylates NPR1 fully.

5.3 Proteasome-mediated turnover of NPR1

NPR1 activity is tightly regulated by post-translational degradation. Proteasome-mediated turnover of NPR1 within the nucleus is a requirement for the full induction of target genes and the establishment of SAR (Spoel et al., 2009). In the absence of pathogen challenge, NPR1 is continuously cleared from the nucleus by the proteasome (Spoel et al., 2009). Inducers of SAR promote NPR1 phosphorylation at residues Ser^{11}/Ser^{15} and facilitate its recruitment to a CUL3-based ubiquitin ligase (Spoel et al., 2009). NPR1 does not interact directly with CUL3, although NPR1 could be pulled down with an antibody against CUL3A or co-immunoprecipitates with CUL3 in *N.benthamiana* extracts (Dieterle et al., 2005; Spoel et al., 2009; Zavaliev et al., 2020). NPR3 and NPR4 function as adaptors of CUL3 E3 ligase to mediate NPR1 degradation in an SA-regulated manner (Fu et al., 2012). Consistent with this, Wang et al. demonstrate that NPR1 is destabilized in NPR4(F426L/T459G) and NPR4 plants when treated with 1 mM SA (Wang et al., 2020).

In support of this, it was found that *npr3 npr4* double mutant accumulates a higher level of EDS1 proteins, indicating that EDS1 is subject to NPR3/4 mediated degradation (Chang et al., 2019; Fu et al., 2012). In addition, NPR3 and NPR4 facilitate the degradation of JAZ proteins to promote ETI (Liu et al., 2016). Interestingly, a bacteria effector AvrPtoB, which is an E3 ligase, also targets NPR1 for degradation via the host 26S proteasome pathway, thereby subverting plant immunity (Chen et al., 2017). In rice, OsCUL3a interacts with and degrades OsNPR1, which acts as a positive regulator of cell death in rice (Liu et al., 2017). The function of NPR1 in plant immunity has been revealed in other species as well (Chen et al., 2012; Malnoy, Jin, Borejsza-Wysocka, He, & Aldwinckle, 2007; L. Wang et al., 2017; Zhang et al., 2012; Zhang, Ni, Ma, & Qiu, 2013). It would be interesting to investigate if NPR1 proteins in other species are also degraded by E3 ligase activity to regulate plant immunity. Interestingly, a recent finding from Fu's group showed that NPR1 protein stability is also controlled by EDS1 (unpublished data). On the other hand, EDS1 protein stability is regulated by PBS3 (Chang et al., 2019). These studies suggest that there are strong physical and biological interactions among the immune proteins in SA signaling pathway. Deeper research will help us to better understand the interplay between these important regulators in SA-mediated plant immunity.

5.4 Polyubiquitination and de-ubiquitination of NPR1

The turnover of NPR1 protein is mediated by 26S proteasome complex through sequential polyubiquitination processes by E3 ligase CUL3 and E4 ligase UBE4 (Figure 3) (Skelly, Furniss, Grey, Wong, & Spoel, 2019). Adult *ube4* mutant displays enhanced expression of immune genes in the absence of pathogen challenge (Skelly et al., 2019),

which is similar to the phenotype observed in mutants in CUL3 E3 ligase that fail to degrade NPR1 (Spoel et al., 2009). UBE4 is involved in polyubiquitination of NPR1. Only when polyubiquitination of NPR1 is enhanced by UBE4, is it targeted for proteasomal degradation (Skelly et al., 2019). The complexity of the ubiquitin-dependent post-translational regulation of NPR1 was further revealed by the identification of ubiquitin-specific protease UBP6 and UBP7 that deubiquitinated NPR1 (Figure 3). Knockout of *UBP6* and *UBP7* resulted in an enhanced turnover and decreased transcriptional output of NPR1 (Skelly et al., 2019). Thus, ubiquitin chain extension and trimming activities can fine-tune transcriptional outputs of transcriptional coactivator NPR1.

5.5 SUMOylation of NPR1

In addition, NPR1 is also regulated by SUMOylation. Small ubiquitin-like modifier 3 (SUMO3) interacts with and SUMOylates NPR1 following SA treatment (Saleh et al., 2015). SUMO-interaction motif (VIL)-(VIL)-x-(VIL) found within the ankyrin repeat domain of NPR1 is required for the interaction between NPR1 and SUMO3 (Saleh et al., 2015). In the absence of SA accumulation, NPR1 is phosphorylated at Ser^{55}/Ser^{59}, which blocks SUMOylation and promotes interaction with WRKY70 in order to repress *PR1* expression (Saleh et al., 2015). Upon induction, Ser^{55}/Ser^{59} of NPR1 is likely dephosphorylated, allowing NPR1 to become SUMOylated. SUMOylation of NPR1 activates defense gene expression by switching NPR1's association with the WRKY transcription repressors to TGA transcription activators. In addition, modification of NPR1 by SUMO3 is required for phosphorylation at Ser^{11}/Ser^{15} to form a signal amplification loop to generate more activated NPR1 (Figure 3) (Saleh et al., 2015). The interplay between SUMOylation and

phosphorylation of NPR1 sheds light on the mystery of why the degradation of NPR1 appeared to be required for the full activity of NPR1 (Spoel et al., 2009).

6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Transcriptional regulation of NPR1 plays a vital role in SA signaling. Although a couple of transcription factors that regulate NPR1 promoter activity have been found, there are still some transcription factors that are yet to be identified. *NPR1* promoter contains a *TL1* (CTGAAGAAGAA) element; thus, the expression of *NPR1* gene may be regulated by *TL1*-binding transcription factor TBF1 (Pajerowska-Mukhtar et al., 2012). It has been shown that CDK8 functions as a bridge between WRKY transcription factors that bind to NPR1 promoter and RNA polymerase II (Chen et al., 2019). CDK8 kinase module subunits MED12 and MED13 also positively regulate NPR1 gene expression (Chen et al., 2019). It would be interesting to investigate whether if MED12 and MED13 also associate with NPR1 promoters through transcription factors such as WRKY18, WRKY6, or others that are yet to be identified (Figure 2). In addition, SARD1 and CBP60g have been found to bind to the promoters of *NPR1* gene through ChIP assay (Sun et al., 2015); however, the underlying molecular mechanism remains cryptic (Figure 2).

Post-translational modifications of NPR1 have been extensively studied. However, there are still some basic questions that remain to be answered. NPR1 interacts with transcription factors, such as TGAs, to regulate defense genes expression; however, how exactly is the transcription coactivity of NPR1 orchestrated, and what genes does NPR1 control during specific time points of immune response? How do specific posttranscriptional modifications of NPR1 affect its interaction with TGAs and other transcription factors such as TCPs, that have been shown to regulate *PR5* gene

expression (Li et al., 2018)? A previous study showed that NPR1 forms a protein complex with HAC1 and TGAs to regulate *PR1* gene expression. NPR1 may interact with other chromatin remodeling proteins as well, which warrants further investigation. In addition, ChIP assay using NPR1 transgenic plant will further help us understand the regulatory role of NPR1 in the expression of defense genes.

Phosphorylation has been shown to regulate multiple NPR1 functions. Two members of the SnRK family of kinases have been shown to interact with and phosphorylate NPR1 (Lee et al., 2015; Xie et al., 2010); however, the kinase responsible for Ser^{11}/Ser^{15} and Ser^{55}/Ser^{59} phosphorylation have not yet been identified. NPR1 contains multiple sites that are potentially phosphorylated (Withers & Dong, 2016). Thus, it would be interesting to identify those sites further to understand the phosphorylation events within the NPR1 protein. Recent studies have suggested that NPR1 is also dephosphorylated (Saleh et al., 2015); however, no phosphatases that directly interact with and regulate NPR1 have been discovered.

The post-translational regulation of NPR1 is well studied; however, it is not known if all NPR1 homologs would undergo similar biochemical processes to NPR1 in response to SA accumulation, such as the transition from oligomer to monomer, translocation from cytosol to nuclear, polyubiquitination, and rapid protein turnover. Although the crystal structure of NPR4 SBC has been revealed, the crystal structures of full-length NPR1/3/4 are still mysteries. The structural determination of full-length NPR1/3/4 in its modified states and/or bound to SA would provide the ultimate understanding of the physical dynamics of NPR1/3/4.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure1. NPR1 promotes survival during ETI. During ETI, infected cells detect pathogen effectors through nucleotide-binding leucine-rich repeat (NB-LRR) proteins. The signal is then transduced through components such as EDS1/PAD4 and WRKY transcription factors. NPR1 monomers enter the nucleus and are subjected to the NPR3/4-CUL3 complex for degradation to remove its inhibitory effect on ETI. In adjacent cells, SA mediates the activation of nuclear NPR1 to induce transcription of SAR genes, including salicylic acid-induced NPR1 condensates (SINC) components, such as NB-LRRs, EDS1, and WRKY54/70. In the presence of SA, NPR1 promotes cell survival by recruiting CUL3 and targeting substrates that are involved in cell death for ubiquitination and degradation through the formation of SINCs.

Figure 2. Transcriptional regulation of *NPR1* gene. Upon pathogen infection, SA accumulates in the plant cell. SA binds to NPR1 protein and promotes the interaction between NPR1 and WRKY18. CDK8 also interacts with WRKY transcription factors WRKY18 and WRKY6, which associate with *NPR1* promoter through W-box motif. In the presence of SA, NPR1 recruits CDK8 to *NPR1* promoter to facilitate its own gene expression. CDK8 kinase module subunits MED12 and MED13 are also involved in the transcriptional regulation of *NPR1* gene. Some unknown transcription factors that interact with MED12 or MED13 may regulate the expression of *NPR1* gene. TBF1 potentially regulates the expression of *NPR1* gene through the *TL1* element. SARD1 and CBP60g associate with *NPR1* promoter to regulate its gene expression through an unknown *cis*-element. Other unidentified transcription factors that regulate *NPR1* gene expression need to be discovered. TF, transcription factor.

Figure 3. Post-translational regulation of NPR1 protein. At the resting stage, NPR1 mainly exists as oligomer in the cytosol. NPR1 is phosphorylated at Ser^{55}/Ser^{59} (S55/59) and interacts with WRKY70 to suppress *PR1* gene induction. NPR1 is constantly degraded by CUL3 and its adapter protein NPR4. Thioredoxins (TRXs) and GSNO mediate the transition of NPR1 between oligomeric and monomer state. Upon pathogen infection (induced condition), SA accumulates in the plant cell. SnRK2.8 phosphorylates NPR1 at S589 and facilitates its translocation from the cytosol to the nucleus. T373 is also required for NPR1 nuclear transport. In the nucleus, SA accumulation promotes dephosphorylation of S55/59 through an unknown mechanism and induces the SUMOylation of NPR1 by SUMO3. SUMOylation promotes phosphorylation of NPR1 at Ser^{11}/Ser^{15} and the interaction between NPR1 and TGAs to facilitate *PR1* gene expression. The turnover of NPR1 protein is mediated by 26S proteasome (26S) complex through sequential polyubiquitination processes by CUL3 and E4 ligase UBE4. On the other hand, NPR1 deubiquitination process is mediated by ubiquitin-specific proteases UBP6 and UBP7, which are closely linked to 26S proteasome. P, phosphorylation. S, SUMOylation.